The high-affinity calcium–calmodulin-binding site does not play a role in the modulation of type 1 inositol 1,4,5-trisphosphate receptor function by calcium and calmodulin

Elena NOSYREVA*, Tomoya MIYAKAWA†, Zhengnan WANG*, Lyuba GLOUCHANKOVA*, Akiko MIZUSHIMA†, Masamitsu IINO† and Ilya BEZPROZVANNY*[1]

*Department of Physiology, UT Southwestern Medical Center at Dallas, Dallas, TX 75390, U.S.A., and †Department of Pharmacology, Graduate School of Medicine, The University of Tokyo, Tokyo 113-0033, Japan

Modulation of the inositol 1,4,5-trisphosphate ($InsP_3$) receptors ($InsP_3R$) by cytosolic calcium (Ca^{2+}) plays an essential role in Ca^{2+} signalling, but structural determinants and mechanisms responsible for the $InsP_3R$ regulation by Ca^{2+} are poorly understood. In the present study, we expressed rat $InsP_3R$ type 1 ($InsP_3R1$) in *Spodoptera frugiperda* cells using a baculovirus-expression system and reconstituted the recombinant $InsP_3R1$ into planar lipid bilayers for functional analysis. We observed only minor effects of 0.5 mM of calmodulin (CaM) antagonist W-7 on the Ca^{2+} dependence of $InsP_3R1$. Based on a previous analysis of mouse $InsP_3R1$ [Yamada, Miyawaki, Saito, Nakajima, Yamamoto-Hino, Ryo, Furuichi and Mikoshiba (1995) Biochem J. **308**, 83–88], we generated the $Trp^{1577} \rightarrow Ala$ (W1577A) mutant of rat $InsP_3R1$ which lacks the high-affinity Ca^{2+}–CaM-binding site. We found that the W1577A mutant displayed a bell-shaped Ca^{2+} dependence similar to the wild-type $InsP_3R1$ in planar lipid bilayers. Activation of B cell receptors resulted in identical Ca^{2+} signals in intact DT40 cells lacking the endogenous $InsP_3R$ and transfected with the wild-type $InsP_3R1$ or the W1577A mutant cDNA subcloned into a mammalian expression vector. In the planar lipid bilayer experiments, we showed that both wild-type $InsP_3R1$ and W1577A mutant were equally sensitive to inhibition by exogenous CaM. From these results, we concluded that the interaction of CaM with the high-affinity Ca^{2+}–CaM-binding site in the coupling domain of the $InsP_3R1$ does not play a direct role in biphasic modulation of $InsP_3R1$ by cytosolic Ca^{2+} or in $InsP_3R1$ inhibition by CaM.

Key words: baculovirus, calcium signalling, DT40 cells, planar lipid bilayer, Sf9 cells, single-channel recordings, structure function.

INTRODUCTION

The inositol (1,4,5)-trisphosphate ($InsP_3$) receptor ($InsP_3R$) is an intracellular calcium (Ca^{2+}) release channel that plays a critical role in Ca^{2+} signalling [1]. Three mammalian isoforms of $InsP_3R$, namely type I ($InsP_3R1$), type II ($InsP_3R2$) and type III ($InsP_3R3$) have been identified [2], with only minimal information available to date about the differences in their functional properties. The $InsP_3Rs$ are subjected to multiple levels of regulation [1,3,4]. Binding of $InsP_3$ triggers the opening of the $InsP_3R$ channel. The activity of $InsP_3R1$ is under biphasic feedback control by cytosolic Ca^{2+}: at lower concentrations Ca^{2+} acts as a co-activator of $InsP_3R1$ and at higher concentrations it inhibits $InsP_3R1$ [5–7]. This property of $InsP_3R1$, called the 'bell-shaped' Ca^{2+} dependence, is essential for the generation of Ca^{2+} waves and oscillations in cells [1,8–10].

Despite the crucial importance for the $InsP_3R$ signalling function, very little is known about the structural determinants and the mechanisms responsible for biphasic regulation of $InsP_3R1$ by cytosolic Ca^{2+} (see [4] for a recent review). Direct association of Ca^{2+} with the intrinsic Ca^{2+}-sensor region appears to be involved in the activation of $InsP_3R1$ by Ca^{2} [11]. Ca^{2+}-binding protein calmodulin (CaM) has been strongly implicated in the inhibition of $InsP_3R1$ by Ca^{2+} [12]. A high-affinity Ca^{2+}–CaM-binding site has been identified in the coupling domain of the $InsP_3R1$ in biochemical experiments [13]. An additional Ca^{2+}-independent low-affinity CaM-binding site was detected in the ligand-binding amino terminal portion of the $InsP_3R1$ in the $InsP_3$-binding experiments [14–16]. Exogenous application of CaM has been reported to inhibit the $InsP_3R1$ activity in a variety of functional assays [12,15–19]. What is the role of high-affinity Ca^{2+}–CaM-binding site in the biphasic modulation of $InsP_3R1$ by Ca^{2+} and inhibition by CaM?

To address this question, we generated a $Trp^{1577} \rightarrow Ala$ (W1577A) mutation in the rat $InsP_3R1$, which eliminated the Ca^{2+}-dependent association of $InsP_3R1$ with CaM. We also compared the Ca^{2+} dependence of the wild-type $InsP_3R1$ with the W1577A mutant. The $InsP_3R1$ used for the present study was expressed in insect *Spodoptera frugiperda* (Sf9) cells using recombinant baculovirus and reconstituted into planar lipid bilayers for single-channel recordings and analysis. We also analysed the B cell receptor (BCR)-mediated Ca^{2+} signals supported by the wild-type $InsP_3R1$ and the W1577A mutant expressed in DT40 cells lacking endogenous $InsP_3R$ [20]. In addition, we evaluated the changes in the Ca^{2+} dependence of $InsP_3R1$ induced by CaM antagonist W-7 and compared the responses of wild-type $InsP_3R1$ and W1577A mutant with exogenous CaM. The results lead us to conclude that the high-affinity Ca^{2+}–CaM-binding site in the coupling domain of $InsP_3R1$ is not involved directly in the biphasic modulation of $InsP_3R1$ by Ca^{2+} or in the inhibition by CaM.

Abbreviations used: BCR, B cell receptor; CaM, calmodulin; $InsP_3$, inositol 1,4,5-trisphosphate; $InsP_3R$, $InsP_3$ receptor; Sf9, *Spodoptera frugiperda*; W1577A, $Trp^{1577} \rightarrow Ala$.

[1] To whom correspondence should be addressed (e-mail Ilya.Bezprozvanny@UTSouthwestern.edu).

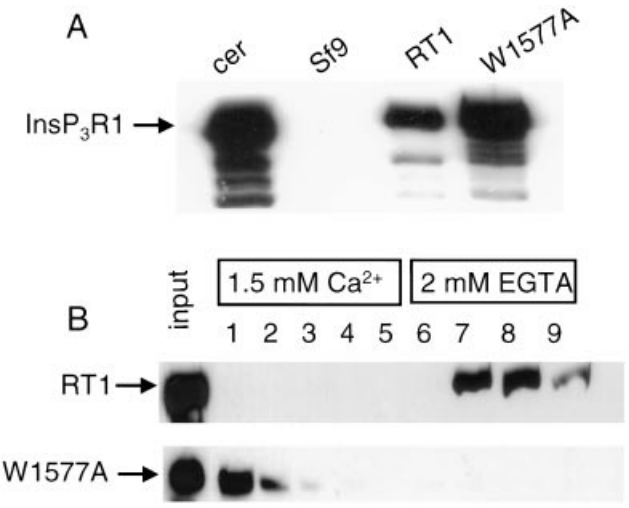

Figure 1 Expression in Sf9 cells and Ca^{2+}–CaM-binding properties of rat wild-type $InsP_3R1$ and W1577A mutant

(**A**) Western-blot analysis of microsomal proteins. Microsomes isolated from rat cerebellar (cer), non-infected Sf9 cells (Sf9), from Sf9 cells infected with RT1 (RT1) and W1577A (W1577A) recombinant baculoviruses were analysed by Western blotting with anti-$InsP_3R1$ polyclonal antibody T_{443}. For each preparation 10 μg microsomal protein was loaded on to the gel. The arrow points to the 260 kDa position expected for the $InsP_3R1$. (**B**) Association of $InsP_3R1$ and W1577A with CaM–Sepharose. Fractions collected from the output of CaM–Sepharose column were collected while washing in high Ca^{2+}-binding buffer (1.5 mM of Ca^{2+}), eluted in low Ca^{2+}-elution buffer (2 mM EGTA) and analysed by Western blotting with anti-$InsP_3R1$ polyclonal antibody T_{443}. An equal volume from each collected fraction was loaded on to each lane. The input lane contains 1/50 of the total sample used for CaM–Sepharose binding experiments. Results obtained with the wild-type $InsP_3R1$ (RT1, top row) and W1577A mutant (W1577A, bottom row) are compared.

EXPERIMENTAL

Generation of recombinant baculoviruses

The full-length neuronal rat $InsP_3R1$ (SI−/SII+) [21] expression construct in pcDNA3 vector ($InsP_3R1$–pcDNA3) was described previously [22]. The coding sequence of the $InsP_3R1$ was excised from pcDNA3 vector with *Xho*I and *Xba*I and subcloned into *Sal*I and *Xba*I sites of pFastBac1 expression vector (Invitrogen, Carlsbad, CA, U.S.A.) with the *Eco*RI, *Bam*HI, *Xho*I, *Kpn*I and *Sph*I sites removed. The generated pFastBac1–$InsP_3R1$ plasmid was transformed into DH10Bac (Invitrogen) *Escherichia coli* strain and baculoviruses expressing $InsP_3R1$ (RT1) were generated using the Bac-to-Bac baculovirus-expression system according to the manufacturer's (Invitrogen) instructions. The generated RT1 baculoviruses were amplified three times in Sf9 cells to yield P3 stock with the titre 10^8–10^9 pfu/ml. The W1577A mutant was generated on the basis of $InsP_3R1$–pcDNA3 construct using the megaprimer PCR method and was verified by sequencing. The 2.5 kb fragment of the $InsP_3R1$ sequence containing W1577A mutation was excised from W1577A–pcDNA3 by *Bam*HI (3797, 6329) and subcloned into *Bam*HI-digested and CIAP-dephosphorylated pFastBac1–$InsP_3R1$. The orientation of the *Bam*HI/*Bam*HI fragment was verified by PCR and the presence of W1577A mutation in the resulting pFastBac1–W1577A plasmid was verified by sequencing. The recombinant W1577A baculovirus encoding the $InsP_3R1$–W1577A mutant was generated and amplified using the Bac-to-Bac system (Invitrogen) as described for the RT1 virus.

Expression of the $InsP_3R1$ in Sf9 cells

Sf9 cells were obtained from ATCC and cultured in suspension culture in supplemented Grace's Insect Media (Invitrogen) with 10% foetal bovine serum at 27 °C. For the $InsP_3R1$ or W1577A expression 150 ml of Sf9 cell culture was infected by RT1 or W1577A baculoviruses at MOI of 5–10. Sf9 cells (66 h post-infection) were collected by centrifugation at 4 °C for 5 min at 800 rev./min (GH 3.8 rotor; Beckman Instruments). The cellular pellet was resuspended in 25 ml of homogenization buffer [0.25 M sucrose/5 mM Hepes (pH 7.4)] supplemented with a cocktail of protease inhibitors [1 mM EDTA, 2 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM benzamidine, 2.2 mM 4-(2-aminoethyl)benzene-sulphonyl fluoride hydrochloride, 10 μg/ml pepstatin and 0.1 mg/ml PMSF]. Cells were disrupted by sonication (Branson Ultrasonics) and manually homogenized on ice with the glass–Teflon homogenizer. The microsomes were isolated from the Sf9 cell homogenate by gradient centrifugation as described previously for HEK-293 cells [22]. The final microsomal preparation was resuspended in 0.5 ml of the storage buffer [10% sucrose/10 mM 3-morpholino-2-hydroxypropanesulphonic acid (pH 7.0)] to typically yield 6 mg/ml of protein (Bradford assay; Bio-Rad), aliquoted, quickly frozen in liquid nitrogen and stored at −80 °C. Expression of $InsP_3R1$ was confirmed by Western-blot analysis. The anti-$InsP_3R1$ rabbit polyclonal antibody (T_{443}) was described previously [22].

Single-channel recordings and analysis of the $InsP_3R1$ activity

Recombinant wild-type $InsP_3R1$ or W1577A mutants expressed in Sf9 cells were incorporated into the bilayer by microsomal vesicle fusion as described previously for native cerebellar $InsP_3R$ and for the $InsP_3R1$ expressed in HEK-239 cells [5,22–24]. Single-channel currents were recorded at 0 mV transmembrane potential using 50 mM Ba^{2+} dissolved in Hepes (pH 7.35) in the *trans* (intraluminal) side as a charge carrier [24]. The *cis* (cytosolic) chamber contained 110 mM of Tris dissolved in Hepes (pH 7.35). To obtain the Ca^{2+} dependence of the $InsP_3R1$ we follow the method described in [5]. Concentration of free Ca^{2+} in the *cis* chamber was controlled over the range 10 nM (pCa 8) to 10 μM (pCa 5) using a mixture of 1 mM EGTA, 1 mM HEDTA and variable concentrations of $CaCl_2$. The resulting concentration of the free Ca^{2+} was calculated using a program described in [25]. All recordings of $InsP_3R1$ activity were performed in the presence of 0.5 mM Na_2ATP [23] and 2 μM $InsP_3$. All additions ($InsP_3$, ATP, $CaCl_2$ and CaM) were made to the *cis* chamber from the concentrated stocks with stirring the solutions for at least 30 s in both chambers. $InsP_3R1$ single-channel currents were amplified (Warner OC-725), filtered at 1 kHz by lowpass 8-pole Bessel filter, digitized at 5 kHz (Digidata 1200; Axon Instruments, Foster City, CA, U.S.A.) and stored on computer hard drive and recordable optical discs. $InsP_3$ was purchased from Alexis (San Diego, CA, U.S.A.), CaM from Calbiochem (La Jolla, CA, U.S.A.), lipids were from Avanti Polar Lipids (Alabaster, AL, U.S.A.) and all other chemicals were from Sigma.

For off-line computer analysis (pClamp 7; Axon Instruments) single-channel data were filtered digitally at 500 Hz and for the presentation of the current traces data were filtered at 200 Hz. Evidence for the presence of 2–3 functional channels in the bilayer was obtained in most of the experiments. The number of active channels in the bilayer was estimated as the maximal number of simultaneously open channels during the course of the experiment [26]. The probability of the closed level, and the 1st and 2nd open levels was determined by using the half-threshold crossing criteria ($t \geqslant 2$ ms) from the records lasting for at least 2 min and 30 s. The single-channel open probability P_o for one

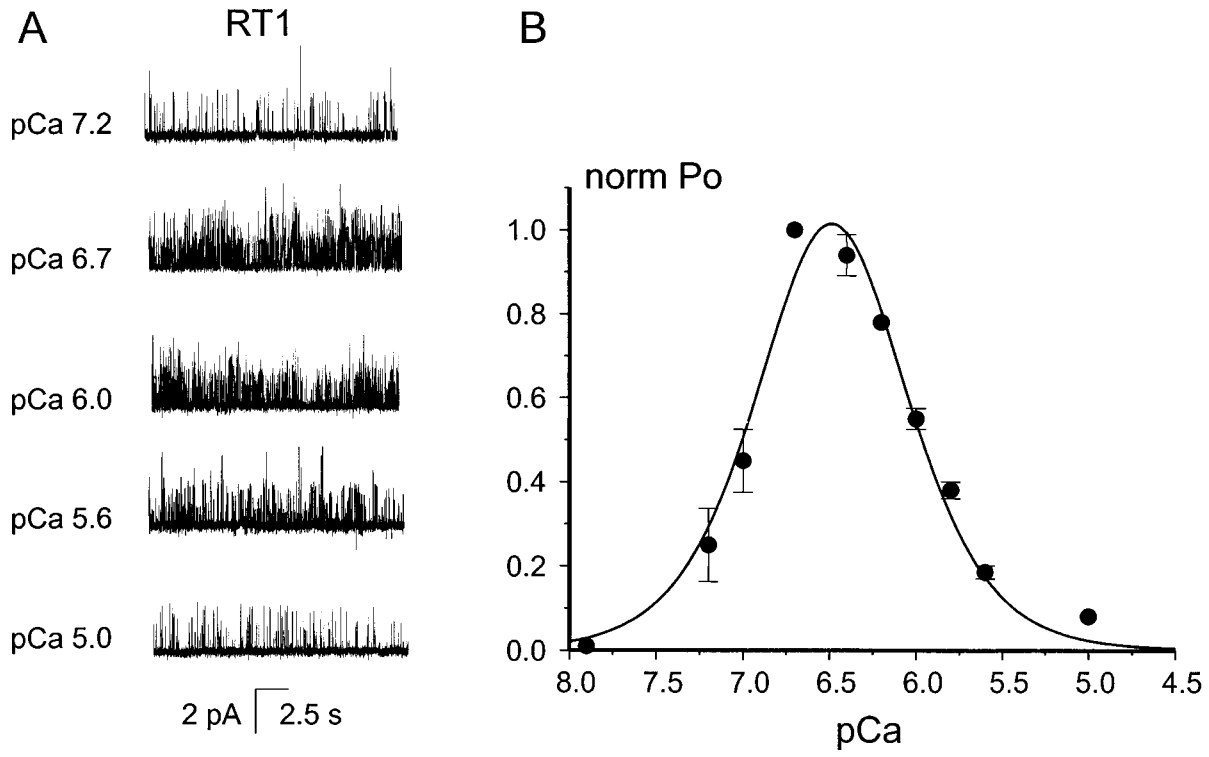

Figure 2 Bell-shaped Ca^{2+} dependence of recombinant $InsP_3R1$

(**A**) Single-channel records of $InsP_3R1$ in planar lipid bilayers. The unitary current recordings from the same experiment are shown at different *cis* (cytosolic) Ca^{2+} concentrations as indicated. (**B**) Bell-shaped Ca^{2+} dependence of recombinant $InsP_3R1$ (●). The channel P_o at each Ca^{2+} concentration was normalized to the maximum P_o observed in the same experiment. The normalized data from independent experiments were averaged together at each Ca^{2+} concentration and shown as means ± S.E.M. ($n = 4$). The maximal $P_o = 17 \pm 5\%$ ($n = 4$).

channel was calculated using the binomial distribution for the levels 0, 1 and 2, and assuming that the channels were identical and independent [27]. Normalizing the P_o to the maximal P_o observed in the same experiment minimized potential errors in absolute P_o values associated with the possible underestimation of the number of active channels in the bilayer. To generate the complete Ca^{2+}-dependence curves, the experiments with persistent $InsP_3R1$ activity for duration of the experiment (> 40 min) were chosen for analysis in order to prevent artifacts related to irreversible channel inactivation that are occasionally observed in the bilayers.

CaM–Sepharose-binding experiments

For CaM–Sepharose-binding experiments, recombinant $InsP_3R1$ and W1577A were extracted from Sf9 microsomes prepared as described above in the extraction buffer [1% (v/v) Triton X-100/100 mM NaCl/20 mM Tris (pH 7.5)/0.2 mM $CaCl_2$/2 mM 2-mercaptoethanol/0.1 mg/ml PMSF]. Protein extracts were clarified by centrifugation for 10 min at 100000 ***g*** (TL-100) and adjusted to 0.2% (v/v) Triton X-100, 200 mM NaCl, 20 mM Tris (pH 7.4), 1.5 mM $CaCl_2$ and 1 mM 2-mercaptoethanol. The resulting protein lysate (0.15 ml) was mixed with 0.3 ml of CaM–Sepharose 4B beads (Amersham–Pharmacia Biotech, Piscataway, NJ, U.S.A.) and incubated for 4 h at 4 °C with gentle stirring. The beads were loaded on to a PD-10 disposable column and washed with 5 bead vols of binding buffer [0.2% (v/v) Triton X-100/200 mM NaCl/20 mM Tris (pH 7.4)/1.5 mM $CaCl_2$/1 mM 2-mercaptoethanol]. The attached proteins were eluted with 4 bead vols of elution buffer [0.2% (v/v) Triton X-100/200 mM NaCl/20 mM Tris (pH 7.4)/2 mM EGTA/1 mM 2-mercaptoethanol]. During the washing and elution stages, 0.4 ml fractions were collected manually and were analysed by SDS/PAGE and Western blotting with anti-$InsP_3R1$-specific antibodies (T_{443}).

Ca^{2+} imaging in DT40 cells

DT40 chicken B lymphoma cells were cultured in RPMI1640 supplemented with 10% fetal calf serum, 1% chicken serum, 100 units/ml penicillin, 100 units/ml streptomycin and 2 mM glutamine. Mutant DT40 cells with all three of their $InsP_3R$ genes disrupted [20] were transfected with the linearized rat $InsP_3R1$–pcDNA3 plasmid (with or without the W1577A mutation) by electroporation (330 V; 250 μF). Several stably expressing clones were isolated in the presence of 2 mg/ml of G418 (Geneticin; Gibco BRL). Ca^{2+} imaging of RT1 and W1577A transfected cells was performed as described previously [11,28]. Briefly, cells on poly-L-lysine and collagen-coated coverslips were loaded with 1 μM fura-2 acetoxymethyl ester in a physiological salt solution [150 mM NaCl/4 mM KCl/2 mM $CaCl_2$/1 mM $MgCl_2$/5 mM Hepes/5.6 mM of glucose (pH 7.4)]. After loading the indicator, cells were stimulated with ligation of the BCR with anti-BCR antibody, which resulted in the generation of $InsP_3$ through tyrosine phosphorylation of phospholipase Cγ. The fluorescence images were captured at room temperature (22–24 °C) with an Olympus IX70 inverted microscope, equipped with a cooled CCD camera (Photometrics, Tucson, AZ, U.S.A.) and a polychromatic illumination system (T.I.L.L. Photonics; Germany), at a rate of one pair of frames with excitation at 345

and 380 nm every 10, 1 or 0.25 s. Intracellular Ca^{2+} concentrations of the fura-2 acetoxymethyl ester-loaded cells were calculated using the equation reported previously [29]. The mutant DT40 cells did not show any response to BCR stimulation before transfection with either wild-type or mutant $InsP_3R1$ [20].

RESULTS

The W1577A mutation of rat $InsP_3R1$ eliminated the Ca^{2+}-dependent association with CaM

To study the role of high-affinity Ca^{2+}–CaM-binding site in the $InsP_3R1$ sequence, we generated baculoviruses encoding wild-type rat $InsP_3R1$ (RT1) and W1577A mutant of rat $InsP_3R1$ (W1577A) as described in Experimental section. Microsomes isolated from RT1 or W1577A-infected Sf9 cells, but not from non-infected cells, contained large quantities of $InsP_3R1$ detectable by Western blotting with T_{443} anti-$InsP_3R1$ polyclonal antibodies (Figure 1A). Small amounts of endogenous $InsP_3R1$ were detected in microsomes from non-infected Sf9 cells when the blots were overexposed (results not shown). Our results agree with the previously reported high-level expression of $InsP_3R1$ in Sf9 cells using the baculovirus-expression system [14,30]. The apparent molecular mass of recombinant $InsP_3R1$ and W1577A mutant was identical to the mass of the $InsP_3R1$ present in rat cerebellar microsomes (Figure 1A). The immunopositive bands of smaller molecular mass detected in samples of rat cerebellar microsomes and microsomes from RT1 or W1577A-infected Sf9 cells most likely correspond to the products of partial $InsP_3R1$ proteolysis. Indeed, if protease inhibitors were omitted from the homogenization buffer, the bands of smaller molecular mass became more pronounced when compared with the full-length band on the anti-$InsP_3R1$ Western blot (results not shown). Similar amount of degradation was observed for native rat cerebellar $InsP_3R$, wild-type recombinant rat $InsP_3R1$ and W1577A mutant (Figure 1A).

Yamada et al. [13] reported that the W1576A mutation in mouse $InsP_3R1$ eliminates the Ca^{2+}-dependent association of $InsP_3R1$ with CaM–Sepharose. The W1577A mutation in rat $InsP_3R1$ was designed to mimic the W1576A mutation in mouse $InsP_3R1$. Is the Ca^{2+}–CaM-binding site eliminated by W1577A mutation in rat $InsP_3R1$? To address this question, we repeated CaM–Sepharose-binding experiments of Yamada et al. with the wild-type rat $InsP_3R1$ and W1577A mutant expressed in Sf9 cells by baculovirus infection. In these experiments, the microsomes isolated from RT1 or W1577A-infected Sf9 cells were solubilized in Triton and incubated with CaM–Sepharose beads in the binding buffer containing 1.5 mM Ca^{2+}. Following incubation, the beads were loaded on to a column and washed using the binding buffer. The bound proteins were eluted from the CaM–Sepharose column by the Ca^{2+}-free elution buffer containing 2 mM EGTA. The samples collected in the output of the column during the washing and elution stages were analysed by Western blotting with T_{443} anti-$InsP_3R1$ antibodies. In agreement with Yamada et al. [13], we observed Ca^{2+}-dependent retention of $InsP_3R1$ on the CaM–Sepharose column (Figure 1B,

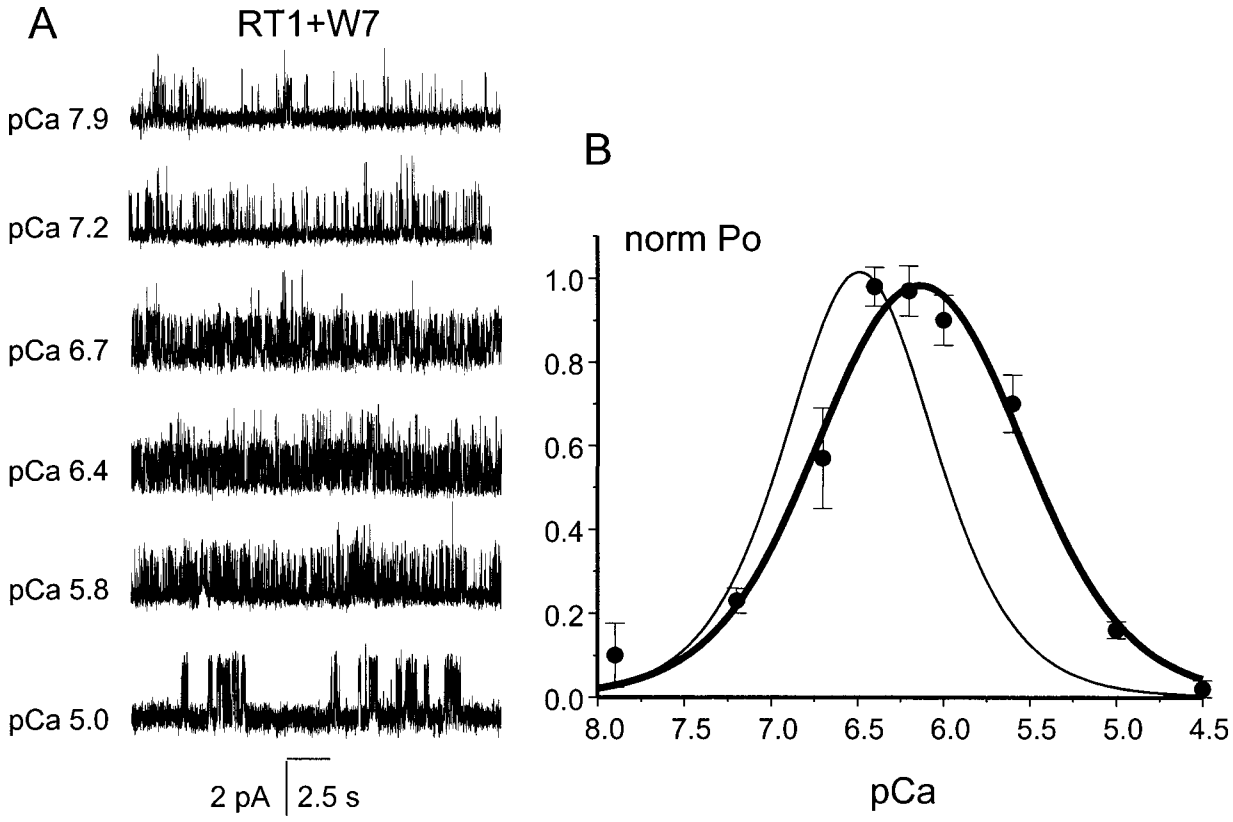

Figure 3 Effect of W-7 on $InsP_3R1$ modulation by cytosolic Ca^{2+}

(**A**) The unitary single-channel recordings of recombinant $InsP_3R1$ channels in the presence of W-7 (0.5 mM) at different Ca^{2+} concentration as indicated. (**B**) Bell-shaped Ca^{2+} dependence of recombinant $InsP_3R1$ channels in the presence of 0.5 mM W-7 (●). The channel P_o at each Ca^{2+} concentration was normalized to maximal P_o in the same experiment, averaged as described in Figure 2(B) and shown as means ± S.E.M. ($n = 3$). The maximal $P_o = 32 \pm 5\%$ ($n = 4$). The thin smooth line represents Ca^{2+} dependence of the wild-type $InsP_3R1$ from Figure 2(B).

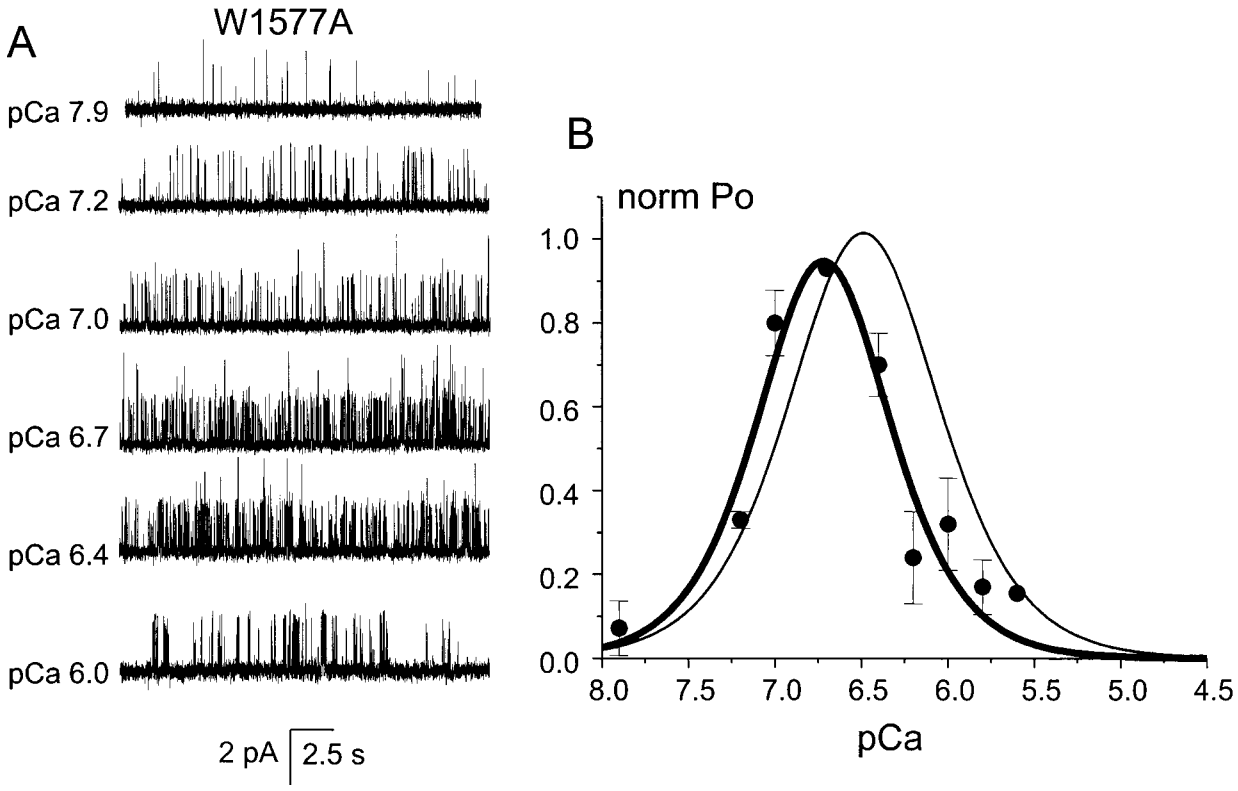

Figure 4 Modulation of the $InsP_3R1$ W1577A mutant by cytosolic Ca^{2+}

(**A**) The unitary single-channel recordings of W1577A mutant at different Ca^{2+} concentrations are indicated. (**B**) Bell-shaped Ca^{2+} dependence of recombinant W1577A mutant (●). The channel P_o at each Ca^{2+} concentration was normalized to maximal P_o in the same experiment, averaged as described for Figure 2(B) and shown as means ± S.E.M. ($n = 3$). The maximal $P_o = 12 \pm 2\%$ ($n = 4$). The thin smooth line represents the Ca^{2+} dependence of the wild-type $InsP_3R1$ from Figure 2(B).

top row). In contrast with the wild-type $InsP_3R1$, the W1577A mutant was not retained on the column (Figure 1B, bottom row). From these results we concluded that a unique high-affinity Ca^{2+}–CaM-binding site present in rat $InsP_3R1$ is indeed eliminated in the W1577A mutant.

Modulation of recombinant $InsP_3R1$ by cytosolic Ca^{2+}

We previously established that $InsP_3R1$ expressed in Sf9 cells form functional $InsP_3$-gated channels upon reconstitution in planar lipid bilayers [31]. Feedback regulation of $InsP_3R1$ by cytosolic Ca^{2+} is one of the most fundamental properties of $InsP_3R1$ responsible for complex spatio-temporal aspects of Ca^{2+} signalling [1]. In the next series of experiments, we evaluated the modulation of recombinant rat $InsP_3R1$ by cytosolic Ca^{2+}. In agreement with the behaviour of native cerebellar $InsP_3R$ [5] and recombinant rat $InsP_3R1$ expressed in HEK293 and COS cells [22,32], rat $InsP_3R1$ expressed in Sf9 cells displays the bell-shaped Ca^{2+} dependence with the maximal P_o at 300 nM Ca^{2+}, half-maximal activation at 100 nM Ca^{2+} and half-inhibition at 1 μM Ca^{2+} (Figures 2A and 2B).

Role of CaM in the $InsP_3R1$ modulation by cytosolic Ca^{2+}

Association of Ca^{2+} and CaM with the $InsP_3R1$ has been proposed to be critical for the Ca^{2+}-dependent inactivation of cerebellar $InsP_3R1$ [12]. In support of this hypothesis, application of the CaM inhibitor W-7 at a concentration of 0.5 mM has been reported to activate cerebellar $InsP_3R1$ [12]. We tested submillimolar concentrations of W-7 and observed an increase in the maximal single-channel P_o of recombinant $InsP_3R1$ from $17 \pm 5\%$ ($n = 4$) in control experiments to $32 \pm 5\%$ ($n = 4$) in the presence of 0.5 mM W-7. These results were in agreement with those of Michikawa et al. [12]. However, the effect of W-7 is unlikely to be related to its inhibition of CaM. First, the 0.5 mM concentration of W-7 required to see the potentiating effect on $InsP_3R1$ activity in our experiments and in experiments of Michikawa et al. [12] is at least 10 times greater than its apparent affinity for CaM observed in other systems [33,34]. Smaller concentrations of W-7 or compound 48/80 had no significant effect on cerebellar $InsP_3R1$ activity in experiments of Michikawa et al. and in our experiments (results not shown; Bezprozvanny, I. and Ehrlich, B. E., unpublished work). Secondly, the potentiating effect of 0.5 mM W-7 on $InsP_3R1$ activity was uniform over the tested range of Ca^{2+} concentrations. Indeed, when P_o values were normalized to the maximal P_o observed in the same experiment, it became apparent that 0.5 mM of W-7 caused only a minor shift in the Ca^{2+} dependence of rat cerebellar $InsP_3R1$ ($n = 3$, results not shown) or recombinant rat $InsP_3R1$ expressed in Sf9 cells (Figure 3, $n = 3$). For recombinant $InsP_3R1$ the peak of the normalized open probability curve was at 300 nM Ca^{2+} in control experiments (Figures 2B and 3B, thin line) and at 1 μM Ca^{2+} in the presence of 0.5 mM W-7 (Figure 3B, thick line).

To further investigate the role of CaM in the $InsP_3R1$ modulation by Ca^{2+}, we analysed the Ca^{2+} dependence of W1577A mutant, which lacks the high-affinity Ca^{2+}–CaM-binding site (Figure 1B). When the W1577A mutant of $InsP_3R1$

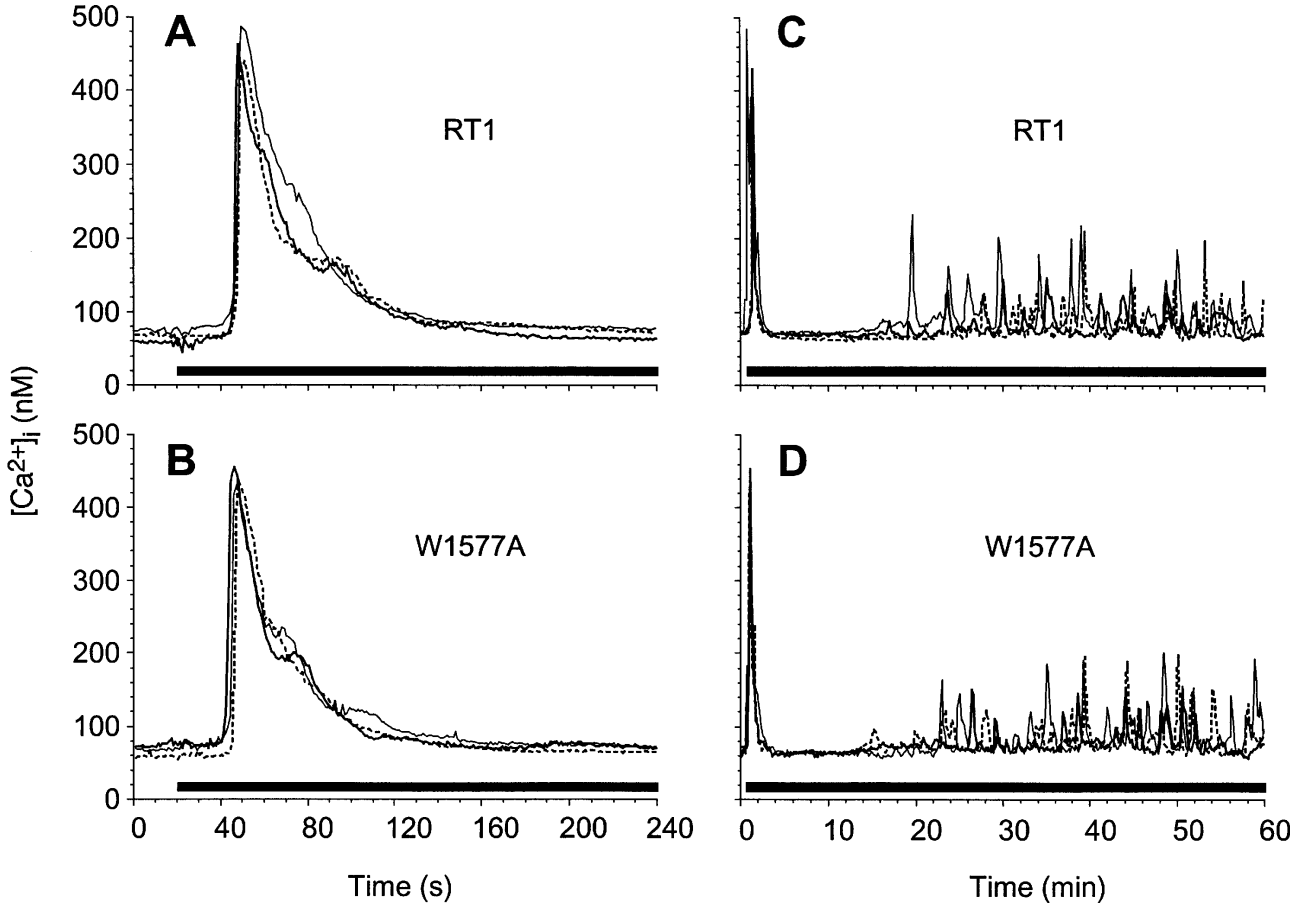

Figure 5 Ca^{2+} signals in DT40 cells expressing $InsP_3R1$ (A, C) and W1577A mutant (B, D) in response to BCR stimulation

Cells were stimulated with ligation of BCR with anti-BCR antibody (1 μg/ml) in physiological salt solution. The data are shown for 4 min (**A**, **B**) and 60 min (**C**, **D**). Data for three independent cells, represented by thin, thick and dotted traces, are shown in each panel.

was expressed in Sf9 cells using the baculovirus-expression system and incorporated in planar lipid bilayers, the $InsP_3$-gated channels were recorded (Figure 4A). The gating and conductance properties of W1577A channels were similar to the wild-type $InsP_3R1$ (results not shown). Similar to wild-type $InsP_3R1$ (Figures 2B and 4B, thin line), the W1577A mutant displayed the bell-shaped Ca^{2+} dependence on cytosolic Ca^{2+} with the peak at 200 nM Ca^{2+} (Figure 4B, thick line).

It is possible that CaM associated with the wild-type $InsP_3R1$ in cells is lost during the planar lipid bilayer reconstitution procedure. To test this hypothesis, we compared Ca^{2+} signals induced by BCR stimulation in DT40 cells lacking endogenous $InsP_3R$ [20] and stably transfected with the wild-type $InsP_3R1$ or with the W1577A mutant expression plasmids. Because Ca^{2+} dependence of the $InsP_3R$ underlies spatio-temporal aspects of Ca^{2+} signalling in cells, we reasoned that the differences in Ca^{2+} modulation of $InsP_3R$ should result in different patterns of observed Ca^{2+} signals. Indeed, DT40 cells expressing mutant $InsP_3R1$ with a decreased Ca^{2+} sensitivity showed significant alterations in the BCR-stimulated Ca^{2+}-signalling patterns, i.e. reduced rate of rise and loss of Ca^{2+} oscillations [11]. However, the temporal pattern of Ca^{2+} signals in DT40 cells expressing the wild-type $InsP_3R1$ and the W1577A mutant was indistinguishable (Figures 5A–5D, $n = 3$ for $InsP_3R1$ and W1577A), arguing against the importance of CaM association with the high-affinity Ca^{2+}–CaM-binding site in the coupling domain of $InsP_3R1$ ([13]; Figure 1B) for $InsP_3R1$ modulation by Ca^{2+} in intact cells.

The $InsP_3R1$ inhibition by CaM

Our results (Figures 2–5) lead us to conclude that the high-affinity Ca^{2+}–CaM-binding site does not play a role in biphasic modulation of $InsP_3R1$ by cytosolic Ca^{2+}. A number of reports [12,15–19] suggest a direct inhibitory influence of CaM on $InsP_3R1$ activity. What is the role of high-affinity Ca^{2+}–CaM-binding site in $InsP_3R1$ inhibition by CaM? To answer this question, we compared the sensitivity of the wild-type $InsP_3R1$ and W1577A mutant with an application of exogenous CaM to planar lipid bilayers. In agreement with the previous reports [12,15–19], the $InsP_3R1$ activity was reduced to $69 \pm 18\,\%$ ($n = 3$) of control by application of 12 μM of CaM (Figure 6, ○). It is highly possible that the relatively modest inhibitory effects of CaM observed in our experiments are related to the high $InsP_3$ concentration (2 μM) and low Ca^{2+} concentration (300 nM) used in recordings of the $InsP_3R1$ activity. The sensitivity to inhibition by CaM was unchanged in W1577A mutant; on an average, the activity of W1577A channels was decreased to $69 \pm 23\,\%$ ($n = 3$) of control by application of 12 μM CaM (Figure 6, ●). Thus we conclude

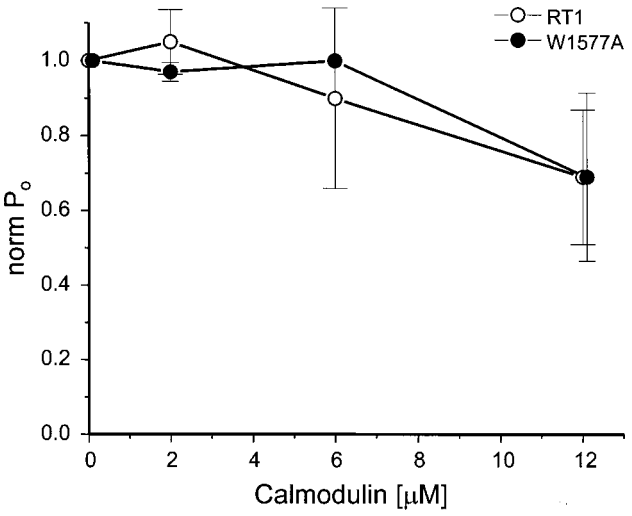

Figure 6 Functional effect of CaM on $InsP_3R1$ and W1577A mutant

The channel activity was determined in the presence of increasing concentrations of CaM as indicated. The P_o at each CaM concentration was normalized to P_o in the absence of CaM in the same experiment. The normalized P_o values from different experiments were averaged together at each CaM concentration and plotted as means ± S.E.M. ($n = 3$) for wild-type $InsP_3R1$ (○) and W1577A (●). In all experiments, channel activity was recorded in the presence of 2 μM $InsP_3$, 300 nM Ca^{2+} and 0.5 mM ATP. In this series of experiments, P_o in the absence of CaM was 3.6 ± 0.1 % ($n = 3$) for the wild-type $InsP_3R1$ and 10.3 ± 0.2 % ($n = 3$) for W1577A.

that the high-affinity Ca^{2+}–CaM-binding site in the $InsP_3R1$ sequence does not play an important role in the modulation of $InsP_3R1$ activity by CaM [12,15–19].

DISCUSSION

Using the baculovirus-expression system we analysed the role of the high-affinity Ca^{2+}–CaM-binding site in the regulation of $InsP_3R1$ activity by Ca^{2+} and CaM in planar lipid bilayers. We found that the recombinant wild-type $InsP_3R1$ expressed in Sf9 cells display a bell-shaped Ca^{2+} dependence with the peak at 300 nM, similar to the Ca^{2+} dependence of native cerebellar $InsP_3R$ [5] and of recombinant $InsP_3R1$ expressed in HEK293 cells [22]. CaM has been strongly implicated in Ca^{2+}-dependent inhibition of cerebellar $InsP_3R$ [12]. Observations of Michikawa et al. (1999) showed that (1) purified cerebellar $InsP_3R$ lacked Ca^{2+} inhibition, but addition of CaM to the purified $InsP_3R$ restored the inhibition, (2) addition of CaM antagonist W-7 at 0.5 mM or CaM-binding peptide activated microsomal cerebellar $InsP_3R$. In the work by Ramos-Franco et al. [32,35] it was shown that solubilized and purified cerebellar $InsP_3R$ and recombinant $InsP_3R1$ display the bell-shaped Ca^{2+} dependence without addition of exogenous CaM. The reason for discrepancy between the results obtained by Michikawa et al. [12] and Ramos-Franco et al. [32,35] is not clear. Similar to Michikawa et al. [12], we found that 12 μM of CaM decreased the activity of $InsP_3R1$ in planar lipid bilayers (Figure 6). However, the inhibitory effect of CaM was evident with $InsP_3R1$ incorporated to bilayers by microsomal vesicle fusion, and not only with solubilized and purified $InsP_3R$ as implied by Michikawa et al. [12]. In the present study, we also observed a 2-fold activation of $InsP_3R1$ by 0.5 mM W-7, but the effect was uniform over the range of Ca^{2+} concentrations tested and resulted only in a minor shift in Ca^{2+} dependence of normalized P_o of rat cerebellar $InsP_3R$ (results not shown) or recombinant $InsP_3R1$ (Figure 3). The 0.5 mM concentration of W-7 required to observe the potentiating effect on $InsP_3R1$ activity [12] is at least 10 times greater than its apparent affinity for CaM observed in other systems [33,34]. Thus the potentiating effect of W-7 on $InsP_3R1$ activity is unlikely to be caused by specific inhibition of the CaM function.

To investigate further the role of CaM in $InsP_3R1$ modulation by Ca^{2+} and CaM, we generated the W1577A mutant of rat $InsP_3R1$, which was designed to mimic the equivalent W1576A mutation in mouse $InsP_3R1$ [13]. In agreement with the data of Yamada et al. [13], we found that the W1577A mutant of rat $InsP_3R1$ lacks the high-affinity Ca^{2+}–CaM-binding site (Figure 1B). The W1577A mutant displayed the bell-shaped Ca^{2+}dependence in bilayers (Figure 4) and supported the Ca^{2+} signalling pattern in DT40 cells (Figure 5) indistinguishable from the ones characteristic of the wild-type $InsP_3R1$. The sensitivity of the W1577A mutant to inhibition by CaM was similar to the sensitivity of the wild-type $InsP_3R1$ (Figure 6). From these results, we conclude that under our assay conditions the Ca^{2+}–CaM-binding site in the $InsP_3R1$ coupling domain [13] does not play a direct role in biphasic modulation of $InsP_3R1$ by Ca^{2+} [5–7] or in the inhibition of $InsP_3R1$ by CaM [15–19].

The high-affinity Ca^{2+}–CaM-binding site investigated in the present study is conserved in $InsP_3R1$ and $InsP_3R2$ isoforms, but is absent in the $InsP_3R3$ isoform [13]. From lipid-bilayer recordings it has been initially suggested that $InsP_3R3$ is not inhibited by Ca^{2+} [36]. However, using Ca^{2+}-flux measurements [37–39] and *Xenopus* nuclear envelope patch-clamp recordings [40] it was later demonstrated that $InsP_3R3$ are biphasically regulated by Ca^{2+}. When effects of exogenous CaM were tested in permeabilized cells, it was concluded that $InsP_3R3$ and $InsP_3R1$ display similar sensitivity to inhibition by CaM [17,41]. Thus our conclusion that the $InsP_3R1$- and $InsP_3R2$-specific Ca^{2+}–CaM-binding sites do not play a direct role in $InsP_3R1$ modulation by Ca^{2+} and CaM agrees with the previous functional comparison of $InsP_3R1$ and $InsP_3R3$ isoforms.

The $InsP_3R1$ mRNA undergoes an alternative splicing in the SII site located in the middle-coupling domain [2]. The SII(+) (neuronal) isoform of $InsP_3R1$ was used in our study. Biochemical data indicate that an additional CaM-binding site is created in the SII(−) isoform of $InsP_3R1$ [42,43]. Thus it is possible that the SII(−) isoform of $InsP_3R1$, expressed outside of the nervous system [44], is more sensitive to modulation by CaM. Indeed, the Ca^{2+}–CaM-dependent inhibition of $InsP_3$-induced Ca^{2+} release was observed in A7r5 cells [19], which primarily express the $InsP_3R1$-SII(−) isoform. In recent planar lipid bilayer experiments [31] we discovered that both $InsP_3R1$-SII(+) and $InsP_3R1$-SII(−) isoforms expressed in Sf9 cells displayed the bell-shaped Ca^{2+} dependence that peaked at 300 nM Ca^{2+} but had different width. The role played by SII(−) splice variant-specific CaM-binding site in the observed differences is unknown.

An additional low-affinity CaM-binding site, which may potentially play a role in $InsP_3R1$ modulation, has been identified in the ligand-binding domain of $InsP_3R1$ [14,15,45]. This site is a Ca^{2+}-independent CaM-interaction site, which decreases the $InsP_3R$ affinity for $InsP_3$. This site is likely to be responsible for CaM-dependent inhibition of $InsP_3R$ function (Figure 6; [15–19]). It is also conceivable that such an interaction could indirectly modulate Ca^{2+} regulation, since it was shown that the sensitivity of cerebellar [46] and *Xenopus* [47] $InsP_3R1$ to Ca^{2+} inhibition is inversely related to the $InsP_3$ concentration.

In summary, the high-affinity Ca^{2+}–CaM-binding site in the coupling region of $InsP_3R1$-SII(+) isoform [13] does not play a

direct role in the biphasic modulation of $InsP_3R1$ by Ca^{2+} [5–7] or in $InsP_3R1$ inhibition by CaM [15–19]. The putative 'inhibitory Ca^{2+}-binding site' responsible for the negative limb of the 'bell-shaped' curve may be a part of the $InsP_3R1$ or it may be provided by another, yet unidentified, accessory protein. The CaM-dependent inhibition of $InsP_3R1$ function is likely to involve the amino terminal-binding site in the $InsP_3R1$ ligand-binding domain [14,15,45]. The functional significance of the high-affinity Ca^{2+}–CaM-binding site in the $InsP_3R1$ coupling domain needs to be clarified in future studies.

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