Ca^{2+} differentially regulates the ligand-affinity states of type 1 and type 3 inositol 1,4,5-trisphosphate receptors

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To elucidate the functional difference between type 1 and type 3 $\text{Ins}(1,4,5)\text{P}_3$ receptors [$\text{Ins}(1,4,5)\text{P}_3\text{R1}$ and $\text{Ins}(1,4,5)\text{P}_3\text{R3}$ respectively] we studied the effect of Ca^{2+} on the ligand-binding properties of both $\text{Ins}(1,4,5)\text{P}_3\text{R}$ types. We expressed full-length human $\text{Ins}(1,4,5)\text{P}_3\text{R1}$ and $\text{Ins}(1,4,5)\text{P}_3\text{R3}$ from cDNA species in insect ovary Sf9 cells, and the membrane fractions were used for $\text{Ins}(1,4,5)\text{P}_3$ -binding assays. The binding of $\text{Ins}(1,4,5)\text{P}_3$ to $\text{Ins}(1,4,5)\text{P}_3\text{R1}$ and $\text{Ins}(1,4,5)\text{P}_3\text{R3}$ was differentially regulated by Ca^{2+} . With increasing concentrations of free Ca^{2+} ($[\text{Ca}^{2+}]$), $\text{Ins}(1,4,5)\text{P}_3$ binding to $\text{Ins}(1,4,5)\text{P}_3\text{R1}$ decreased, whereas that to $\text{Ins}(1,4,5)\text{P}_3\text{R3}$ increased. Alteration of $\text{Ins}(1,4,5)\text{P}_3$ binding to $\text{Ins}(1,4,5)\text{P}_3\text{R1}$ was observed at $[\text{Ca}^{2+}]$ ranging from less than 1 nM to more than 10 μM . The EC_{50} of $\text{Ins}(1,4,5)\text{P}_3$ binding was 100 nM Ca^{2+} for $\text{Ins}(1,4,5)\text{P}_3\text{R1}$. In contrast, $\text{Ins}(1,4,5)\text{P}_3$ binding to $\text{Ins}(1,4,5)\text{P}_3\text{R3}$ was changed at high $[\text{Ca}^{2+}]$ with an EC_{50} value

of 872 nM, and steeply between 100 nM and 10 μM . These Ca^{2+} -dependent alterations of $\text{Ins}(1,4,5)\text{P}_3$ binding to both $\text{Ins}(1,4,5)\text{P}_3\text{R}$ types were reversible. Scatchard analyses revealed that Ca^{2+} changed the affinity of both $\text{Ins}(1,4,5)\text{P}_3\text{R}$ types but not the total number of $\text{Ins}(1,4,5)\text{P}_3$ -binding sites. The K_d values of $\text{Ins}(1,4,5)\text{P}_3\text{R1}$ for $\text{Ins}(1,4,5)\text{P}_3$ were 78.5 nM with 3 nM free Ca^{2+} , and 312 nM with 1.4 μM free Ca^{2+} . In contrast, $\text{Ins}(1,4,5)\text{P}_3\text{R3}$ exhibited an affinity for $\text{Ins}(1,4,5)\text{P}_3$ with K_d values of 116 nM with 3 nM free Ca^{2+} , and 62.2 nM with 1.4 μM free Ca^{2+} . These results indicate that (1) both $\text{Ins}(1,4,5)\text{P}_3\text{R1}$ and $\text{Ins}(1,4,5)\text{P}_3\text{R3}$ have at least two affinity states, (2) Ca^{2+} regulates interconversions between these states, and (3) Ca^{2+} regulates the binding of $\text{Ins}(1,4,5)\text{P}_3$ to $\text{Ins}(1,4,5)\text{P}_3\text{R1}$ and $\text{Ins}(1,4,5)\text{P}_3\text{R3}$ in opposite manners.

INTRODUCTION

The second messenger $\text{Ins}(1,4,5)\text{P}_3$ plays one of the major roles in intracellular Ca^{2+} signalling. $\text{Ins}(1,4,5)\text{P}_3$ is generated by phosphoinositide hydrolysis by phospholipase C after many extracellular stimuli [1] and liberates Ca^{2+} from intracellular Ca^{2+} stores by binding to a specific receptor/ Ca^{2+} -releasing channel, $\text{Ins}(1,4,5)\text{P}_3$ receptor [$\text{Ins}(1,4,5)\text{P}_3\text{R}$] [2–5]. Structural and functional analyses revealed that the structure of $\text{Ins}(1,4,5)\text{P}_3\text{R}$ can be divided into three functionally different domains: an N-terminal ligand-binding domain, a modulatory domain and a channel domain near the C-terminus [6,7]. Molecular cloning studies showed that there are at least three types of $\text{Ins}(1,4,5)\text{P}_3\text{R}$ derived from distinct genes: type 1 [$\text{Ins}(1,4,5)\text{P}_3\text{R1}$] [8–11], type 2 [$\text{Ins}(1,4,5)\text{P}_3\text{R2}$] [12–14] and type 3 [$\text{Ins}(1,4,5)\text{P}_3\text{R3}$] [14–16]. These three types share both 60–70% identity in amino acid sequences and differential distributions of putative sites of modulation: namely calmodulin (CaM) binding, phosphorylations, ATP binding and glycosylations [17]. The three types of $\text{Ins}(1,4,5)\text{P}_3\text{R}$ were shown to be distributed differentially between tissues (T. Monkawa and M. Yamamoto-Hino, unpublished work) [18–20]. The functional differences between the members of the $\text{Ins}(1,4,5)\text{P}_3\text{R}$ family still remain unclear.

Ca^{2+} is a versatile second messenger, which regulates the functions of a vast variety of its target molecules. Even Ca^{2+} -handling molecules (e.g. $\text{Ins}(1,4,5)\text{P}_3\text{R}$, phospholipase C, voltage-dependent Ca^{2+} channel) are regulated by Ca^{2+} . Thus Ca^{2+} forms an intracellular signalling web exquisitely controlled through feedback regulation, and shows complex spatiotemporal dy-

namics [1,21]. $\text{Ins}(1,4,5)\text{P}_3\text{Rs}$ prepared from distinct tissues were reported to be regulated differentially by Ca^{2+} . Ca^{2+} was reported to inhibit, at concentrations of more than 100 nM, the binding of $\text{Ins}(1,4,5)\text{P}_3$ to $\text{Ins}(1,4,5)\text{P}_3\text{Rs}$ from cerebella [22–25], smooth muscle [26] and a promyelocytic leukaemia cell line HL-60 [27], but to promote the binding of $\text{Ins}(1,4,5)\text{P}_3$ to the $\text{Ins}(1,4,5)\text{P}_3\text{Rs}$ from livers [28–30] and a leukaemic basophilic granulocyte line, RBL [31]. Given the differential distribution of $\text{Ins}(1,4,5)\text{P}_3\text{Rs}$, this difference among tissues might be due to differential regulation of distinct $\text{Ins}(1,4,5)\text{P}_3\text{R}$ types by Ca^{2+} . However, many tissues tested so far, except for cerebella, which contain predominantly $\text{Ins}(1,4,5)\text{P}_3\text{R1}$, co-express all the three types (T. Monkawa, unpublished work) [32–34], which together can form a heterotetrameric complex [35,36]. Furthermore the intracellular milieu surrounding the $\text{Ins}(1,4,5)\text{P}_3\text{Rs}$ (e.g. $\text{Ins}(1,4,5)\text{P}_3\text{R}$ -binding proteins, state of phosphorylation) differ among the tissues. Therefore it remains uncertain whether each member of the $\text{Ins}(1,4,5)\text{P}_3\text{R}$ family is differentially regulated by Ca^{2+} .

To elucidate the differences in functional properties between $\text{Ins}(1,4,5)\text{P}_3\text{R1}$ and $\text{Ins}(1,4,5)\text{P}_3\text{R3}$, we expressed full-length human $\text{Ins}(1,4,5)\text{P}_3\text{R}$ species of both types from cDNA in insect ovary Sf9 cells by using a baculovirus expression vector system, then analysed the effect of Ca^{2+} on the binding of $\text{Ins}(1,4,5)\text{P}_3$ to each $\text{Ins}(1,4,5)\text{P}_3\text{R}$ type. In the present study the functional characters of the two types of $\text{Ins}(1,4,5)\text{P}_3\text{R}$ could be compared without any bias caused by other $\text{Ins}(1,4,5)\text{P}_3\text{R}$ types contaminating the preparations or differences in intracellular milieu between the original tissues. By using the full-length proteins we could avoid overlooking any effects of the modulations on the

Abbreviations used: AEBF, 4-(2-aminoethyl)-benzenesulphonyl fluoride; B_{max} , total number of $\text{Ins}(1,4,5)\text{P}_3$ -binding sites; $[\text{Ca}^{2+}]$, concentration of free Ca^{2+} ; CaM, calmodulin; IICR, $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release; $\text{Ins}(1,4,5)\text{P}_3\text{R}$, $\text{Ins}(1,4,5)\text{P}_3$ receptor; $\text{Ins}(1,4,5)\text{P}_3\text{R1}$ (R2, R3), type 1 (2, 3) $\text{Ins}(1,4,5)\text{P}_3\text{R}$; mAb, monoclonal antibody.

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Ins(1,4,5) P_3 Rs, even if the primary target sites of the modulations are not located in the ligand-binding domain. Here we report the differential regulation of Ins(1,4,5) P_3 binding to Ins(1,4,5) P_3 R1 and Ins(1,4,5) P_3 R3 by Ca^{2+} .

EXPERIMENTAL

Expression of Ins(1,4,5) P_3 R types

Overlapping cDNA clones of human Ins(1,4,5) P_3 R1 [10] were used to construct the full-length cDNA. The full-length cDNA species of human Ins(1,4,5) P_3 R1 and Ins(1,4,5) P_3 R3 [14] were subcloned into the baculovirus transfer vectors pVL1392 and pVL1393 (Invitrogen) to create recombinant plasmids pVL-h1 and pVL-h3 respectively. Sf9 cells (kindly provided by Dr. M. Amagai, Keio University, Tokyo, Japan) were cultured in TNM-FH medium supplemented with 10% (v/v) heat-inactivated fetal calf serum. The pVL1392, pVL-h1 and pVL-h3 plasmids were co-transfected into Sf9 cells with linearized baculovirus DNA (BaculoGold, Pharmingen) to create recombinant baculoviruses b-vec, b-h1 and b-h3 respectively. The recombinant viruses were amplified by infecting Sf9 cells.

Preparation of membrane fractions

Sf9 cells infected with amplified recombinant viruses were harvested 2 days after infection by centrifugation at 1000 *g* for 5 min at room temperature. The cell pellets were resuspended in 10 vol. of a buffer containing 0.25 M sucrose, 1 mM EDTA, 1 mM D,L-dithiothreitol, 200 μ M 4-(2-aminoethyl)-benzenesulphonyl fluoride (AEBSF), 1 μ M pepstatin A, 10 μ M leupeptin, 10 μ M E-64 and 5 mM Hepes/KOH, pH 7.4, at 1 °C, and homogenized in a glass-Teflon Potter homogenizer with 10 strokes at 1000 rev./min on ice-water. The homogenates were centrifuged at 1000 *g* for 5 min at 4 °C. The supernatants were centrifuged at 105000 *g* for 60 min at 2 °C to sediment membrane proteins. The pellets were resuspended in 139 mM KCl/12 mM NaCl/2 mM KH_2PO_4 /1 mM D,L-dithiothreitol/200 μ M AEBSF/1 μ M pepstatin A/10 μ M leupeptin/10 μ M E-64/50 mM Hepes/KOH, pH 7.10, at 1 °C (cytosol-like buffer) containing 50 μ M EGTA. The suspensions were recentrifuged at 379000 *g* for 15 min at 2 °C to sediment the membrane proteins. The pellets were resuspended in cytosol-like buffer containing 50 μ M EGTA to give a final protein concentration of 5–10 mg/ml and stored at –80 °C after rapid freezing in liquid nitrogen. Protein concentrations were determined with the Protein Assay kit (Bio-Rad) with BSA as standard. Membrane fraction from human T cell line Jurkat was prepared by the same method as the Sf9 cells.

Western blot analysis

Membrane fractions were mixed with SDS/PAGE sampling buffer to give a final concentration of 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol and 62.5 mM Tris/HCl, pH 6.8, and then boiled for 3 min. The samples were subjected to SDS/PAGE [4.5% (w/v) gel] in the buffer system of Laemmli [37] and transferred electrophoretically to nitrocellulose filters (Hybond-ECL, Amersham). Ins(1,4,5) P_3 R types were probed with the type-specific monoclonal antibodies (mAbs) KM1112 [anti-Ins(1,4,5) P_3 R1], KM1083 [anti-Ins(1,4,5) P_3 R2] and KM1082 [anti-Ins(1,4,5) P_3 R3] [38], and bound antibodies were detected by the enhanced chemiluminescence Western blotting system (Amersham).

Ins(1,4,5) P_3 binding assay

Membrane fractions (0.5 mg/ml protein) in the cytosol-like buffer containing specified concentrations of free Ca^{2+} and Ca^{2+} -free EGTA were incubated with 4.8 or 9.6 nM [3H]Ins(1,4,5) P_3 (New England Nuclear) and either various concentrations of unlabelled Ins(1,4,5) P_3 (Dojindo) or other unlabelled inositol phosphates at 10 μ M (Calbiochem or Dojindo) for 10 min on ice. In some experiments for Scatchard analyses we have included points where we added only [3H]InsP3 to make the InsP3 concentration 1.2, 2.4, 4.8 or 9.6 nM. We found no higher-affinity component with these analyses. The total reaction volume was 100 or 200 μ l. After incubation the samples were centrifuged at 19000 *g* for 5 min at 1 °C and the supernatants were immediately removed by aspiration. The pellets were solubilized and the radioactivity associated with the solubilized pellets was measured in scintillation cocktail with a liquid-scintillation counter. Non-specific binding was determined in the presence of 20 μ M unlabelled Ins(1,4,5) P_3 .

Calcium buffering

The concentrations of free Ca^{2+} ($[Ca^{2+}]$), Ca^{2+} -free EGTA and total EGTA in the cytosol-like buffer were controlled by adding specified amounts of K_2H_2EGTA and/or $K_2CaEGTA$ [39]. The stock solutions of K_2H_2EGTA and $K_2CaEGTA$ were prepared from the same batch of dried H_4EGTA powder to minimize errors in estimation of $[Ca^{2+}]$ in the buffers. H_4EGTA powder (Dojindo) was baked at 150 °C for 5 h just before preparation of the stock solutions to eliminate impurities in the H_4EGTA powder [40]. The stock solution of $K_2CaEGTA$ was prepared by pH metric titration of Ca^{2+} against EGTA [39]. The apparent dissociation constant of EGTA for Ca^{2+} in the cytosol-like buffer (168 mM ionic strength, pH 7.10 at 1 °C) was calculated to be 379 nM by the method of Bers et al. [41] on the basis of the association constants and their ΔH values of EGTA for Ca^{2+} and H^+ [42,43]. The concentration of total calcium contaminating cytosol-like buffer was determined to be 5 μ M by using 2 μ M fura 2. The standards were made by using the calcium-buffering system described above. The standards contained 2 mM Ca^{2+} -free EGTA to make the contaminating calcium negligible (at an order of micromolar concentrations). We measured $[Ca^{2+}]$ in cytosol-like buffer containing 50 μ M total EGTA and then calculated the total Ca^{2+} concentration: $[Ca^{2+}]$ in cytosol-like buffer containing 500 μ M total EGTA was calculated to be 3 nM, considering the contamination.

To examine the reversibility of the effect of free Ca^{2+} on Ins(1,4,5) P_3 binding to Ins(1,4,5) P_3 R types, $[Ca^{2+}]$ in cytosol-like buffer was changed to specified values by alternately adding $K_2CaEGTA$ and K_2H_2EGTA . The Ca^{2+} -free EGTA and total EGTA concentrations were within the range indicated in Figure 3 even after final adjustment of $[Ca^{2+}]$ by the addition of $K_2CaEGTA$ or K_2H_2EGTA . An aliquot containing the same amount of membrane fraction was taken after each addition of $K_2CaEGTA$ or K_2H_2EGTA , and the specific binding of Ins(1,4,5) P_3 was measured.

RESULTS AND DISCUSSION

Functional expression of full-length Ins(1,4,5) P_3 R1 and Ins(1,4,5) P_3 R3

Western blot analysis with the Ins(1,4,5) P_3 R-type-specific mAbs [38] indicated that the Sf9 cells infected with the recombinant baculoviruses bearing the cDNA species of full-length human Ins(1,4,5) P_3 R1 (b-h1) or Ins(1,4,5) P_3 R3 (b-h3) expressed ex-

Figure 1 Expression of human Ins(1,4,5) P_3 R1 and Ins(1,4,5) P_3 R3 in Sf9 cells

Membrane fractions from human T-cell leukaemia Jurkat cells (10 μ g per lane; Jurkat), insect ovary Sf9 cells (10 μ g per lane; Sf9), Sf9 infected with b-vec, a recombinant baculovirus bearing no cDNA (10 μ g per lane; vec/Sf9), Sf9 infected with b-h1, a recombinant baculovirus bearing the cDNA of full-length human Ins(1,4,5) P_3 R1 (10 μ g per lane; IP₃R1/Sf9), and Sf9 infected with b-h3, a recombinant baculovirus bearing the cDNA species of full-length human Ins(1,4,5) P_3 R3 (10 μ g per lane; IP₃R3/Sf9) were analysed by Western blot with mAb KM1112 (anti-Ins(1,4,5) P_3 R1; IP₃R1), mAb KM1083 (anti-Ins(1,4,5) P_3 R2; IP₃R2) and mAb KM1082 (anti-Ins(1,4,5) P_3 R3; IP₃R3). The positions of molecular mass markers (in kDa) are indicated at left and right.

Table 1 Ligand specificity of expressed Ins(1,4,5) P_3 R1 and Ins(1,4,5) P_3 R3

Membrane fractions (50 μ g) from Sf9 expressing Ins(1,4,5) P_3 R1 and Ins(1,4,5) P_3 R3 were incubated with 9.6 nM [3 H]Ins(1,4,5) P_3 and various unlabelled inositol phosphates (10 μ M each) in cytosol-like buffer containing 50 μ M total EGTA. The specific binding of [3 H]Ins(1,4,5) P_3 to the membrane fractions was determined as described in the Experimental section. Binding in the absence of any competitors (buffer) was defined as 100%, and binding in the presence of 10 μ M unlabelled Ins(1,4,5) P_3 was defined as 0%. Results are means $\pm$ S.E.M. for three experiments.

Competitor	Specific [3 H]Ins(1,4,5) P_3 binding (%)	
	Ins(1,4,5) P_3 R1	Ins(1,4,5) P_3 R3
Buffer	100 $\pm$ 2	100 $\pm$ 3
Ins(1,4) P_2	100 $\pm$ 1	95.2 $\pm$ 1.0
Ins(4,5) P_2	71.5 $\pm$ 1.3	71.4 $\pm$ 0.1
Ins(1,4,5) P_3	0.00 $\pm$ 0.821	0.00 $\pm$ 0.10
Ins(2,4,5) P_3	8.63 $\pm$ 1.36	23.9 $\pm$ 0.5
Ins(1,3,4,5) P_4	56.5 $\pm$ 3.7	68.5 $\pm$ 3.4
Ins(1,3,4,5,6) P_5	85.5 $\pm$ 2.1	86.4 $\pm$ 3.3
Ins(1,2,3,4,5,6) P_6	81.2 $\pm$ 4.6	71.9 $\pm$ 3.0

clusively the cDNA-derived Ins(1,4,5) P_3 R type (Figure 1). Human T-cell line Jurkat cells, as a positive control, expressed all three Ins(1,4,5) P_3 R types. There was no detectable protein with these mAbs either in the membrane fractions from uninfected Sf9 cells or in those from Sf9 cells infected with recombinant virus bearing no cDNA (b-vec). The formation of a tetrameric complex of the expressed Ins(1,4,5) P_3 R types as in cerebellum [5] was also confirmed by Western blot analysis after cross-linking (C. Fujiwara-Hirashima, unpublished work).

The Ins(1,4,5) P_3 binding assay with [3 H]Ins(1,4,5) P_3 indicated that the membrane fractions from Sf9 cells infected with either b-h1 or b-h3 had high specific Ins(1,4,5) P_3 binding, whereas the

Figure 2 Effect of $[Ca^{2+}]$ on [3 H]Ins(1,4,5) P_3 binding to Ins(1,4,5) P_3 R1 and Ins(1,4,5) P_3 R3

Membrane fractions from Sf9 expressing Ins(1,4,5) P_3 R1 (100 μ g per point, $\bullet$) and Ins(1,4,5) P_3 R3 (50 μ g per point, $\blacktriangle$) were incubated with 4.8 nM [3 H]Ins(1,4,5) P_3 in cytosol-like buffer containing 500 μ M Ca^{2+} -free EGTA and various concentrations of free Ca^{2+} . In a series of experiments, membrane fractions from Sf9 expressing Ins(1,4,5) P_3 R1 (100 μ g per point, $\blacklozenge$) were incubated with 4.8 nM [3 H]Ins(1,4,5) P_3 in cytosol-like buffer containing 20 mM, 5 mM, 1 mM, 250 μ M, 50 μ M, 200 μ M and 50 μ M total EGTA (consecutively) to give various concentrations of free Ca^{2+} . The specific binding of [3 H]Ins(1,4,5) P_3 to membrane fractions was determined as described in the Experimental section. The specific bindings at 3 nM and 7.3 μ M $[Ca^{2+}]$ were defined as 100% for Ins(1,4,5) P_3 R1 and Ins(1,4,5) P_3 R3 respectively. Results are the means $\pm$ S.E.M. for three determinations.

membrane fraction from b-vec-infected Sf9 cells had no significant specific binding (results not shown).

We next analysed the ligand specificity of the expressed Ins(1,4,5) P_3 R types. Various inositol phosphates at a concentration of 10 μ M competed with the 9.6 nM [3 H]Ins(1,4,5) P_3 for binding sites on the membrane fractions from the infected cells. The order of potency of competition with Ins(1,4,5) P_3 by various inositol phosphates was Ins(1,4,5) P_3 > Ins(2,4,5) P_3 > Ins(1,3,4,5) P_4 > Ins(4,5) P_2 > Ins(1,2,3,4,5,6) P_6 > Ins(1,3,4,5,6) P_5 > Ins(1,4) P_2 for the membrane fractions from both the b-h1- and b-h3-infected cells (Table 1), in good agreement with that of Ins(1,4,5) P_3 R types prepared from native tissues [2,22,44–46] and from other cDNA-derived Ins(1,4,5) P_3 R expression systems [4,12,14,16,32].

Effects of free Ca^{2+} on apparent binding of Ins(1,4,5) P_3 to Ins(1,4,5) P_3 R types

To elucidate whether each type of Ins(1,4,5) P_3 R is differentially regulated by Ca^{2+} , the apparent binding of Ins(1,4,5) P_3 to the expressed Ins(1,4,5) P_3 R types was measured at various $[Ca^{2+}]$ values ranging from 100 pM to 100 μ M (Figure 2). $[Ca^{2+}]$ was controlled by adding increasing amounts of $K_2CaEGTA$ to the buffers maintaining the Ca^{2+} -free EGTA concentration at 500 μ M (see the Experimental section). In a series of experiments (represented by $\blacklozenge$ in Figure 2), Ins(1,4,5) P_3 binding to Ins(1,4,5) P_3 R1 assayed with 20 mM, 5 mM, 1 mM, 250 μ M, 50 μ M, 200 μ M or 50 μ M total EGTA to give various $[Ca^{2+}]$ values. In all experiments, total EGTA concentrations were within the range indicated in Figure 3 (see below). Surprisingly, Ins(1,4,5) P_3 binding to Ins(1,4,5) P_3 R1 and Ins(1,4,5) P_3 R3 was regulated reciprocally by $[Ca^{2+}]$. As a function of $[Ca^{2+}]$, Ins(1,4,5) P_3 binding to Ins(1,4,5) P_3 R1 decreased monotonically, whereas that to Ins(1,4,5) P_3 R3 increased monotonically. A sigmoid curve could be fitted to each plot. Alternation of Ins(1,4,5) P_3 binding to

Figure 3 Effect of total EGTA concentration on [^3H]Ins(1,4,5) P_3 binding to Ins(1,4,5) P_3 R1 and Ins(1,4,5) P_3 R3

Membrane fractions from Sf9 expressing Ins(1,4,5) P_3 R1 [100 μg per point ($\bullet$)] from the same data as indicated in Figure 2 as the left five points of ($\blacklozenge$) and Sf9 expressing Ins(1,4,5) P_3 R3 (50 μg per point, ($\blacktriangle$)) were incubated with 4.8 nM [^3H]Ins(1,4,5) P_3 in cytosol-like buffer containing various concentrations of total EGTA. The Ca^{2+} -free EGTA concentrations were almost equal to the total EGTA concentrations because K_2CaEGTA was not added. The specific binding of [^3H]Ins(1,4,5) P_3 to the membrane fractions was determined as described in the Experimental section. The specific binding at 50 μM total EGTA concentration was defined as 100%. Results are the means of duplicate determinations.

Ins(1,4,5) P_3 R1 was observed at [Ca^{2+}] values covering the physiological range in the cytosol (from less than 1 nM to more than 10 μM , slope factor -0.55). The EC_{50} of the effect of Ca^{2+} was 100 nM for Ins(1,4,5) P_3 R1. In contrast, Ins(1,4,5) P_3 binding to Ins(1,4,5) P_3 R3 changed steeply at high [Ca^{2+}] with an EC_{50} of 872 nM (between 100 nM and 10 μM , slope factor 1.8).

Although Ca^{2+} buffering systems using Ca^{2+} chelators are generally employed to control [Ca^{2+}] accurately below 100 μM , some Ca^{2+} chelators themselves have been reported to have inhibitory effects on Ins(1,4,5) P_3 binding to Ins(1,4,5) P_3 Rs as well as on Ins(1,4,5) P_3 -induced Ca^{2+} release (IICR) [47,48]. Chelators in Ca^{2+} -free form are thought to be responsible for these effects on Ins(1,4,5) P_3 Rs [49]. To test the effect of EGTA, Ins(1,4,5) P_3 binding to Ins(1,4,5) P_3 R1 and Ins(1,4,5) P_3 R3 was assayed at various total EGTA concentrations (Figure 3). In these experiments the Ca^{2+} -free EGTA concentration was almost equal to the total EGTA concentration because K_2CaEGTA was not added. Ins(1,4,5) P_3 binding to Ins(1,4,5) P_3 R3 was not significantly affected by EGTA. Ins(1,4,5) P_3 binding to Ins(1,4,5) P_3 R1 was apparently increased as the total EGTA concentration was increased. Considering 5 μM total calcium contaminating the cytosol-like buffer, [Ca^{2+}] is 42 μM and 0.1 nM with 50 μM and 20 mM total EGTA respectively. In the presence of 2 μM free Ca^{2+} , however, Ins(1,4,5) P_3 binding to Ins(1,4,5) P_3 R1 was not affected significantly by the Ca^{2+} -free EGTA concentration (results not shown). Thus the apparent increase in Ins(1,4,5) P_3 binding to Ins(1,4,5) P_3 R1 shown in Figure 3 can be attributed to the decrease in [Ca^{2+}] from contaminating calcium.

Reversible effects of Ca^{2+} on ligand affinities of Ins(1,4,5) P_3 R types

To clarify by what mechanism(s) the effects of Ca^{2+} shown in Figure 2 were exerted, we next performed Scatchard analyses and examined the reversibility of these effects of Ca^{2+} .

Figure 4 shows the Scatchard analyses of saturable Ins(1,4,5) P_3 binding to Ins(1,4,5) P_3 R1 and Ins(1,4,5) P_3 R3 with 3 nM or 1.4 μM free Ca^{2+} . Calculated binding parameters are summarized

Figure 4 Scatchard analyses of saturable [^3H]Ins(1,4,5) P_3 binding to Ins(1,4,5) P_3 R1 and Ins(1,4,5) P_3 R3 with 3 nM and 1.4 μM free Ca^{2+}

Membrane fractions from Sf9 expressing (A) Ins(1,4,5) P_3 R1 (100 μg per point) and (B) Ins(1,4,5) P_3 R3 (50 μg per point) were incubated with 9.6 nM [^3H]Ins(1,4,5) P_3 and various concentrations of unlabelled Ins(1,4,5) P_3 in cytosol-like buffer containing 500 μM Ca^{2+} -free EGTA and 3 nM ($\bullet$, $\blacktriangle$) or 1.4 μM ($\circ$, $\triangle$) free Ca^{2+} . The specific binding of [^3H]Ins(1,4,5) P_3 to the membrane fractions was determined as described in the Experimental section. Results are the means of duplicate determinations in a typical experiment. B, Ins(1,4,5) P_3 bound to membrane fraction; F, free Ins(1,4,5) P_3 .

Table 2 Effects of Ca^{2+} on the parameters of saturable [^3H]Ins(1,4,5) P_3 binding to Ins(1,4,5) P_3 R1 and Ins(1,4,5) P_3 R3

Binding parameters were determined from saturation binding experiments as described in the legend to Figure 4. Results are means $\pm$ S.E.M. for n experiments.

	[Ca^{2+}] (nM)	K_d (nM)	B_{max} (pmol/mg)	n
Ins(1,4,5) P_3 R1	3	78.5 ± 18.8	5.78 ± 0.05	5
	1400	312	5.03	2
Ins(1,4,5) P_3 R3	3	116 ± 9	89.5 ± 0.2	3
	1400	62.2	79.6	2

in Table 2. For both the Ins(1,4,5) P_3 R types, Scatchard plots with 3 nM or 1.4 μM free Ca^{2+} gave single but distinct values for K_d , and the total number of Ins(1,4,5) P_3 -binding sites (B_{max}) was slightly affected by the differences in [Ca^{2+}]. Ins(1,4,5) P_3 R1 exhibited a higher affinity for Ins(1,4,5) P_3 with 3 nM free Ca^{2+} (K_d 78.5 ± 18.8 nM; mean $\pm$ S.E.M.) and a lower affinity for Ins(1,4,5) P_3 with 1.4 μM free Ca^{2+} (K_d 312 nM). In contrast, Ins(1,4,5) P_3 R3 exhibited a lower affinity for Ins(1,4,5) P_3 with 3 nM free Ca^{2+} (K_d 116 ± 9 nM; mean $\pm$ S.E.M.) and a higher affinity for Ins(1,4,5) P_3 with 1.4 μM free Ca^{2+} (K_d 62.2 nM). The order of affinity of Ins(1,4,5) P_3 R types for Ins(1,4,5) P_3 was reported to be Ins(1,4,5) P_3 R2 > Ins(1,4,5) P_3 R1 > Ins(1,4,5) P_3 R3 by using cDNA-derived truncated Ins(1,4,5) P_3 R with 1 mM EDTA (probably with less than nanomolar free Ca^{2+}) [12,32]. In

Table 3 Reversible effects of $[Ca^{2+}]$ on $[^3H]$ Ins(1,4,5) P_3 binding to Ins(1,4,5) P_3 R1 and Ins(1,4,5) P_3 R3

Membrane fractions from Sf9 expressing Ins(1,4,5) P_3 R1 (100 μ g per point) and Ins(1,4,5) P_3 R3 (50 μ g per point) were incubated with 4.8 nM $[^3H]$ Ins(1,4,5) P_3 in cytosol-like buffer. At each session, K_2H_2EGTA or $K_2CaEGTA$ was added to give $[Ca^{2+}]$ as specified, and the reaction mixture was incubated at 1 °C for 10 min. Neither the Ca^{2+} -free EGTA concentration nor the total EGTA concentration exceeded the range indicated in Figure 3. The specific binding of $[^3H]$ Ins(1,4,5) P_3 to the membrane fraction was determined as described in the Experimental section. The specific binding in session I was determined as 100%. Results are means $\pm$ S.E.M. for four determinations. The specific binding was significantly different from that in the previous session (* $P < 0.05$; ** $P < 0.002$ by Student's t test). Abbreviation: SB, specific binding.

Session	Ins(1,4,5) P_3 R1		Ins(1,4,5) P_3 R3	
	$[Ca^{2+}]$ (nM)	SB (%)	$[Ca^{2+}]$ (nM)	SB (%)
I	0	100 $\pm$ 3	0	100 $\pm$ 1
II	600	74.7 $\pm$ 4.5*	2000	201 $\pm$ 4**
III	30	93.3 $\pm$ 0.0*	250	114 $\pm$ 1**
IV	600	71.4 $\pm$ 0.1**	2000	165 $\pm$ 7**

the present study the order derived with 3 nM free Ca^{2+} was also Ins(1,4,5) P_3 R1 > Ins(1,4,5) P_3 R3. However, with 1.4 μ M free Ca^{2+} , Ins(1,4,5) P_3 R3 exhibited a higher affinity for Ins(1,4,5) P_3 than did Ins(1,4,5) P_3 R1.

Alternate addition of $K_2CaEGTA$ and K_2H_2EGTA (see the legend to Table 3) allowed $[Ca^{2+}]$ to be increased and decreased respectively to specified values repetitively. As shown in Table 3, Ins(1,4,5) P_3 binding to both Ins(1,4,5) P_3 R1 and Ins(1,4,5) P_3 R3 was changed reversibly and repetitively by $[Ca^{2+}]$.

The results in Tables 2 and 3 and in Figure 4 are in good agreement quantitatively with the results in Figure 2, indicating that the effects of Ca^{2+} on the apparent binding of Ins(1,4,5) P_3 to both the Ins(1,4,5) P_3 R types shown in Figure 2 were exerted not through alterations of the number of total binding sites but through alterations of the affinities of the Ins(1,4,5) P_3 R types for Ins(1,4,5) P_3 , and also that the affinities can be changed in a reversible manner by Ca^{2+} . The possibility of irreversible modification, such as degradation of the Ins(1,4,5) P_3 R proteins, is excluded because such modification cannot explain the slight change in the B_{max} values in the Scatchard analyses (Figure 4 and Table 2) or the reversibility of Ins(1,4,5) P_3 binding (Table 3). Another possibility, that of Ins(1,4,5) P_3 production and/or metabolism, is also excluded in these experiments, because changing the background Ins(1,4,5) P_3 concentration could not result in the completely opposite effects observed on the apparent binding of Ins(1,4,5) P_3 to Ins(1,4,5) P_3 R1 and Ins(1,4,5) P_3 R3. With the results presented above we demonstrate that (1) both Ins(1,4,5) P_3 R1 and Ins(1,4,5) P_3 R3 have at least two affinity states, (2) Ca^{2+} regulates interconversions between these states, and (3) Ca^{2+} regulates the binding of Ins(1,4,5) P_3 to Ins(1,4,5) P_3 R1 and Ins(1,4,5) P_3 R3 in opposite manners.

Putative target sites of various modulatory mechanisms reside in the modulatory and channel domains of Ins(1,4,5) P_3 Rs. Recently, Joseph et al. [50] reported the association of the N- and C-termini of cerebellar Ins(1,4,5) P_3 R. The mAb 18A10, which recognizes the C-terminal 12 amino acid residues of Ins(1,4,5) P_3 R1 [51], augmented the affinity of cerebellar Ins(1,4,5) P_3 R for Ins(1,4,5) P_3 but inhibited IICR [52]. These reports indicated that the C-terminal cytosolic stretch of Ins(1,4,5) P_3 R1 played some role in Ins(1,4,5) P_3 binding as well as in IICR. In the present study we used full-length Ins(1,4,5) P_3 R types. We thereby avoided overlooking any effects of modulations

on the Ins(1,4,5) P_3 R types, even if the mechanisms require other sites than the ligand-binding domain to exert their effects. The expressed Ins(1,4,5) P_3 R types exhibited the same properties as the native ones [high and specific binding of Ins(1,4,5) P_3 , tetramer formation and inhibition by Ca^{2+} of Ins(1,4,5) P_3 binding to Ins(1,4,5) P_3 R1].

Previously the binding of Ins(1,4,5) P_3 to cerebellar Ins(1,4,5) P_3 Rs [almost exclusively Ins(1,4,5) P_3 R1] were shown to be inhibited by Ca^{2+} [22–25]. These observations are consistent with the result of the present study with Ins(1,4,5) P_3 R1. In contrast, Ca^{2+} was reported to promote Ins(1,4,5) P_3 binding to Ins(1,4,5) P_3 R types from livers [28–30] and RBL cells [31]. Liver was shown to contain predominantly Ins(1,4,5) P_3 R1 and Ins(1,4,5) P_3 R2 but hardly any Ins(1,4,5) P_3 R3 [35]. All established cell lines tested so far have expressed significant amounts of Ins(1,4,5) P_3 R3 [35]. Ins(1,4,5) P_3 R2 and Ins(1,4,5) P_3 R3 might be responsible for the promotion of Ins(1,4,5) P_3 binding by Ca^{2+} in livers and RBL cells respectively. Distinct types of Ins(1,4,5) P_3 R can form heterotetrameric complexes [35,36]. Such complexes of Ins(1,4,5) P_3 R types might be responsible for the promotion of Ins(1,4,5) P_3 binding by Ca^{2+} in livers and RBL cells. The properties of Ins(1,4,5) P_3 R2 and heterotetramers, however, remains to be elucidated.

The mechanism by which Ca^{2+} regulates the interconversions of the affinity states of the Ins(1,4,5) P_3 R types also remains to be elucidated. Free Ca^{2+} were reported to regulate IICR in a bell-shaped manner [53–57]. Although Marshall and Taylor [30] suggested that liver Ins(1,4,5) P_3 R has two distinct target sites of effect of Ca^{2+} on Ins(1,4,5) P_3 binding and IICR, the relation between the effects of Ca^{2+} on Ins(1,4,5) P_3 binding to Ins(1,4,5) P_3 R types and that on IICR remains obscure. Ins(1,4,5) P_3 R1 and Ins(1,4,5) P_3 R2 were shown to bear a region that directly bound Ca^{2+} [58]. The corresponding region is conserved also in Ins(1,4,5) P_3 R3 [14]. Regulation might be exerted by direct binding of Ca^{2+} to Ins(1,4,5) P_3 R types and/or via Ca^{2+} -sensitive molecule(s) that in turn regulate(s) Ins(1,4,5) P_3 R types.

The effects of Ca^{2+} on Ins(1,4,5) P_3 binding to cerebellar Ins(1,4,5) P_3 R were reported to be mediated by a Ca^{2+} -sensitive molecule, 'calmodin' [2,25], and IICR through purified cerebellar Ins(1,4,5) P_3 Rs was not affected significantly by Ca^{2+} [3,54,59]. We showed previously that cerebellar Ins(1,4,5) P_3 R bound CaM in a Ca^{2+} -dependent manner [5], and that the cDNA-derived Ins(1,4,5) P_3 R3 did not bind CaM [60]. However, Ins(1,4,5) P_3 binding to purified cerebellar Ins(1,4,5) P_3 R was not inhibited by 500 μ M Ca^{2+} even after the addition of CaM [2]. The sensitivity of the Ins(1,4,5) P_3 R types to Ca^{2+} shown in the present study might be mediated by these Ca^{2+} -sensitive molecule(s).

Cerebellar Ins(1,4,5) P_3 Rs were reported to be phosphorylated by protein kinase C [61] and Ca^{2+} /CaM-dependent kinase II [61,62] in a Ca^{2+} -dependent manner. Phosphorylation of Ins(1,4,5) P_3 R by protein kinase A was reported not to promote Ins(1,4,5) P_3 binding to cerebellar Ins(1,4,5) P_3 R by itself [63] but to enhance the promotion of Ins(1,4,5) P_3 binding by Ca^{2+} in rat hepatocytes [29]. Activation and inhibition of IICR in fibroblasts were suggested to be mediated by Ca^{2+} /CaM-dependent protein kinase II at low $[Ca^{2+}]$ and by a Ca^{2+} -activated protein phosphatase, calcineurin, at high $[Ca^{2+}]$ respectively [56]. Calcineurin was also reported to associate with cerebellar Ins(1,4,5) P_3 R via an immunodepressant FK506-binding protein, FKBP12, to reverse the activation of IICR by protein kinase C [64]. However, because all the binding assays in the present study were performed on ice without the addition of ATP, cAMP or Mg^{2+} (required in phosphorylation or dephosphorylation), Ca^{2+} -activated phosphorylation and/or dephosphorylation of

Ins(1,4,5) P_3 R proteins and/or of Ins(1,4,5) P_3 R-associated proteins is not likely to be responsible for the effect of Ca^{2+} presented here.

The effects of free Ca^{2+} on Ins(1,4,5) P_3 binding to Ins(1,4,5) P_3 R1 and Ins(1,4,5) P_3 R3 were both monophasic; however, the directions (promotion or inhibition), the EC_{50} values and the slope factors differed for each type, which might indicate that Ins(1,4,5) P_3 R1 and Ins(1,4,5) P_3 R3 are regulated by Ca^{2+} through a single distinct mechanism for each type. Both the Ins(1,4,5) P_3 R types were expressed in the same intracellular milieu of Sf9 cells in the present study. Therefore the difference among the regulation of the two Ins(1,4,5) P_3 R types by Ca^{2+} is not attributable to a difference in the availability of modification(s) of each Ins(1,4,5) P_3 R type but to the distinct responses or the distinct susceptibility of each Ins(1,4,5) P_3 R type to a certain modification mechanism.

The present study suggests that the difference between the Ca^{2+} regulation of the ligand-affinity states of Ins(1,4,5) P_3 R1 and that of the states of Ins(1,4,5) P_3 R3 is responsible for the differential effects of Ca^{2+} on Ins(1,4,5) P_3 R functions between tissues reported so far. This diverse regulation of Ins(1,4,5) P_3 R types by Ca^{2+} might point to the molecular basis underlying the complex spatiotemporal dynamics of intracellular Ca^{2+} .

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