The calmodulin-binding domain in the mouse type 1 inositol 1,4,5-trisphosphate receptor

Maki YAMADA,* Atsushi MIYAWAKI,*¶ Kazuki SAITO,† Terumi NAKAJIMA,† Miki YAMAMOTO-HINO,‡ Yoshimi RYO,* Teiichi FURUICHI* and Katsuhiko MIKOSHIBA*§

*Department of Molecular Neurobiology, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108, †Suntory Institute for Bioorganic Research, 1-1-1 Wakayamadai, Shimamoto-cho, Mishima-gun, Osaka 618, ‡Tokyo Research Laboratories, Kyowa Hakko Kogyo Co., Ltd., 30606 Asahimachi, Machida-shi, Tokyo 194, §Molecular Neurobiology Laboratory, Institute of Physical and Chemical Research (RIKEN), Tsukuba Life Science Center, 3-1-1 Koyadai, Tsukuba-shi, Ibaraki 305, Japan

We determined the amino acid sequence responsible for the calmodulin (CaM)-binding ability of mouse type 1 Ins(1,4,5)P_3 receptor (IP_3R1). We expressed various parts of IP_3R1 from deleted cDNA and examined their CaM-binding ability. It was shown that the sequence stretching from Lys-1564 to Arg-1585 is necessary for the binding. The full-length IP_3R1 with replacement of Trp-1576 by Ala lost its CaM-binding ability. Antibody against residues 1564–1585 of IP_3R1 inhibited cerebellar IP_3R1 from binding CaM. The fluorescence spectrum of the peptide that corresponds to residues 1564–1585 shifted when Ca^{2+}-CaM was added. From the change in the fluorescence spectrum, we estimated the dissociation constant (K_D) between the peptide and CaM to be 0.7 μM. The submicromolar value of K_D suggests an actual interaction between CaM and IP_3R1 within cells. The CaM-binding ability of other types of IP_3Rs was also examined. A part of the type 2 IP_3R, including the region showing sequence identity with the CaM-binding domain of IP_3R1, also bound CaM, while the expressed full-length type 3 IP_3R did not.

INTRODUCTION

The Ins(1,4,5)P_3 receptor (IP_3R) is a pivotal molecule for cytosolic Ca^{2+} mobilization. It forms a tetramer on the membrane of intracellular Ca^{2+} pools such as the endoplasmic reticulum [1,2]. IP_3R works as an Ins(1,4,5)P_3 (IP_3)-gated Ca^{2+} channel [3] when IP_3 is produced in response to extracellular signals such as hormones and neurotransmitters. This IP_3-induced Ca^{2+} release (IICR) triggers various Ca^{2+}-dependent cellular responses. Molecular cloning studies have shown there are at least three homologous types of IP_3R, designated type 1 (IP_3R1) [4–6], type 2 (IP_3R2) [7,8] and type 3 (IP_3R3) [8–10]. Each type of IP_3R is differentially expressed among tissues and cell types ([11–14a]; T. Monkawa, unpublished work) and is thought to have different characteristics.

Calmodulin (CaM) is a ubiquitous Ca^{2+}-binding protein, which has been shown to be present at micromolar concentrations in almost all mouse tissues [15]. CaM binds to a wide variety of proteins in a Ca^{2+}-dependent manner and regulates their functions [16].

Several studies have shown the blockage of IICR by CaM inhibitors, suggesting regulation of IICR by CaM. Hill et al. [17] reported that CaM inhibitors (W7, W13 and CGS9343B) blocked IICR in a permeabilized liver epithelial cell line. Yuzaki and Mikoshiba [18] reported that W7 inhibited Ca^{2+} release from caffeine-insensitive pools in cultured cerebellar Purkinje cells. Somogyi and Stucki [19] showed that the CaM inhibitors calmidazolium and CGS9343B inhibited hormone-induced Ca^{2+} oscillations in intact hepatocytes. However, these studies did not specify the target molecules of CaM. Maeda et al. [1] have demonstrated that the purified cerebellar IP_3R (predominantly IP_3R1) binds CaM in a Ca^{2+}-dependent manner. To pursue the possibility that CaM regulates the function of the IP_3R-Ca^{2+} channel directly, we defined the CaM-binding domain in the IP_3R1. By using the synthetic peptide corresponding to the domain, (i) we analysed the kinetics of the CaM binding and (ii) we have made a polyclonal antibody which prevents CaM binding to the IP_3R. We discuss here the molecular features and biological significances of CaM binding to the IP_3R protein.

MATERIALS AND METHODS

Expression of deleted IP_3R1 and IP_3R3 in NG108-15 and preparation of the proteins

The plasmids encoding mutant proteins of IP_3R1 were constructed from the plasmid pBactS-C1 described previously [20]. The mutant proteins were transiently expressed in the neuroblastoma/glioma hybrid cell line NG108-15. Full-length cDNA of human IP_3R3 [8] was also expressed using the same vector, pBactS.

For soluble proteins, cells were homogenized in an equal volume of buffer S [0.25 M sucrose/0.2 M NaCl/20 mM Tris/HCl (pH 7.4)/1 mM 2-mercaptoethanol]. The homogenates were centrifuged at 700 *g* for 3 min, and the resultant supernatants were re-centrifuged at 250000 *g* for 8 min. The soluble fractions were supplemented with $CaCl_2$ to 1.5 mM.

For membrane proteins, cells were homogenized in 9 vol. of buffer M (buffer S plus 1 mM EDTA, 100 μM phenylmethanesulphonyl fluoride, 10 μM pepstatin A and 10 μM leupeptin).

The abbreviations used are: CaM, calmodulin; IP_3, inositol 1,4,5-trisphosphate; IP_3R, inositol 1,4,5-trisphosphate receptor; IP_3R1, type 1 IP_3R; IP_3R2, type 2 IP_3R; IP_3R3, type 3 IP_3R; K_D, dissociation constant; IICR, IP_3-induced Ca^{2+} release; MBP, maltose-binding protein; PKA, cyclic AMP-dependent protein kinase; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration

¶ To whom all correspondence should be addressed.

The homogenates were centrifuged at 1200 *g* for 3 min and the supernatants were re-centrifuged at 250000 *g* for 15 min. The pellets were resuspended in buffer BT [1% Triton X-100/0.2 M NaCl/20 mM Tris/HCl (pH 7.4)/1 mM 2-mercaptoethanol/1.5 mM $CaCl_2$] to 3 mg of protein/ml, and were stirred at 4 °C for 30 min for solubilization. The solubilized membrane proteins were centrifuged at 18000 *g* for 15 min to remove insoluble materials.

To confirm whether the expressed protein had the expected molecular mass, each sample was subjected to immunoblotting by either 4C11, the anti-IP_3R1 monoclonal antibody [4], or KM1082, the monoclonal antibody to the C-terminus of IP_3R3 [47].

Preparation of plasmids for production of fusion proteins

We subcloned the *Pst*I–*Ava*I fragment of mouse IP_3R1 (coding amino acid residues Gln-1502–Glu-1638) into a protein-fusion vector pMal-c (New England Biolabs), so that maltose-binding protein (MBP), mouse IP_3R1 (Gln-1502–Glu-1638) and β-galactosidase were expressed as an in-frame fusion protein. In detail, we cloned the *Pst*I–*Ava*I fragment of mouse IP_3R1 into *Pst*I and *Eco*RV sites of pBluescript (the recessed end of *Ava*I had been filled), then the *Pst*I–*Hin*dIII fragment of the resultant plasmid was ligated to the *Pst*I and *Hin*dIII sites of pMal-c. To truncate the IP_3R1-derived amino acid sequence from its N-terminus, we deleted the inserted part of IP_3R1 from its 5′ end with exonuclease III/mung-bean nuclease. The frame-matched clones produced fusion protein with β-galactosidase, and were obtained by the method of blue/white selection. The MBP sequence toward the fused portion with the IP_3R1-derived peptide was -AQTNSSS.

The following procedures were for mutants C80 and C85. At first, the cDNA fragment coding residues from Gln-1557 to Glu-1638 was generated by PCR from mouse renal cDNA. The PCR was performed with the two oligonucleotides: 5′-GGGATATC(C/T)TGGACAGCCA(A/G)GTCAACA-3′ and 5′-CCGAATTCTCGGGGAAGAGCAG-3′ as 5′ and 3′ primers respectively. The PCR product was digested with *Eco*RI and *Eco*RV, whose recognition sites were introduced in the PCR primers, and was cloned into *Stu*I and *Eco*RI sites of the pMal-c vector. The plasmid was cleaved at the 3′ side of the insert by *Eco*RI and *Pst*I, and the insert was deleted uni-directionally by exonuclease III/mung-bean nuclease. The recircularized plasmid produced a fusion protein (including Gln-1557–Ser-1588), which bound CaM. Then the plasmid was cleaved by *Hin*dIII at the 3′ end of the insert and further deleted by BAL-31S bidirectionally. The deleted fragments were blunted, digested by *Sca*I, and ligated to the *Xba*I (filled)–*Sca*I fragment of pMal-c vector. (The *Sca*I recognition site is in the sequence coding β-lactamase, and *Stu*I, *Eco*RI, *Xba*I, *Pst*I and *Hin*dIII recognition sites are in the multicloning site of pMal-c in this order.) By this method, deleted portions of the vector were recovered, and the translation of some fusion proteins was terminated at the stop codon in the *Xba*I site. Each translation of C80 or C85 was terminated at the C-terminus of the IP_3R1-derived sequence. All constructions were confirmed by DNA-sequencing.

Expression and solubilization of the MBP fusion proteins

After 2 h of induction with 0.3 mM isopropyl β-D-thiogalactopyranoside, the expressed MBP fusion proteins in *Escherichia coli* were transported to the inclusion bodies. The fusion proteins were solubilized by the method described by Takazawa and Erneux [21]. In brief, 1% Triton X-100-insoluble proteins were dissolved in buffer containing 0.5% SDS and 5% 2-mercaptoethanol for 5 min at 4 °C, then centrifuged at 18000 *g* for 5 min. The supernatants were immediately diluted 50-fold in buffer [1% Triton X-100/0.1 M NaCl/20 mM Tris/HCl (pH 7.5)/0.2 mM $CaCl_2$/2 mM 2-mercaptoethanol/0.2 mM phenylmethanesulphonyl fluoride/50 μM leupeptin/20 μM pepstatin A] and were re-centrifuged at 55000 *g* for 10 min to remove aggregates. The solubilized proteins were applied to the CaM–Sepharose column immediately. The proteins from 200 ml of bacterial culture was used per 1 ml of column volume.

CaM-binding assay

A sample of solubilized proteins (0.5 column vol.) was applied to a CaM–Sepharose 4B (Pharmacia) column equilibrated with buffer B [0.2% Triton X-100/0.2 M NaCl/20 mM Tris/HCl (pH 7.4)/1.5 mM $CaCl_2$/1 mM 2-mercaptoethanol]. After being washed by 3.5 (Figure 1b below) or 5.5 (others) column vol. of buffer B, CaM-binding proteins were eluted by 4 column vol. of buffer E (buffer B containing 2 mM EGTA instead of 1.5 mM $CaCl_2$). In Figures 2–6 (below), we collected 3 column vol. of effluents after applying samples and buffer E, which were referred to as 'flow-through' (F) and eluate with buffer E (E) respectively. The relevant proteins in the fractions were detected by immunoblotting. MBP fusion proteins were detected with anti-MBP serum (New England Biolabs).

Site-directed mutagenesis of the Trp-1576 residue of IP_3R1 to Ala

Site-directed mutagenesis was performed with Mutan-K system (Takara). The complementary oligonucleotide, 5′-GA TAA CCG CGC GTT CAG GG-3′, was used.

Preparation of anti-(1564–1585 peptide) antibody

The immunogenic peptide used was identical with residues 1564–1585 of mouse IP_3R1, except for the addition of one cysteine residue to the N-terminus (CKSHNIVQKTALNWRLSARNAAR). The peptide (synthesized with a Millipore type 9050 peptide synthesizer and purified by HPLC) was cross-linked to keyhole-limpet haemocyanin (Pierce) via the N-terminal Cys residue by using m-maleimidobenzoyl-*N*-hydroxysuccinimide ester (Pierce). The cross-linked product was used to immunize rabbits (Japan White; Japan SLC), along with Freund's adjuvant (Sigma). An IgG fraction was prepared with Ampure PA (Amersham).

Inhibition of the CaM binding of cerebellar IP_3R1 by the antibody

Mouse (ICR; Japan SLC) cerebellar microsomal fraction was prepared as described previously [22]. The microsomes were preincubated with the IgG from immune or preimmune serum for 30 min at 4 °C in buffer B, then 1% Triton X-100 was added and the mixture was stirred for 30 min at 4 °C for solubilization. The samples pretreated with the IgGs were centrifuged at 18000 *g* for 15 min, and the supernatants were applied to a CaM–Sepharose 4B column. The concentration of the IP_3R in the cerebellar microsomes (predominantly IP_3R1 [23]) was estimated by calculating the number of IP_3 binding sites [24]; in 1 mg of the cerebellar microsomal protein, the amount of IP_3R was estimated to be about 6 ng.

Homology search

Protein homology with non-redundant Swiss-Plot, PIR, PRF and GenPept databases was searched with the computer program TFASTA. The definition of similar amino acids (in Figure 6a below) was 0 or more in the protein matrix PAM250.

Cloning of the partial cDNA sequence of IP_3R2

The partial cDNA fragment of IP_3R2 that includes the region homologous with the CaM-binding domain of IP_3R1 was isolated by PCR from mouse renal cDNA. The PCR was performed with the two oligonucleotides: 5′-GGGATATC(C/T)TGGACAG-CCA(A/G)GTCAACA-3′ and 5′-GGGAATTCATGCTGGC-CCTCCAAGAGC-3′ as 5′ and 3′ primers respectively. The PCR product was digested with *Eco*RI and *Eco*RV, whose recognition sites were introduced into the PCR primer, and was cloned into *Stu*I and *Eco*RI sites of the pMal-c vector. The DNA sequence of the clone was determined; the amino acid sequence was identical with that of rat IP_3R2(1558–1596) [7]. This clone expressed a protein with expected molecular mass and which reacted with anti-MBP antibody. Another protein of lower molecular mass (40–44 kDa) was detected by the antibody (Figure 6b below). For three reasons we assume the protein is almost equivalent to the MBP that might be generated by degradation at a site near its end. First, the extra proteins with same molecular mass also appeared in all lysates of *E. coli* (in Figure 2 below, including the 'vector'), regardless of the length and sequence of the fused portions. Secondly, they had the same molecular mass as MBP (42 kDa). Thirdly, the proteins did not react with the antibody to the residues 1564–1585 of IP_3R1 used in Figure 4 (below).

Fluorescence measurements of the peptide

The CaM-binding activity of the peptide used for antibody generation was analysed by fluorescence measurements. The quantity of the peptide was analysed by amino acid analysis. Decalcified CaM was purified from bovine brain as described in [25], with minor modification. Both peptide (5.0 mM) and CaM at appropriate concentrations were dissolved in buffer [0.1 M KCl/20 mM Hepes/KOH (pH 7.4)/5 mM 2-mercaptoethanol/2 mM $CaCl_2$ or 1 mM EGTA]. Fluorescence spectra were obtained with a Hitachi F-2000 fluorescence spectrophotometer. The excitation wavelength used was 300 nm, where the emission from CaM became negligible. K_D values were estimated by non-linear regression analysis of emission intensities at 326 nm by the least-squares method, where stoichiometry of CaM binding of the peptide was assumed to be 1:1.

RESULTS

Determination of amino acid sequence responsible for CaM binding of IP_3R1

In many cases, CaM-binding domains have been defined as a stretch of about 20 amino acid residues [16]. First, we produced various deletion-mutant proteins of IP_3R1 (Figure 1a) in NG108-15 cells from the cDNA and investigated their ability to bind to a CaM–Sepharose column. Figure 1(b) shows the elution profiles of the mutant proteins. The expressed wild-type IP_3R1 bound to the CaM–Sepharose column and was eluted by EGTA, while the mutant ΔBam (lacking residues 1267–2110) did not. In addition, the mutant EL (residues 1–2217 of IP_3R1) bound to

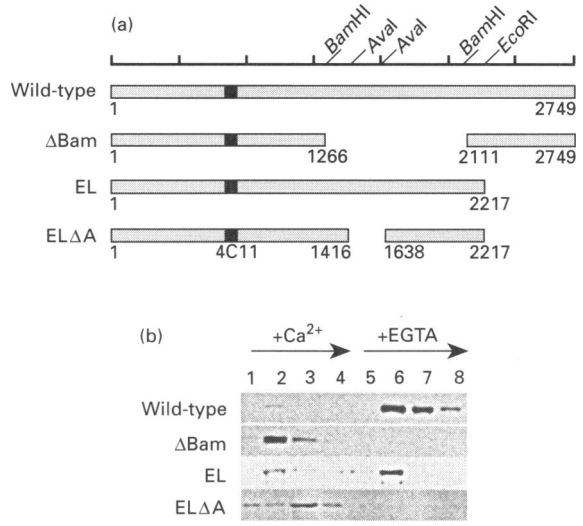

Figure 1 CaM-binding experiment on the mutant proteins of IP_3R1

(**a**) The deletion mutant proteins of IP_3R1 used for CaM-binding experiment. The restriction-enzyme sites used for the introduction of the deletions are shown at the top. (**b**) The elution profiles of expressed mutant proteins from CaM–Sepharose 4B. A 0.5 column vol. of sample was applied to a CaM–Sepharose 4B column. The effluents collected from every 1 column vol. are numbered. The buffer containing Ca^{2+} was applied until up to four fractions had been collected, then the buffer was changed to that containing EGTA. The proteins were detected by rat monoclonal antibody raised against the purified cerebellar IP_3R: 18A10 for wild-type and 4C11 for mutant proteins. The recognition site of the 4C11 is shown by solid bars in (**a**).

CaM–Sepharose, while the mutant ELΔA (lacking residues 1417–1637 of EL) did not bind. These results indicate that the region stretching from amino acid residues 1417 to 1637 is necessary for the CaM binding of IP_3R1. Then we expressed residues 1502–1638 in *E. coli* as an MBP fusion protein, MBP–IP_3R1 (1502–1638) and found that the protein bound to CaM–Sepharose in a Ca^{2+}-dependent manner (results not shown). We further truncated the IP_3R1-derived part of the fusion protein from the N- and C- termini and examined their CaM-binding ability (Figure 2). A truncated mutant, N64 (including residues 1564–1638 of IP_3R1), bound to CaM–Sepharose and was eluted by EGTA (E in Figure 2b), while most N69 (1569–1638) mutant protein flowed from the column in the presence of Ca^{2+} (F in Figure 2b). The C-terminal deletion mutant protein, C85 (1554–1585), bound to CaM–Sepharose,

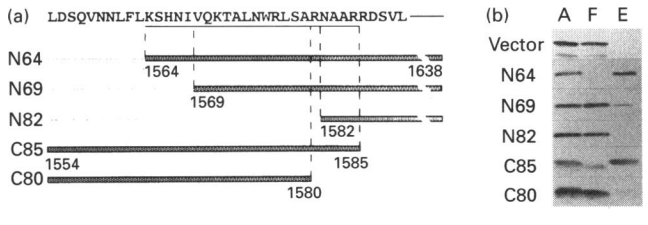

Figure 2 CaM binding of the fusion proteins, including various lengths of peptides derived from IP_3R1

(**a**) The portions of IP_3R1 expressed in *E. coli* as MBP fusion proteins. The corresponding amino acid sequence including Lys-1564–Arg-1585 (underlined) is shown at the top. (**b**) The elution profiles of the fusion proteins from CaM–Sepharose when the *E. coli* extracts were applied. A, applied sample; F, flow-through in the buffer including Ca^{2+}; E, eluates obtained with the buffer including EGTA. The expressed proteins were detected by anti-MBP serum.

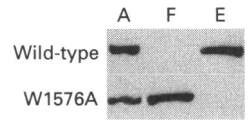

Figure 3 CaM binding of the mutant IP_3R1 in which Trp-1576 is replaced by Ala

Solubilized membrane proteins containing the full-length wild-type IP_3R1 or mutant IP_3R1 with Ala-1576, W1576A, were applied to a CaM–Sepharose 4B column. A, F and E are the same as in Figure 2(b). The expressed proteins were specifically detected by antiserum to residues 1718–1731 of IP_3R1, which do not react with the endogenous IP_3R1 in NG108-15 (splicing variant).

while the mutant C80 (1554–1580) lost its binding ability. Therefore the amino acid sequence responsible for the CaM binding was thought to be included in residues 1564–1585:

KSHNIVQKTALNWRLSARNAAR

CaM binding of IP_3R1 with Trp-1576 replaced by Ala

It has been shown that a Trp residue is indispensable for binding of several CaM-binding proteins [26,27]. In residues 1564–1585 of IP_3R1, we found a Trp residue at position 1576. We mutated this Trp-1576 to Ala by site-directed mutagenesis. In the MBP–IP_3R1 (1502–1638) fusion protein, the replacement of Trp resulted in a loss of the CaM-binding ability (results not shown). The Trp residue is probably indispensable also for the CaM binding of the region. The full-length mutant IP_3R1 with Ala-1576, W1576A, lost its ability to bind to CaM–Sepharose (Figure 3). There should be no other regions capable of binding CAM than the residues 1564–1585.

Inhibition of CaM binding of the intact IP_3R1 by IgG against peptide 1564–1585

We raised rabbit polyclonal antibody against the synthetic peptide corresponding to the residues 1564–1585 of IP_3R1. In an immunoblotting analysis with the cerebellar microsomal protein, IgG from the immune serum gave one protein signal of about 250 kDa, which disappeared when the IgG was preincubated with peptide 1564–1585 used as the immunogen (Figure 4a). The signal was then thought to represent principally IP_3R1. When 10 μg of the cerebellar microsomal protein was incubated with 1 μg of IgG, the CaM binding of the cerebellar IP_3R1 was completely blocked (Figure 4b). On the other hand, 15 μg of IgG from preimmune serum had no effect. These results support the importance of residues 1564–1585 in the CaM binding of the native IP_3R1 in the cerebellar microsomal protein.

Fluorescence measurement of peptide 1564–1585

Since the fluorescence spectrum is very sensitive to the environment of the fluorophore, it becomes a good index for the interaction of fluorescent compounds with other molecules. The peptide corresponding to residues 1564–1585 of IP_3R1 has one Trp residue at 1576, while mammalian CaM contains no Trp. This Trp residue is a good fluorophore to monitor the binding of the peptide with CaM. As the concentration of CaM was increased, the fluorescence intensity increased (Figure 5) and the peak shifted to a shorter wavelength (by up to about 20 nm). This is direct evidence that this peptide definitely bound to the CaM in the solution. From the change in fluorescence intensity at 326 nm, the K_D was obtained by regression analysis by the least-squares method (Figure 5). If equimolar (1:1) binding was

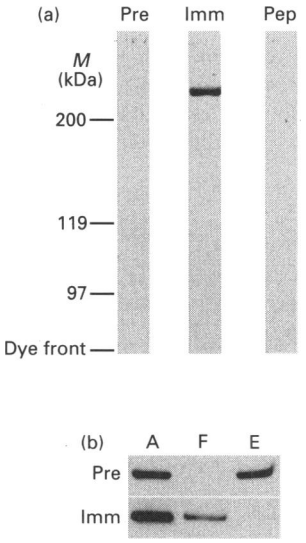

Figure 4 (a) Specificity of the IgG against peptide 1564–1585, and (b) inhibition of the CaM binding of cerebellar IP_3R1 by IgG

(**a**) Immunoblotting was performed with mouse cerebellar microsomal protein (5 μg) using immune IgG (1 μg/ml) against synthetic peptide (1564–1585) of IP_3R1 (Imm) or preimmune IgG (1μg/ml) (Pre). The immune IgG (1μg/ml) was preabsorbed with immunizing peptide (1 μM) (Pep). The positions of marker proteins are shown (*M* is molecular mass). The position of the dye front is shown. (**b**) Microsomal protein (10 μg) was incubated for 1 h with 1 μg of immune IgG (Imm) or 15 μg of preimmune IgG (Pre) and applied to CaM–Sepharose 4B. A, F and E are the same as in Figure 2B. IP_3R1 was detected by monoclonal antibody 18A10, which is specific to IP_3R1 [22].

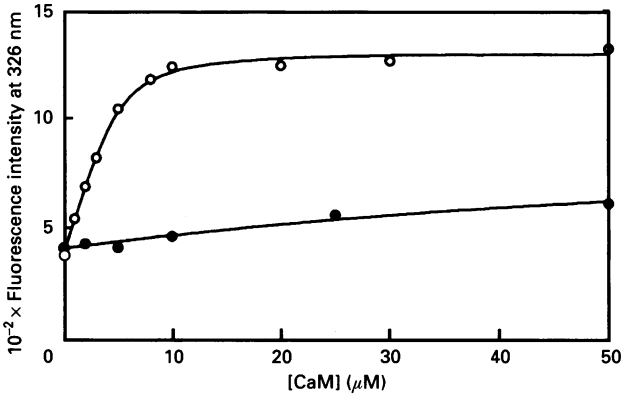

Figure 5 Changes in fluorescence intensity of peptide 1564–1585 of IP_3R1 mixed with various concentrations of CaM

The peptide (5.0 μM) and CaM at appropriate concentrations were dissolved in buffer containing 2 mM $CaCl_2$ (open circles) or 1 mM EGTA (closed circles). Fluorescence spectra were obtained by the excitation at 300 nm and the emission intensities at 326 nm were plotted as a function of CaM concentration. The continuous lines represent regression curves after the least-squares fitting of the data under the assumption of 1:1 binding.

assumed between the peptide and CaM, the K_D value was estimated to be 0.7 μM in the presence of 2 mM Ca^{2+}. On the other hand, without Ca^{2+}, the peptide scarcely bound to CaM; the estimated K_D value was greater than 0.1 mM. Thus the peptide interacts with CaM in a Ca^{2+}-dependent manner.

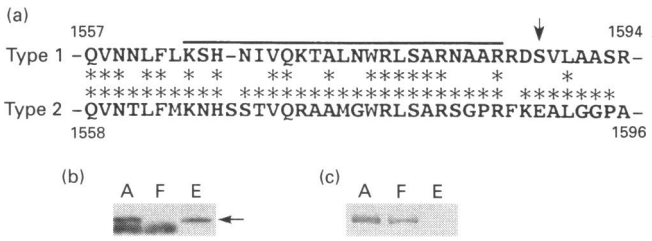

Figure 6 CaM-binding experiment with a part of IP_3R2 and full-length IP_3R3

(**a**) Alignment of the region including residues 1564–1585 of mouse IP_3R1 (overlined) with the corresponding region of IP_3R2. The identical residues are shown by double asterisks and similar residues by a single asterisk. The arrow shows the site of phosphorylation (Ser-1588 of IP_3R1) by PKA. (**b**) Elution profiles of MBP fusion protein including Gln-1558–Ala-1596 of IP_3R2, detected by anti-MBP serum. A, F and E are the same as in Figure 2(b). The protein indicated by an arrow had the expected size of the fusion protein. The protein of lower molecular mass was thought to be almost equivalent to MBP (see the Materials and methods section). (**c**) Elution profiles of full-length IP_3R3 which was produced in NG108-15 cells from the cDNA. A, F and E are the same as in Figure 2(b). IP_3R3 was detected by type-specific monoclonal antibody KM1082 [47].

CaM-binding ability of IP_3R2 and IP_3R3

There are three types of IP_3R that exhibit 60–70% amino-acid-sequence identity. IP_3R2 [7,8] was the only molecule found to carry a region showing considerable amino-acid-sequence identity with residues 1564–1585 of IP_3R1 in the homology search (see the Materials and methods section). The region in IP_3R2 is located in a similar position, namely residues 1565–1587. Figure 6(a) shows a comparison of the amino acid sequences of the regions of IP_3R1 and IP_3R2. Within the 23 amino acids, 12 are identical and 10 are similar. We constructed an MBP fusion protein including residues 1558–1596 of IP_3R2, MBP–IP_3R2(1558–1596). This fusion protein also bound to CaM–Sepharose in a Ca^{2+}-dependent manner (Figure 6b).

On the other hand, IP_3R3 shows no significant similarities to the amino acid sequence 1564–1585 of IP_3R1. Full-length IP_3R3 expressed in NG108-15, did not bind to CaM–Sepharose (Figure 6c).

DISCUSSION

From the present study it is concluded that the amino acid sequence Lys-1564–Arg-1585 is responsible for the CaM-binding ability of mouse IP_3R1. The sequence can form a CaM-binding domain by itself with a K_D for CaM of about 0.7 μM in the presence of Ca^{2+}.

The K_D between two molecules allows us to estimate to what extent they interact in cells. In the cerebellum, the reported concentration of CaM in the soluble fraction is about 19 μM (19 μmol/litre of tissue) [15], and both CaM and IP_3R1 occur predominantly in the Purkinje cells [28,29]. Furthermore, biochemical studies on subcellular fractions and immunoelectron microscopy have shown that CaM is found on the smooth endoplasmic reticulum in the post synapse [29,30], where IP_3R1 is highly concentrated [2,31–34]. These findings, together with the submicromolar value of the K_D between CaM and the CaM-binding domain in IP_3R1, suggest that CaM binds to IP_3R1 in response to the increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$).

The CaM-binding domain was found in the modulatory region, the spacer region between the IP_3-binding region [20,35] and the Ca^{2+}-channel-forming region. This agrees with the fact that CaM does not affect IP_3 binding of a purified receptor directly [36]. CaM is thought to regulate the Ca^{2+} channel activity of IP_3R1 allosterically. Within the modulatory region of IP_3R1, there are two sites of phosphorylation by protein kinase A (PKA), at Ser-1588 and at Ser-1755 [37], and the phosphorylation was shown to modulate the channel activity of IP_3R1 [23,38]. In the case of myosin-light-chain kinase and some other CaM-binding proteins, CaM binding and phosphorylation of the neighbouring serine residue inhibit each other [39]. It is interesting that Ser-1588 of IP_3R1 is close to the CaM-binding domain (1564–1585). The close proximity of these two sites may indicate interactions between phosphorylation and CaM binding.

Several studies using CaM inhibitors suggested up-regulation of IICR by CaM [17–19]. On the other hand, many lines of evidence have shown that IICR is regulated biphasically by $[Ca^{2+}]_i$ [40–43]: Ca^{2+} enhances IICR at a low level of $[Ca^{2+}]_i$ (lower than 0.2–0.3 μM), while at a high level of $[Ca^{2+}]_i$ (over 0.3 μM), Ca^{2+} exerts an inhibitory effect. This biphasic feedback regulation is thought to play a crucial role in cellular Ca^{2+} dynamics such as Ca^{2+} waves and Ca^{2+} oscillations [19]. Since observations on feedback regulation have been made only with crude materials like permeabilized cells or microsomes, there is a possibility that CaM or some other Ca^{2+}-mediated proteins participate in the feedback regulation of IICR. CaM-dependent kinase II and calcineurin have been proposed to be involved in the feedback regulations of IICR [44], which is still controversial [45,46]. The detailed mechanisms of the feedback regulations should be re-investigated in terms of the direct interaction between IP_3R and CaM.

So far, three types of IP_3Rs have been cloned. We have demonstrated that IP_3R1 and IP_3R2 bind CaM, whereas IP_3R3 does not (Figure 6). The effects of CaM on IP_3R are probably observed in cells expressing IP_3R1 or IP_3R2, but not in cells expressing only IP_3R3. The difference in CaM-binding ability may lead to the heterogeneity of IICR observed among many cell types.

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