The Interaction of Calmodulin with Alternatively Spliced Isoforms of the Type-I Inositol Trisphosphate Receptor*

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A 592-amino acid segment of the regulatory domain of the neuronal type-I inositol 1,4,5-trisphosphate receptor (IP3R) isoform (type-I long, amino acids 1314–1905) and the corresponding 552-amino acid alternatively spliced form present in peripheral tissues (type-I short, amino acids 1693–1733) were expressed as glutathione S-transferase fusion proteins. These domains encompass a putative calmodulin (CaM) binding domain and two protein kinase A phosphorylation sites. Both long and short forms of the protein retained the ability to bind CaM in a Ca2+-dependent manner as measured by CaM-Sepharose chromatography or a dansyl-CaM fluorescence assay. Both assays indicated that the short fusion protein bound twice the amount of CaM than the long form at saturating concentrations of CaM. In addition, the binding of the short form to CaM-Sepharose was inhibited by phosphorylation with protein kinase A, whereas the binding of the long form was unaffected. Full-length cDNAs encoding type-I long, type-I short, and type-III IP3R isoforms were expressed in COS cells, and the Ca2+-sensitivity of [3H]IP3 binding to permeabilized cells was measured. The type-I long isoform was more sensitive to Ca2+ inhibition (IC50 = 0.55 μM) than the type-I short (IC50 = 5.7 μM) or the type-III isoform (IC50 = 3 μM). In agreement with studies on the fusion proteins, the full-length type-I short bound more CaM-Sepharose, and this binding was inhibited to a greater extent by protein kinase A phosphorylation than the type-I long IP3R. Although type-III IP3R did not bind directly to CaM-Sepharose, hetero-oligomers of type-III IP3R retained the ability to interact with CaM. We conclude that the deletion of the SII splice site in the type-I IP3R results in the differential regulation of the alternatively spliced isoforms by Ca2+, CaM, and protein kinase A.

Inositol 1,4,5-trisphosphate receptors (IP3Rs) are a family of intracellular Ca2+ channels responsible for the mobilization of Ca2+ from intracellular stores in response to an agonist-mediated elevation of IP3 (reviewed in Refs. 1 and 2). In mammals three different IP3Rs isoforms are expressed, each encoded by a distinct gene. All 3 isoforms share approximately 70% homology and are organized into three functional domains: an N-terminal ligand binding domain, a central regulatory domain, and a C-terminal channel domain. Functional channels are believed to be tetrameric. Individual cell types can express more than one isoform, which may be present as homo- or heterotetrameric populations (3–5). The functional role of isoform diversity has not been established.

The gene encoding the type-I isoform is subject to alternative splicing and gives rise to three splice variants that have been denoted as S1, S2, and S3 (6–8). The S1 splice site is located in the ligand binding domain, whereas the S2 and S3 splice sites are in the regulatory domain. Functional studies comparing the S1(−) and S1(+) forms of the type-I IP3R have not revealed any marked differences in ligand binding or channel function (9, 10), and the functional consequences of the S3 insertion/deletion have not been investigated. Both short and long forms of S1 and S3 appear to co-exist in the same tissues (7, 8, 11). In contrast, the expression of the S2 insert is more stringent with the S2 insert being present in neurons and absent from the type-I IP3Rs of peripheral tissues (7, 12).

The S2 splice site encodes a region of 40 amino acids and is located between serine 1589 and serine 1756. Both serine residues can be phosphorylated by protein kinase A (13), and serine 1756 in cerebellum IP3R is phosphorylated by G-kinase (14). In addition, binding sites for Ca2+ (15), CaM (16), ATP (17), and FKBP-12 (18) are all found in the vicinity of the S2 splice site (see Fig. 1A). Hence, the presence or absence of the S2 splice site may modify regulation of type-I IP3Rs. For example, there is evidence that the deletion of the S2 region can alter the serine that is preferentially phosphorylated by protein kinase A (12). There is also experimental evidence to suggest that the effects of protein kinase A phosphorylation on IP3Rs-gated channels in neuronal and peripheral tissues are different (reviewed in Joseph et al. (19)). The Ca2+- regulation of [3H]IP3 binding to cerebellar membranes (S2(−)) is also different from that observed in a number of tissues that express high amounts of the S2(−) form of the type-I IP3R, e.g. vas deferens (20). However, it is difficult to definitively attribute such differences solely to the presence of the alternatively spliced forms of the type-I IP3R, and the basis for these differences is not well understood. In the present study we have utilized GST fusion proteins encoding IP3R regulatory domains and full-length S2 long and short recombinant IP3Rs expressed in COS-7 cells to compare the Ca2+/CaM and protein kinase A regulation of the S2 alternatively spliced isoforms of the type-I IP3R. The data suggest that the regulatory properties of these two alternatively spliced isoforms may be markedly influenced by differences in their interactions with CaM.
**Experimengal Procedures**

**Materials**

Pfu polymerase was obtained from Stratagene (La Jolla, CA). Stas- 
larized acrylamide solution (Protogel) for the preparation of SDS gels was obtained from National Diagnostics (Atlanta, GA). Dulbecco's minimal essential medium and LipofectAMINE were from Life Technologies, Inc. Polyamine transfection reagent LT-1 was from PanVerca Corp. (Madison, WI). Horseradish peroxidase-conjugated secondary antibod- 
ies were from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). Pico and Dura enhanced chemiluminescence reagents were from Pierce. Bovine calmodulin, isopropyl-β-thiogalactopyranoside, cata- 
lytic subunit of protein kinase A, protein A-Sepharose, and calmodulin- 
Sepharose was obtained from Sigma. Danysl calmodulin was purchased from Veritas (Potomac, MD). T4 DNA ligase was from Roche Molecular Biologicals, and T4 polynucleotide kinase was from New England Biologicals (Bedford, MA). [3H]IP3 and [γ-32P]ATP were from NEN Life Science Products. The type-I IP3R cDNA was kindly given by Dr. Thomas Sudhof (University of Texas Southwestern Medical Center, Dallas, TX) and was in the expression plasmid pCMVI-9 (6). It should be noted that this plasmid is S1(1). The 3152 base pair fragment was gel-purified and further digested with KpnI and ligated to the NcoI fragment obtained from pGEX2T-RDS. The resulting NcoI fragment was ligated into KpnI/NarI-digested type-I IP3R DNA plasmid.

**Construction of Plasmids**

pGEX-RDL—The region encoding the SII + splice site corresponding to amino acids 1314–1906 was PCR-amplified using Pfu polymerase. Full-length rat type-I IP3R cDNA was used as a template. The forward primer (SK-forward) encoded an BamHI site (5′-ggttgcattctattgtcaaggc-3′), and the reverse primer (SK-reverse) encoded an EcoRI site (5′-gggaaattgtgctttcttggc-3′). The PCR product was digested with BamHI/EcoRI and ligated into BamHI/EcoRI-digested pGEX-2T plasmid. We refer to the GST fusion protein encoded by this plasmid as regulatory domain short (GST-RDL).

pGEX-RDS—The construct from which SII had been deleted was made by ligating two fragments (F1 and F2) made by PCR using rat type-I cDNA as a template. F1 corresponding to amino acids 1314–1692 was made using the SK-forward primer and an F1 reverse primer (5′-ctttcttgctatggacggcagasan-3′). F2 corresponding to amino acids 1733– 
1906 was made using an F2 forward primer (5′-ggagggcctctgaactgcatttg-3′) and the SK-reverse primer. Both F1 and F2 primers were used in the PCR reaction after phosphorylation with T4 polynucleotide ki- 

nase. The F1 and F2 PCR products were ligated together, digested with BamHI/EcoRI, and ligated into BamHI/EcoRI-digested pGEX-2T plasmid. We refer to the GST fusion protein encoded by this plasmid as regulatory domain short (GST-RDS).

**Cell Culture and Transfection**

WB rat liver epithelial cells (23) were grown to confluence in 100-mm dishes in Richter's minimal essential medium containing 5% fetal bovine serum. COS-7 cells were grown to approximately 70% confluency in Dulbecco's modified Eagle's medium supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 5% fetal bovine serum. Cells were transfected with TransIT-LT-1 reagent (PanVerca, Madison, WI) using the procedure recommended by the manufacturer. Routinely, 20–30 μg of DNA was used for transfecting a single 75-cm² flask. Unless otherwise stated, lysates were prepared 48 h after transfection using WB solubilization buffer (150 mM NaCl, 50 mM Tris-HCl (pH 7.8), 1% Triton X-100 (w/v), 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 μl dilution of a commercial mixture of protease inhibitors (Roche Molecular Biochemicals)).

**Calmodulin-Sepharose Binding Assay**

Fusion proteins (20 μg of protein) or lysates from transfected cells were incubated with 50 μl of CaM-Sepharose (50% v/v slurry) in a final volume of 500 μl in buffer A (0.2 M NaCl, 20 mM Tris-HCl (pH 7.4), 0.2% Triton X-100, and 0.5 mM CaCl2) or buffer B (buffer A in which 0.5 mM CaCl2 was replaced by 0.5 mM EGTA). After 90 min at 4 °C, the samples were centrifuged (12,000 × g) and the CaM-Sepharose beads were washed once in each buffer A or B. The fusion protein remaining in the supernatant was subjected to the addition of 30 μl of Streatacin beads (Stratagene). After 30 min at 4 °C, the supernatants were filtered and subjected to the binding assay. Bound proteins were solubilized and analyzed by SDS-PAGE and immunoblotting. Fusion proteins were detected on the immunoblotting with a type I IP3R Ab (ABR-Ab) recog- 

nizing an epitope common to both GST fusion proteins (corresponding to amino acids 1885–1903 in type-1 long) (Affinity Bioreagents, Golden, CO). Full-length type-I IP3Rs were detected on immunoblots with CT-1 Ab. The concentration of type-I long and short IP3R isoforms in COS cell lysates was determined as described previously (25). Briefly, lysates from transfected COS cells were subjected to two consecutive rounds of immunoprecipitation with CT-1 Ab; the first, overnight, and the second, for 2 h. The recombinant IP3Rs in the pooled immunoprecipitates could be easily visualized by Coomassie staining of 5% SDS-PAGE gels. The magnitude of the staining in different amounts of lysate protein was quantitated by densitometry and calibrated with reference to a standard curve of myosin run on the same gel. The concentration of IP3R was calculated after correction for the difference in the molecular weight of myosin and IP3Rs. Values for expression ranged from 24 to 46 fmol of IP3R/μg of lysozomal protein, which is in the range of values observed in the cerebellum (25).

**Fluorescence Assay for the Binding of Danysl-Calmodulin**

Spectra were collected between 400 and 600 nm with a PTI Alphap- 
can fluorimeter (Photon Technology Instruments, Princeton, NJ) using an excitation wavelength of 340 nm. The buffer used for these assays (2 ml) contained 20 mM Tris-HCl (pH 8.0), 250 mM NaCl, 5 mM MgCl2, and 100 mM EGTA.

**32P Incorporation**

The GST-RDL and -RDS were incubated in 50 μl of the phosphory- 
lation reaction buffer (120 mM KCl, 50 mM Tris-HCl (pH 7.2), 0.3 mM MgCl2, 5 μCi of [γ-32P]-ATP) with or without protein kinase A (5 units) for 30 min at 37 °C. The reaction was terminated with SDS-PAGE solubilization buffer. The sample was run on 10% polyacrylamide gels and transferred to nitrocellulose membranes, and labeled polypeptides were visualized by autoradiography. In some experiments, the nitrocel- 

lulose was immunoblotted with ABR-Ab to locate the fusion proteins. An aliquot of the permeabilized cells (0.8 ml) was incubated with 0.8 ml of a label medium containing 120 mM KCl, 20 mM Tris-Hepes (pH 7.2), 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of aprotinin, leupeptin, and soybean trypsin inhibitor. After 1 h, the sample was analyzed by SDS-PAGE and autoradiography. An ali- 

quot of the permeabilized cells (0.8 ml) was incubated with 0.8 ml of a label medium containing 120 mM KCl, 20 mM Tris-Hepes (pH 7.2), 10 mM [γ-32P]-ATP and different amounts of CaCl2. The Ca2⁺ concentrations required to generate a range of free Ca2⁺ concentrations were measured with a Ca2⁺-sensitive mini-electrode calibrated with reference to com- 

mercial standards (WPI, Sarasota, FL). After incubation on ice for 5
Characteristics of the GST fusion proteins encoding the short and long splice variants of the type-I IP_3R. Panel A shows a schematic diagram of the boundaries of the type-I IP_3R regulatory domain encoded by the GST-long construct used in the present study together with the SII region that is deleted in the GST-short construct (SII; amino acids 1692–1732). Also shown are the c-AMP regulatory domain encoded by the GST-long construct used in the present study together with the SII region that is deleted in the GST-short construct. Panel B shows a schematic diagram of the boundaries of the type-I IP_3R.
short and long forms, respectively. The corresponding $E_{\text{max}}$ values (expressed as $F_F_0$) were 1.22 and 1.14. The changes in dansyl CaM fluorescence were diminished when the fusion proteins were preincubated using the buffer conditions required for phosphorylation (data not shown). This precluded the use of this method for reliable measurements of the effects of phosphorylation on dansyl-CaM binding.

**Ca\textsuperscript{2+} Sensitivity of Ligand Binding to Transfected IP\textsubscript{3}Rs**—Previous studies have shown that COS-7 cells have predominantly type-III and type-II IP\textsubscript{3}Rs with negligible levels of type-I IP\textsubscript{3}Rs (25). The plasmid encoding the GST-RDS was used to construct a full-length type-I short form of IP\textsubscript{3}R as described under “Experimental Procedures.” This cDNA was transiently transfected into COS-7 cells, and the $Ca\textsuperscript{2+}$ sensitivity of $[^{3}H]IP_3$ binding in saponin-permeabilized cells was measured and compared with COS-7 cells transfected with the full-length type-I long or type-III IP\textsubscript{3}R (Fig. 4). The levels of $[^{3}H]IP_3$ binding to mock-transfected cells was low (72 fmol/mg of protein) when compared with cells transfected with IP\textsubscript{3}Rs (230–1350 fmol/mg of protein). Increasing concentrations of $Ca\textsuperscript{2+}$ inhibited $[^{3}H]IP_3$ binding to mock-transfected cells and expressing type-I short, type-I long, or the type-III IP\textsubscript{3}R isoform. Binding to the type-I long isoform was more sensitive to $Ca\textsuperscript{2+}$ inhibition than the type-I short isoform (Fig. 4A). Low concentrations of $Ca\textsuperscript{2+}$ have been reported to stimulate $[^{3}H]IP_3$ binding to the type-III IP\textsubscript{3}R isoform expressed in Sf9 cells (27, 28). Only an inhibition of binding was observed in COS-7 cells transiently transfected with the type-III IP\textsubscript{3}R isoform (Fig. 4B). The $IC_{50}$ for $Ca\textsuperscript{2+}$ inhibition of the transfected IP\textsubscript{3}Rs isoforms were $0.55 \pm 0.05 \mu M$ (type-I long), $5.7 \pm 0.3 \mu M$ (type-I short), and $3.3 \pm 0.1 \mu M$ (type-III) (mean $\pm S.E., n = 3$). Although the absolute levels of $[^{3}H]IP_3$ binding achieved in these experiments varied widely, the $IC_{50}$ values for each IP\textsubscript{3}R isoform was independent of the absolute level of binding (Fig. 4C).

**CaM Binding to Full-length IP\textsubscript{3}Rs**—We compared the CaM binding properties of full-length type-I long and short isoforms expressed in COS cell lysates (Fig. 5A). For the comparison to be valid it is necessary to add equivalent concentrations of both isoforms. Silver staining of gels containing immunoprecipitated IP\textsubscript{3}Rs has been used as a method to quantify IP\textsubscript{3}Rs in several cell types (25). We utilized the same approach, with the exception that the high levels of IP\textsubscript{3}Rs in transfected COS cell lysate allowed the use of Coomassie Blue to detect protein. Using equivalent concentrations of type-I short and long isoforms, it was apparent that the short isoform bound more CaM than the long isoform (Fig. 5A). The binding data for the short isoform could be fitted by an equation describing association with a single class of binding sites having a $K_{d}$ for CaM of 0.9 $\pm$ 0.03 $\mu M$ (based on the concentration of CaM immobilized to the Sepharose beads) and a maximal binding of 47 $\pm$ 3.8% of the total IP\textsubscript{3}R. By contrast, the binding to the long isoform was sigmoidal and had a much lower affinity for CaM-Sepharose ($EC_{50} = 3.1 \pm 0.3 \mu M$) with a maximal binding of 25 $\pm$ 1.6% of the total IP\textsubscript{3}R. The $Ca\textsuperscript{2+}$ concentration dependence of the CaM binding measured in the presence of a saturating amount of CaM-Sepharose was not different between the two isoforms (Fig. 5B). Fig. 5C shows data from experiments in which equivalent concentrations of IP\textsubscript{3}Rs in COS cell lysates were incubated in the presence and absence of protein kinase A before measurement of binding to CaM-Sepharose. The data show that incubation with protein kinase A caused a 26% inhibition of CaM binding of the short isoform but did not significantly affect CaM binding to the long isoform.

**CaM Binding to IP\textsubscript{3}R Hetero-oligomers**—The CaM binding sequence motif identified in the type-I IP\textsubscript{3}R is conserved in the type-II IP\textsubscript{3}R but is absent in the type-III IP\textsubscript{3}R isoform (16). To address the question of whether type-I/III IP\textsubscript{3}R hetero-oligomers would retain the ability to bind to CaM, we carried out experiments using WB cell lysates that contain both homo- and heterotetrameric type-I and type-III IP\textsubscript{3}Rs (3) but no immunodetectable type-II IP\textsubscript{3}Rs (data not shown). WB cell lysates were incubated with CaM-Sepharose, and the fraction remaining in the supernatant was immunoprecipitated with either type-I or type-III IP\textsubscript{3}R Ab (Fig. 6A). Immunoblotting of the fractions with type-I IP\textsubscript{3}R Ab indicated that approximately 70% of the type-I and 50% of the type-III IP\textsubscript{3}R bound to CaM, and the binding was entirely dependent on the presence of Ca\textsuperscript{2+}. There are several reasons for believing that the fraction of type-III IP\textsubscript{3}Rs bound to CaM is likely to represent the type-I-containing hetero-oligomeric pool. First, calmodulin-Sepharose selectively depleted the fraction of type-III IP\textsubscript{3}Rs immunoprecipitated by type-I Ab (Fig. 6A, lower panel, compare lanes 2 and 5). The amount of type-III IP\textsubscript{3}R bound to CaM-Sepharose (Fig. 6A, lower panel, lane 1) was also quantitatively similar to the type-III IP\textsubscript{3}Rs present as type-I/III hetero-oligomers (Fig. 6A, lower panel, lane 5). Fig. 6B shows data from experiments in which type-I homo- and heterotetramers were immunodepleted from the lysates by repeated immunoprecipitation with type-I IP\textsubscript{3}R Ab. This protocol eliminated the binding of type-I IP\textsubscript{3}Rs to CaM-Sepharose (Fig. 6B, upper panel, lane 3). Under these conditions, the remaining type-III IP\textsubscript{3}Rs would be expected to be predominantly homo-tetrameric. Fig. 6B shows...
that the immunodepletion treatment markedly reduced the binding of type-III IP₃Rs to CaM-Sepharose (Fig. 6B, lower panel, compare lanes 1 and 3). A small proportion of the homo-tetrameric type-III IP₃Rs in WB cells (lane 3) and transfected type-III IP₃Rs in COS cells (data not shown) continued to bind to CaM-Sepharose, which may indicate the presence of additional low affinity CaM binding sites on this isoform.

**DISCUSSION**

The present study is the first to compare the properties of the S2 alternatively spliced forms of the type-I IP₃R using the recombinant isoforms expressed in a single cell type. The main findings of the present study were that the S2(-) short form of the type-I IP₃R was different in three respects from the long form of the receptor. (a) The short form bound more CaM, (b) ligand binding to the short form was less sensitive to inhibition by Ca²⁺, and (c) CaM binding to the short form was inhibited by protein kinase A phosphorylation, whereas CaM binding to the long form was insensitive to protein kinase A phosphorylation.

The observation that almost twice as much CaM bound to the short than to the long form using saturating concentrations of CaM was made using both fusion proteins and full-length IP₃Rs. We conclude that deletion of the S2 domain must create an additional binding site for CaM and that this site must lie within the sequences delimited by the fusion protein. One possibility is that the deletion of the S2 region may generate a new CaM binding site from the sequences flanking the splice site, as previously suggested by Islam et al. (11). Analysis of the sequence of the regulatory domain using multiple computer programs that predict tertiary structure indicates that the sequences immediately proximal to the S2 splice site form a helix with hydrophobic residues clustered on one side and positively charged residues clustered on the other (data not shown). Such amphipathic helices have been implicated as CaM binding domains (30), and it is possible that this proximal helix may be differentially exposed in the short and long isoforms.

We have measured the effect of Ca²⁺ on [³²P]IP₃ binding to the type-I long and short IP₃Rs transiently expressed in COS cells. Under the conditions of these experiments the contribution of endogenous IP₃Rs to ligand binding was low. It is well established that ligand binding to the native type-I long IP₃R isoform present in cerebellar membranes is markedly inhibited by Ca²⁺ by a mechanism that involves an accessory protein (2, 31, 32). Inhibition of ligand binding by Ca²⁺ was also observed for both the recombinant type-I long and short IP₃Rs expressed in COS cells. However, the inhibition of the type-I short IP₃R isoform occurred with a 10-fold lower sensitivity. The lower sensitivity to Ca²⁺ is unlikely to be due to altered CaM binding sites, since the short isoform actually bound more CaM and with higher affinity than the long isoform. It is also unlikely that the differences arise from depletion of calmodulin or other endogenous accessory proteins, since the differences in IC₅₀ for Ca²⁺ were maintained over a wide range of expression levels (Fig. 4C). At least eight Ca²⁺ binding sites have been identified in the primary sequence of the type-I IP₃R (15), including one in the vicinity of the S2 domain (Fig. 1A). It is possible that one of these sites is responsible for modulation of ligand binding and that the affinity of this site for Ca²⁺ is different in the alternatively spliced isoforms. An alternative possibility is raised by the recent finding that the addition of CaM inhibits [³²P]IP₃ binding to full-length type-I IP₃Rs (33, 34) and to the ligand binding domain expressed as a fusion protein (35). The affinity for Ca²⁺/CaM at an interaction site in the ligand binding domain of the receptor could be different in the two alternatively spliced isoforms. It should be noted that the effect of CaM on ligand binding occurs in the absence of added Ca²⁺ (33–35). We observe negligible binding of CaM-Sepharose to IP₃Rs in the absence of Ca²⁺, suggesting that the CaM interaction site in the ligand binding domain is not detected by our assay conditions.

We have found that Ca²⁺ inhibited [³²P]IP₃ binding to type-III IP₃Rs expressed in COS cells with an IC₅₀ intermediate between type-I long and short isoforms. This result is in contrast to the stimulatory (27) or biphasic (28) effects of Ca²⁺ on ligand binding reported in studies using the SF9 expression system. The reasons for these differences are unclear but may be due to differences in the ancillary regulatory proteins present in the COS and insect cell expression systems. It is apparent that in COS cells, the behavior of the type-III IP₃R is qualitatively similar to that observed for the type-I IP₃R isoforms. Homo-tetrameric IP₃Rs expressed in SF9 cells do not bind to CaM-Sepharose, presumably because the CaM binding sequence present the regulatory domain of the type-I and type-II isoforms is missing in the type-III IP₃R (16). This suggests that Ca²⁺ binding sites or other CaM interaction sites (such as those identified in the ligand binding domain of the type-I isoform) may be responsible for the modulation of the
equal amounts of long or short IP₃R (0.7 pmol) were incubated in a buffer containing 0.5 mM Ca²⁺ or 1 mM EGTA for 30 min at 4 °C. After centrifugation of the CaM-Sepharose beads, equal aliquots of the supernatants were immunoprecipitated with either type-I IP₃R Ab (lanes 2 and 5) or type-III IP₃R Ab (lanes 3 and 6). The IP₃Rs bound to CaM-Sepharose (lanes 1 and 4) and present in the immunoprecipitates were analyzed by SDS-PAGE and immunoblotting with either type-I or type-III IP₃R Ab. Panel B, lysates (200 µg of protein) were immunodepleted of type-I containing homo- and heterotrimers by three rounds of immunoprecipitation with type-I IP₃R Ab. The IP₃R bound to CaM-Sepharose (lanes 1 and 3) and remaining in the supernatant (lanes 2 and 4) were analyzed by SDS-PAGE and immunoblotting.

**Fig. 5. Binding of full-length type-I long and short IP₃Rs to calmodulin-Sepharose.** Lysates were prepared from COS cells transfected with the long (open circles) and short (closed circles) isoforms of the type-I IP₃R. The amount of IP₃R expressed in the lysates was quantitated after immunoprecipitation and Coomassie staining as described under “Experimental Procedures.” Panel A, lysates containing equal amounts of long or short IP₃R (0.7 pmol) were incubated in a buffer containing 0.5 mM Ca²⁺ and different concentrations of CaM-Sepharose beads for 90 min at 4 °C. The calmodulin-Sepharose beads were centrifuged, and the IP₃Rs remaining in the supernatants were immunoprecipitated overnight with IP₃R Ab and protein A-Sepharose. The amount of IP₃R bound to CaM-Sepharose (P) and in the immunoprecipitates (S) were quantitated by immunoblotting and expressed as a percentage of the total (P/(P + S)). The data shown are the mean ± S.E. of three experiments, with each assay performed in duplicate. Nonlinear regression of the data was carried out using the program Graphpad (Graphpad software Inc, San Diego, CA). The data for the short form were fit using the equation for binding to a single site: Y = Bₘₐₓ × X/(Kₐ₊ X). For the long form, the data was best fit by an equation for a sigmoidal dose response curve: Y = Bₘₐₓ/[1 + 10^((logEC₅₀ - X)/Hill slope)]. In these equations Y is the amount of CaM bound, and X is the CaM concentration. Panel B, lysates containing equal amounts of short or long IP₃R (0.7 pmol) were incubated in buffers containing different concentrations of free Ca²⁺ buffered with 2 mM EGTA and 1 mM HEDTA. Calibration of the Ca²⁺ buffers was carried out with a Ca²⁺ mini-electrode as described previously (29). The amount of IP₃R bound to CaM-Sepharose (50 µl) was quantitated by immunoblotting and expressed as a percentage of the maximum bound. The data show the mean of duplicate assays and is representative of two experiments. Panel C, lysates prepared from COS cells transfected with type-I long and short isoforms were incubated in the presence and absence of protein kinase A (A-kinase) for 30 min at 30 °C. After completion of the phosphorylation reaction, aliquots were incubated with CaM-Sepharose in a Ca²⁺–containing medium for 90 min at 4 °C, and the amount bound in the pellet and remaining in the supernatant was determined as described above. The data shown is the mean ± S.E. of three separate experiments.
dicted that protein kinase A phosphorylation may have distinctly different effects on the functional activity of the type-I short and type-I long IP$_3$R channels. Experiments to examine the effect of phosphorylation on the ion channel activity of individual isoforms remain to be carried out.

The functional activity of IP$_3$-gated ion channels shows a biphasic dependence on Ca$^{2+}$ concentration (reviewed in Ref. 2). An emerging theme in the feedback regulation of many other Ca$^{2+}$ ion channels is that CaM may be constitutively attached to these proteins (43). In the case of L-type voltage-gated Ca$^{2+}$ channel, it is proposed that elevations of Ca$^{2+}$ cause CaM, initially attached at a tethering site, to bind to a separate regulatory site and cause channel inactivation (44–46). The regulatory site has been proposed to be an isoleucine-glutamine (IQ) motif. Two IQ-like CaM binding sites have been identified in the consensus sequences of IP$_3$Rs (47). CaM can be glutamine (IQ) motif. Two IQ-like CaM binding sites have been identified in the consensus sequences of IP$_3$Rs (47). CaM can be detected in immunoprecipitates of IP$_3$Rs obtained from COS cell lysates, suggesting that CaM may also be constitutively bound to IP$_3$Rs (data not shown).

In a recent study Michikawa et al. (48) have shown that the purified cerebellar IP$_3$R channel reconstituted into bilayers does not show an inhibition by Ca$^{2+}$ unless CaM is added. It has also been reported that exogenously added CaM potentiates the Ca$^{2+}$ inhibition of IP$_3$-mediated Ca$^{2+}$ release from permeabilized A7r5 cells (49). This suggests that the biphasic Ca$^{2+}$ dependence is not an intrinsic property of IP$_3$Rs and that CaM may mediate the Ca$^{2+}$-dependent inhibition of these channels. Whether the CaM binding examined in the present study is a regulatory site involved in the inhibitory effects of Ca$^{2+}$ on IP$_3$R channel function is presently unknown. However, our data suggest that the number and affinity of CaM binding sites may be different between IP$_3$R isoforms and may be additionally modulated by other regulatory factors, such as protein kinase A phosphorylation.

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