Modulation of Inositol 1,4,5-Trisphosphate Binding to the Recombinant Ligand-biding Site of the Type-1 Inositol 1,4,5-Trisphosphate Receptor by Ca$^{2+}$ and Calmodulin*

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A recombinant protein (Lbs-1) containing the N-terminal 581 amino acids of the mouse type 1 inositol 1,4,5-trisphosphate receptor (IP$_3$R-1), including the complete IP$_3$-binding site, was expressed in the soluble fraction of *E. coli*. The characteristics of IP$_3$ binding to this protein were similar as observed previously for the intact IP$_3$R-1. Ca$^{2+}$ dose-dependently inhibited IP$_3$ binding to Lbs-1 with an IC$_{50}$ of about 200 nM. This effect represented a decrease in the affinity of Lbs-1 for IP$_3$, because the $K_d$ increased from 115 ± 15 nM in the absence to 196 ± 18 nM in the presence of 5 μM Ca$^{2+}$. The maximal effect of Ca$^{2+}$ on Lbs-1 (5 μM Ca$^{2+}$, 42.0 ± 6.4% inhibition) was similar to the maximal inhibition observed for microsomes of insect Sf9 cells expressing full-length IP$_3$R-1 (33.8 ± 10.2%). Conceivably, the two contiguous Ca$^{2+}$-binding sites (residues 304–450 of mouse IP$_3$R-1) previously found by us (Sienaert, I., Missiaen, L., De Smedt, H., Parys, J.B., Sipma, H., and Casteels, R. (1997) *J. Biol. Chem.*, 272, 25899–25906) mediate the effect of Ca$^{2+}$ on IP$_3$ binding to IP$_3$R-1. Calmodulin also dose-dependently inhibited IP$_3$ binding to Lbs-1 with an IC$_{50}$ of about 3 μM. Maximal inhibition (10 μM calmodulin, 43.1 ± 5.9% inhibition) was similar as observed for Sf9-IP$_3$R-1 microsomes (35.8 ± 8.7%). Inhibition by calmodulin occurred independently of Ca$^{2+}$ and was additive to the inhibitory effect of 5 μM Ca$^{2+}$ (together 74.5 ± 5.1%). These results suggest that the N-terminal ligand-binding region of IP$_3$R-1 contains a calmodulin-binding domain that binds calmodulin independently of Ca$^{2+}$ and that mediates the inhibition of IP$_3$ binding to IP$_3$R-1.

Most cell types express inositol 1,4,5-trisphosphate receptors (IP$_3$R) mediating IP$_3$-induced release of Ca$^{2+}$ from internal Ca$^{2+}$ stores. Three IP$_3$R isoforms that differ in structure, IP$_3$ binding characteristics, and regulation have been identified (1). Submicromolar [Ca$^{2+}$]$_i$ inhibits IP$_3$ binding to cerebellar microsomes (2–7) and to microsomes of Sf9 insect cells expressing IP$_3$R-1 (8, 9). Inhibition of IP$_3$ binding to IP$_3$R-1 by Ca$^{2+}$ might constitute one of the components inducing the descending phase of the bell-shaped dependence of IP$_3$-induced Ca$^{2+}$ release on cytoplasmic Ca$^{2+}$ (10–13). There is still controversy about the molecular mechanism responsible for the inhibitory effect of Ca$^{2+}$ on IP$_3$ binding, because experiments on purified IP$_3$R-1 have given conflicting results. It was suggested that Ca$^{2+}$ acts directly on a Ca$^{2+}$-binding site on IP$_3$R-1 (7). On the other hand, indirect inhibition via an accessory Ca$^{2+}$-binding protein like calmedin was also reported (4). Moreover, it is still a matter of debate whether Ca$^{2+}$-induced inhibition is caused by a decrease in the affinity of IP$_3$R-1 for IP$_3$ (5, 7, 8) or by a Ca$^{2+}$-induced reduction in IP$_3$-binding sites (9).

Recently, calmodulin was reported to cause inhibition of IP$_3$ binding (14, 15). Calmodulin would in this case bind to a site different from the Ca$^{2+}$-dependent calmodulin-binding site found earlier (16), because calmodulin was able to bind to IP$_3$R-1 and inhibit IP$_3$ binding to IP$_3$R-1 in a Ca$^{2+}$-independent manner (14, 15).

The N-terminal part of the IP$_3$R contains all the structural determinants responsible for specific and selective binding of its physiological agonist, IP$_3$ (17–20). We have therefore expressed the N-terminal ligand-binding site (first 581 amino acids) of the mouse IP$_3$R-1 in *Escherichia coli*, using a strategy of growth and expression at low temperatures, as described previously by Yoshikawa et al. (20). This protein contains a previously identified Ca$^{2+}$-binding region located between amino acids 304–450 (21). We now demonstrate that Ca$^{2+}$ and calmodulin can both inhibit IP$_3$ binding to this recombinant protein and that these inhibitors act independently and additively. Our data indicate that the N-terminal ligand-binding domain of IP$_3$R-1 contains regulatory regions directly interacting with Ca$^{2+}$ and calmodulin.

**EXPERIMENTAL PROCEDURES**

**Expression of IP$_3$R-1 in Sf9 Insect Cells**—The full-length neuronal mouse IP$_3$R-1 cDNA clone containing the S1 splice domain in p400C1 plasmid vector (22) was kindly provided by Drs. K. Mikoshiba and A. Miyawaki (University of Tokyo, Tokyo, Japan). The 5′-untranslated region of the original p400C1 clone was removed by polymerase chain reaction (PCR) by amplification of the 5′-terminal part up to the Cel-II restriction site (nucleotide 555), before subcloning the IP$_3$R-1 cDNA in the baculovirus (*Autographa californica*) transfer vector pVL 1393 (Invitrogen). Recombinant virus was produced in *Spodoptera frugiperda* (Sf9) cells by cotransfection of the pVL 1393 IP$_3$R-1 construct and the linearized *A. californica* nuclear polyhedrosis virus DNA (BaculoGold, Pharmingen). The recombinant viruses were purified by isolating individual plaques of transfected cells. These clonal viral populations were amplified by infecting Sf9 cells. The recombinant protein was harvested.
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2 days after infection of the SF9 cells with the amplified virus at a multiplicity of infection of 2–6.

Construction of a Vector Encoding the Ligand-Binding Site of IP$_3$R-1—A bacterial expression vector (pET 21b+; Novagen Inc.) was used to express the N-terminal 581 amino acids of the mouse IP$_3$R-1, including the coding sequence for 1) amino acid 1–64 and 2) amino acid 471–581, using SalI cut p400CI containing cDNA (9448 base pairs) encoding the mouse IP$_3$R-1 (22) as a template. Fragment 1 was produced with forward primer 5’-CCGCATATGCTGACAAATTGCGG-3’ (containing an NdeI site including the start codon) and reverse primer 5’-GTATGGGGCCGTTACATGAGG-CAAAACTGCTTG-3’ (containing the codon for methionine-581, directly followed by a stop codon and a NdeI site). PCR fragment 2 was cut with NdeIII and NolI and ligated into the NdeIII/NolI site of pcDNA3.1 (Invitrogen), yielding pcDNA-P2. The SalI digests of p400CI containing a 329-base pair 5’ noncoding region and the complete coding region of IP$_3$R-1 was first subcloned in the pCI expression plasmid (Promega) to yield pCI-IP$_3$R-1. A 1751-base pair fragment was obtained from pCI-IP$_3$R-1 by partial NheI digest and ligated into the NheI/NolIIII sites of the pcDNA-P2 construct. Subsequently, the 5’-end of this fragment of pcDNA-P2 was ligated into the EcoRI/NolI sites of the bacterial expression vector pET 21b+.

To remove the 5’ noncoding sequences, the resulting construct and PCR fragment 1 were digested with NdeI/NolI and ligated, yielding pET-581. The sequences of pET-581 and of the PCR-amplified part of the pVL 1393 IP$_3$R-1 construct were confirmed by double-stranded sequencing using the Automated Laser Fluorescent sequencing system (Amersham Pharmacia Biotech).

Expression in E. coli—The expression of the recombinant N-terminal 581 amino acids of the IP$_3$R-1 (Lbs-1 (ligand-binding site-1)) was performed essentially as described by Yoshikawa et al. (20). A single colony of E. coli BL21(DE3) transformed with pET-581 was resuspended in 2 ml of LB medium containing 100 μg/ml ampicillin and grown overnight at 27 °C. 1 ml of this culture was diluted in 50 ml of fresh medium (100 μg/ml ampicillin) and grown at 21 °C for 10 h to an A$_{600}$ of about 1.5. Subsequently, the expression of the recombinant protein was induced in the presence of isopropyl-1-thio-β-D-galactopyranoside (0.75 mM) for 20 h at 14–16 °C. Cells were harvested by centrifugation and washed with a buffer containing 10 mM KH$_2$PO$_4$, 30 mM NaHPO$_4$, 153 mM NaCl, pH 7.5.

Preparation of the Soluble Fraction of E. coli—The cell pellet was resuspended in 5 ml of homogenization buffer (HB) containing 10 mM Tris-HCl, pH 7.4, 1 mM β-mercaptoethanol, 0.5% benzamidine, 0.2 μM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 1 μM pepstatin A, and 75 mM aprotinin. This cell suspension was digested with lysosome (0.1 mg/ml) for 30 min at 4 °C, followed by six cycles of freeze-thawing (in liquid nitrogen and at 37 °C) and sonication at 12 kHz, twice for 15 s (probe sonicator, MSE Ltd., Westminister, UK). After centrifugation (1 h, 15000 g), the supernatant containing the soluble fraction of E. coli was stored at −80 °C.

Partial Purification of the Recombinant Protein—The soluble fraction containing the recombinant protein (Lbs-1) was supplemented with 0.3 mM NaCl. The column was washed with 6 column volumes HB supplemented with 0.3 mM NaCl, and Lbs-1 was eluted with 2.5 volumes HB supplemented with 0.8 mM NaCl.

IP$_3$ Binding—[3H]IP$_3$ binding was performed in 100 μl of a solution containing 50 mM Tris-HCl, pH 7.0 or 7.8, 50 mM NaCl, 1 mM EGTA or BAPTA, 10 mM β-mercaptoethanol, 2.5 μM of partially purified Lbs-1, and variable concentrations of [3H]IP$_3$ (see figure legends) at 0 °C for 30 min. Subsequently, 10 μg of γ-globulin (20 mg/ml) and 110 μl of 10% polyethylene glycol in IP$_3$ binding buffer was added for 10 min, and the mixture was quickly filtered through glass fiber filters and washed using a Combi Cell Harvester (Skatron). Activity on the filters was quantified with a Beckman scintillation counter. Non-specific binding was determined in the presence of 10 μM unlabeled IP$_3$ Routinely, specific [3H]IP$_3$ binding amounted to more than 95% of total binding. Scavenging of the Kell Reactor-program (version 5.0.4, Biosoft, Cambridge, UK). A Student’s t test (paired or unpaired) was used for statistical analyses. Values were considered statistically different when p < 0.05.

Microsomes, Antibodies, and Western Blotting—Microsomes of rabbit cerebellum and RBL-2H3 cells (F3 fraction) were prepared as described by Parys et al. (23) and Vanlengen et al. (24). Microsomes of 16HBE14o- cells and SF9 insect cells were prepared as described by Sienaert et al. (25) and Yoneshima et al. (8), respectively. Full-length IP$_3$R-1 was detected with the polyclonal antibody Rbt03. This antibody is directed against the same epitope as the previously described Rbt04 (23–25) and has specificity and affinity identical to those of Rbt04. A second rabbit, directed against the Ca$^{2+}$-binding domain of the C144 epitope present between amino acids 378 and 450 of the mouse IP$_3$R-1 (21). Two rabbits were injected subcutaneously and intramuscularly with Freund’s complete adjuvant containing 0.5 mg of cyt3b fused to glutathione S-transferase. Animals were boosted 2 weeks later with the same antigen in Freund’s incomplete adjuvant and regularly thereafter. Antibodies that produced high titers of antibody. Both these antibodies (anti-cyt3b-1 and anti-cyt3b-2) reacted specifically with IP$_3$R-1 from multiple species including rat, rabbit, and Xenopus. The partially purified soluble fraction of E. coli was analyzed by SDS-PAGE on a 3–12% linear gradient and either stained with Coomasie Blue or SyproTM Orange or transferred to Immobilon-P (Millipore). Blots were blocked for 1 h in a buffer containing 10 mM Na$_2$PO$_4$, 50 mM Na$_2$HPO$_4$, pH 7.5, 154 mM NaCl, 0.1% Tween 20, and 5% milk powder and incubated with the primary antibody for 1 h in the same buffer without milk powder. Alkaline phosphatase-coupled anti-rabbit antibody was used as secondary antibody. The immunoreactivity was visualized by conversion of the VistraTM ECF substrate (Amersham Pharmacia Biotech) using the Storm 540 FluorImager, equipped with the Imagequant NT24 software (Molecular Dynamics) as described previously (24, 26).

Materials—Adenophostin-A was a gift of Dr. S. Takahashi (27). IP$_3$ was obtained from Roche Molecular Biochemicals. [3H]IP$_3$ was from Amersham Pharmacia Biotech. Restriction enzymes were from New England Biolabs Inc. or Roche Molecular Biochemicals. T4-DNA ligase was from Life Technologies, Inc. High purity bovine brain calmodulin was from Calbiochem. Polyethylene glycogen, γ-globulins, and heparinagarose beads were obtained from Sigma. SyproTM Orange was from Bio-Rad. Ethylene glycolbis(sulfosuccinimidylsuccinate) was from Pierce.

RESULTS AND DISCUSSION
Expression of the Full-length Mouse IP$_3$R-1 in SF9 Insect Cells—Microsomes of SF9 insect cells transfected with pVL 1393 IP$_3$R-1 (SF9-IP$_3$R-1) were immunoreactive to an antibody specifically recognizing a C-terminal epitope of mouse IP$_3$R-1 (Fig. IA). Under identical conditions, no IP$_3$R was detected in control pVL 1393 transfected SF9 cells. The expressedIP$_3$R-1 migrated on SDS-PAGE with the same apparent molecular mass as observed for IP$_3$R-1 from rabbit cerebral microsomes and amounted to 2.5 times the value of the latter (Fig. IA). In the presence of 5 mM [3H]IP$_3$, microsomes of SF9 cells expressing IP$_3$R-1 specifically bound IP$_3$. The binding activity (589 ± 83 fmol/mg at pH 7.8) was also about 2.5 times the value found for rabbit cerebellar microsomes (252 ± 43 fmol/mg). Microsomes of control pVL 1393 transfected SF9 cells showed no significant IP$_3$ binding under these conditions.

Expression of the IP$_3$-binding Domain of the IP$_3$R-1—We constructed a bacterial expression vector containing the coding sequence of the N-terminal 581 amino acids of the mouse IP$_3$R-1 (pET-581). The recombinant protein (Lbs-1) was expressed in E. coli using a strategy described earlier by Yoshikawa et al. (20). Lbs-1 was partially purified on a heparinagarose column and migrated with an apparent molecular mass of 66 kDa on a SDS-polyacrylamide gel (Fig. 1C). The protein reacted with the anti-cyt3b-1 polyclonal antibody, which specifically recognized an epitope in the N terminus of the IP$_3$R-1 (Fig. 1, B and C). Although some degradation of the 66-kDa protein occurred, it was determined by quantitative analysis of the fluorescein signal obtained in immunoblots (Fig. 1C) and of the proteins separated by SDS-PAGE and stained with the fluorescent dye SyproTM (data not shown) that at least 80% of the recombinant protein was in the intact form. Degradated proteins missing the primary antibody-epitope (amino acids 378–450 (21)) or smaller than 40 kDa will not bind IP$_3$ (20) and will therefore not influence IP$_3$ binding.
Measurements. The isolated IP$_3$-binding domain was likely to exist as a monomer, because it did not contain structural elements necessary for multimerization (18, 28). We investigated possible multimerization of Lbs-1 by chemical cross-linking experiments. After treatment with the bifunctional cross-linker glutathione S-transferase (data not shown), a protein known to form dimers (29, 30) and used here as positive control. However, this treatment failed to covalently link molecules of Lbs-1 with each other or with other proteins in the suspension, either in the presence or the absence of 5 mM IP$_3$, 1 mM adenosphin-A, or 5 mM Ca$^{2+}$ (data not shown). These results strongly suggest that Lbs-1 is a monomeric polypeptide.

Characterization of IP$_3$ Binding to Lbs-1—Fractions of partially purified Lbs-1 specifically bound [H]$[^3]$IP$_3$ at a pH of 7.8, whereas a similar fraction from E. coli that was only transformed with host pET 21b+ did not bind IP$_3$ under these conditions. [H]$[^3]$IP$_3$ was displaced from Lbs-1 by unlabeled IP$_3$ with an IC$_{50}$ of 60 nM (Fig. 2). The displacement data could best be fitted using a single-site Scatchard model, yielding a $K_d$ of 46 ± 4 nM and a $B_{max}$ of 280 ± 60 pmol/mg (n = 3).

Effect of Ca$^{2+}$ on IP$_3$ Binding to Sf9-IP$_3$-R-1 and Lbs-1—Submicromolar [Ca$^{2+}$] was found to inhibit IP$_3$ binding to IP$_3$-R-1 from cerebellum (2–7) or expressed in insect Sf9 cells (8, 9). Lbs-1 contains two amino acid sequences (304–381 and 308–460) that were found to bind Ca$^{2+}$ (21). These sites could be found for the intact purified mouse cerebellum IP$_3$-R-1, obtained under similar experimental conditions (IC$_{50}$, 76 nM; Hill coefficient, 1.1 (31); $K_d$, 37 nM (20)). Furthermore, we have previously reported a $K_d$ of 46 ± 17 nM for the purified Xenopus IP$_3$-R-1 under identical experimental conditions but at a slightly higher pH (13). These findings are in agreement with earlier reports, demonstrating that recombinant proteins, containing the first 788 (17), 734 (20), or 576 amino acids (19) of the IP$_3$-R-1 showed similar specificity for inositol phosphates and similar affinities for IP$_3$ as the intact IP$_3$-R-1. These observations indicate that Lbs-1 is in the right conformation to act as a bona fide IP$_3$-binding pocket.
values of 115 ± 15 and 196 ± 18 nm IP₃, respectively, whereas the Bₘₐₓ values were not significantly different (330 ± 30 and 410 ± 60 pmol/mg, respectively) (Fig. 5). This indicates that Ca²⁺ reduced the affinity of the IP₃-binding site without an effect on the number of binding sites. Furthermore, because Lbs-1 was isolated in the absence of Ca²⁺ chelators, the protein was exposed to micromolar Ca²⁺ concentrations for some time. The fact that after this treatment IP₃ binding was higher in the presence of only Ca²⁺ chelators than in the presence of chelators and/or calmodulin for 5 min before adding [³H]IP₃. Specific binding of IP₃ to microsomes of Sf9-IP₃R-1 cells amounted to 151 ± 18 fmol/mg (about 1000 dpm), whereas microsomes of pVL 1393-transfected Sf9 cells did not significantly bind IP₃. Specific binding to Lbs-1 was 8.5 ± 0.7 pmol/mg (2000 dpm, no binding to soluble fraction of control bacteria treated the same way as the Lbs-1 containing fraction). *, significantly different from binding in the absence of modulators. Data are expressed as the means ± S.E. of at least four experiments, consisting of independent quadruplicates.

![Fig. 3. Binding of IP₃ to Lbs-1 and microsomes of Sf9-IP₃R-1 insect cells. ([³H]IP₃ binding to microsomes of Sf9-IP₃R-1 cells (100 µg, A) and partially purified Lbs-1 (2.5 µg, B) was measured in the presence or absence of Ca²⁺ (5 µM) and/or calmodulin (10 µM) and was expressed as the percentage of binding in the absence of these modulators (control). Binding was measured at pH 7.0 in the presence of 1 mM EGTA, 3.6 nM [³H]IP₃. Sf9-IP₃R-1 microsomes and partially purified Lbs-1 were pre-exposed to Ca²⁺ and/or calmodulin for 5 min before adding [³H]IP₃. Specific binding of IP₃ to microsomes of Sf9-IP₃R-1 cells amounted to 151 ± 18 fmol/mg (about 1000 dpm), whereas microsomes of pVL 1393-transfected Sf9 cells did not significantly bind IP₃. Specific binding to Lbs-1 was 8.5 ± 0.7 pmol/mg (2000 dpm, no binding to soluble fraction of control bacteria treated the same way as the Lbs-1 containing fraction). *, significantly different from binding in the absence of modulators. Data are expressed as the means ± S.E. of at least four experiments, consisting of independent quadruplicates.]

![Fig. 4. The effect of different [Ca²⁺] on IP₃ binding to Lbs-1. ([³H]IP₃ binding to partially purified Lbs-1 (3.75 µg) in the presence of the indicated concentrations of Ca²⁺ was expressed as a percentage of the binding measured in Ca²⁺-free buffer with 1 mM EGTA at pH 7.0 (A) or 1 mM BAPTA at pH 7.8 (B). The different Ca²⁺ concentrations were buffered with 1 mM EGTA or BAPTA and calculated with the Maxchelator software (Dr. C. Patton, Stanford University, Stanford, CA). *, significantly different from binding in Ca²⁺-free buffer. Data are expressed as the means ± S.E. of at least four experiments, consisting of independent quadruplicates.]

![Fig. 5. Scatchard analysis of IP₃ binding to Lbs-1 in the presence and absence of Ca²⁺. Partially purified Lbs-1 (3.75 µg) was incubated with 3.6 nM [³H]IP₃ at pH 7.0 and increasing concentrations of unlabeled IP₃ in the absence ([]) or presence ([ ]) of 5 µM Ca²⁺. Data are expressed as means of three independent determinations of one experiment repeated twice with similar results.]

Because there are at least five additional potential interaction sites with Ca²⁺ in the cytosolic domains (21), a more complex dependence on Ca²⁺ for the intact receptor is not unexpected. Our data indicate that there is a direct interaction of Ca²⁺ with the IP₃-binding domain, but this interaction may represent only part of the feedback mechanism that controls IP₃-induced Ca²⁺ release.

Remarkably, the effect of Ca²⁺ on IP₃ binding was pH-dependent. No inhibition was observed at a pH of 7.8 (Fig. 4B). In these experiments, BAPTA was used as chelating agent. It has been suggested that high doses of Ca²⁺-free chelators, espe-
cially BAPTA, can inhibit IP$_3$ binding (32, 33). The inhibitory effect of Ca$^{2+}$ on IP$_3$ binding might therefore be shielded by a stimulatory effect of Ca$^{2+}$ in relieving inhibition by the chelator. IP$_3$ binding to Lbs-1 (pH 7.8) was, however, identical in the presence of 1 mM BAPTA, 0.1 mM BAPTA, 0.1 mM BAPTA and 5 $\mu$m free Ca$^{2+}$, and 1 mM EGTA (data not shown). Therefore, we can exclude the possibility that the absence of an effect of Ca$^{2+}$ on IP$_3$ binding at pH 7.8 was caused by effects of BAPTA. As shown above, lowering the pH from 7.8 to 7.0 caused an increase of the $K_d$ value for the binding of IP$_3$ to pET-581 from 46 to 115 nM. The enhancement of IP$_3$ binding to the IP$_3$-R-1 at a higher pH is a well documented phenomenon (5, 34). It can be suggested that the different conformational states of the IP$_3$-R-1 that apparently accompany changes in pH are unequally susceptible to inhibition by Ca$^{2+}$.

Because Lbs-1 was expressed in a bacterial environment, the inhibitory effect of Ca$^{2+}$ on IP$_3$ binding to Lbs-1 strongly suggests direct binding of Ca$^{2+}$ to the N-terminal IP$_3$-binding domain of IP$_3$-R-1 and strongly disfavors the idea of involvement of accessory proteins, such as calmedin (4). The hypothesis of direct binding of Ca$^{2+}$ to IP$_3$-R-1 is in agreement with results obtained by Picard et al. (7), who showed that Ca$^{2+}$ could still inhibit IP$_3$ binding to rat cerebellar microsomes after removal of peripheral membrane proteins with high alkaline treatment and to immunopurified sheep cerebellar IP$_3$-R.

We have previously demonstrated direct Ca$^{2+}$ binding (21) to a stretch of amino acids (304–450) located in the "core" IP$_3$-binding domain (20). Most likely, Ca$^{2+}$ exerts its inhibitory effect on IP$_3$ binding by interacting with this particular region. Our data indicate that the modulation of the IP$_3$ affinity by cytosolic Ca$^{2+}$ is an inherent property of the IP$_3$-binding domain. Unfortunately, it may be very difficult, if not impossible, to determine the amino acid residues critically involved in the inhibitory effect of Ca$^{2+}$ on IP$_3$ binding because mutations and deletions in this region will almost certainly also affect the characteristics of IP$_3$ binding or eliminate IP$_3$ binding (20).

Our data give further strong support to the idea that Ca$^{2+}$, in a physiological range, is able to inhibit IP$_3$ binding to IP$_3$-R-1. This mechanism is likely to contribute to feedback inhibition of IP$_3$-induced Ca$^{2+}$ release by (sub)micromolar [Ca$^{2+}$] (10–13).

**Effect of Calmodulin on IP$_3$ Binding to Lbs-1**—In the presence of Ca$^{2+}$, calmodulin binds to the regulatory domain of the type-1 and -2 IP$_3$-R (16). Recently, it was shown that calmodulin can also bind to IP$_3$-R-1 in the absence of Ca$^{2+}$, thereby inhibiting IP$_3$ binding to the purified cerebellar IP$_3$-R (14) and to microsomes of insect Sf9 cells expressing rat IP$_3$-R-1 (15). In our hands, calmodulin (10 $\mu$m) inhibited IP$_3$ binding to microsomes of Sf9 cells expressing IP$_3$-R-1 and to Lbs-1 by 35.8 ± 8.7 and 43.1 ± 5.9%, respectively (Fig. 3, A and B). These values were similar to maximal inhibition (10 $\mu$m calmodulin) reported for Sf9 microsomes (40% (15)) and rat cerebellum microsomes (36% (14)). In the presence of both Ca$^{2+}$ (5 $\mu$m) and calmodulin (10 $\mu$m), IP$_3$ binding was inhibited by 74.5 ± 5.1% (i.e. 50% of the level in the presence of only Ca$^{2+}$). Therefore, inhibition by calmodulin was Ca$^{2+}$-independent and was additive to inhibition by Ca$^{2+}$, as suggested previously for the intact IP$_3$-R-1 (14, 15). As was also observed for the effect of Ca$^{2+}$, inhibition of IP$_3$ binding by calmodulin does not seem to depend on the presence or absence of the S1 splice domain. The effect of calmodulin on IP$_3$ binding to Lbs-1 was concentration-dependent (Fig. 6A). Calmodulin half-maximally inhibited IP$_3$ binding at a concentration of about 3 $\mu$m, assuming maximal inhibition of IP$_3$ binding at 10 $\mu$m (14, 15). This value is similar to the one found for the purified cerebellar IP$_3$-R (14) but is three times higher than that observed for IP$_3$-R-1 expressed in Sf9 cells (15). The inhibitory effect of calmodulin was completely abolished at higher pH (Fig. 6B). This was also reported by Patel et al. (14) and is most likely due to an altered conformation of calmodulin at higher pH. High pH is also known to block Ca$^{2+}$-independent interaction of calmodulin with the ryanodine receptor (35).

Our results on calmodulin therefore confirm and extend the results obtained for the intact IP$_3$-R-1 by Taylor and co-workers (14, 15) and suggest that a Ca$^{2+}$-independent interaction site for calmodulin is located in the N-terminal ligand-binding domain of the IP$_3$-R-1.

**Conclusions**—We have expressed the N-terminal 581 amino acids of IP$_3$-R-1 containing the complete IP$_3$-binding site in E. coli. Ca$^{2+}$-dose-dependently inhibited IP$_3$ binding to this protein by decreasing its affinity for IP$_3$. Conceivably, this inhibition is mediated by one of the Ca$^{2+}$-binding sites that we have previously located within the core IP$_3$-binding pocket of the receptor. Furthermore, calmodulin inhibited IP$_3$ binding to the recombinant ligand-binding site independently of Ca$^{2+}$. In conclusion, we found functional evidence for both a Ca$^{2+}$-binding site and a calmodulin-binding site in the N-terminal ligand-binding domain of IP$_3$-R-1.

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