CIB1, a Ubiquitously Expressed Ca\(^{2+}\)-binding Protein Ligand of the InsP\(_3\) Receptor Ca\(^{2+}\) Release Channel\(^*\)**

Received for publication, March 7, 2006, and in revised form, May 23, 2006 Published, JBC Papers in Press, May 24, 2006, DOI 10.1074/jbc.M602175200

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A family of Ca\(^{2+}\)-binding proteins (CaBPs) was shown to bind to the inositol 1,4,5-trisphosphate receptor (InsP\(_3\)-R) Ca\(^{2+}\) release channel and gate it in the absence of InsP\(_3\), establishing them as protein ligands (Yang, J., McBride, S., Mak, D.-O. D., Vardi, N., Palczewski, K., Haeseeler, F., and Foskett, J. K. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 7711—7716). However, the neuronally restricted expression of CaBP and its inhibition of InsP\(_3\)-R-mediated Ca\(^{2+}\) signaling when overexpressed (Kasri, N. N., Holmes, A. M., Bullynck, G., Parys, J. B., Bootman, M. D., Riedtbro, K., Missiaen, L., McDonald, F., De Smedt, H., Conway, S. J., Holmes, A. B., Berridge, M. J., and Roderick, H. L. (2004) EMBO J. 23, 312—321; Haynes, L. P., Tepikin, A. V., and Burgoyne, R. D. (2004) J. Biol. Chem. 279, 547—555) have raised questions regarding the functional implications of this regulation. We have discovered the Ca\(^{2+}\)-binding protein CIB1 (calmyrin) as a ubiquitously expressed ligand of the InsP\(_3\)-R. CIB1 binds to all mammalian InsP\(_3\)-R isoforms in a Ca\(^{2+}\)-sensitive manner dependent on its two functional EF-hands and activates InsP\(_3\)-R channel gating in the absence of InsP\(_3\). In contrast, overexpression of CIB1 or CaBP1 attenuated InsP\(_3\)-R-dependent Ca\(^{2+}\) signaling, and in vitro pre-exposure to CIB1 reduced the number of channels available for subsequent stimulation by InsP\(_3\). These results establish CIB1 as a ubiquitously expressed activating and inhibiting protein ligand of the InsP\(_3\)-R.

Calcium mobilization from the endoplasmic reticulum (ER)\(^2\) through the inositol 1,4,5-trisphosphate (InsP\(_3\)) receptor (InsP\(_3\)-R) Ca\(^{2+}\) release channel is a ubiquitous signaling system that is central to the regulation of numerous cellular processes, ranging from transcription to synaptic plasticity (1). InsP\(_3\)-mediated Ca\(^{2+}\) signals display complex spatial and temporal features that vary among cell types and have been attributed to the subcellular distribution and expression levels of three InsP\(_3\)-R genes with alternatively spliced isoforms (2—4) and complex regulation of the channel by its ligands InsP\(_3\) and Ca\(^{2+}\) (5—7), as well as modulation by ATP (8, 9), phosphorylation (10—12), and protein interactions (7).

The InsP\(_3\)-liganded InsP\(_3\)-R is regulated by [Ca\(^{2+}\)], in a complex manner. Increases of [Ca\(^{2+}\)], from resting levels (50 nM) to \(\sim \)1 \(\mu\)M stimulate channel gating (5, 13—15), enabling the channel to participate in Ca\(^{2+}\)—induced Ca\(^{2+}\) release, which couples the activities of individual InsP\(_3\)-R within channel clusters and transforms local [Ca\(^{2+}\)] signals to global and propagating ones. High [Ca\(^{2+}\)] (>10 \(\mu\)M) achieved in close proximity to the mouths of Ca\(^{2+}\) channels inhibit InsP\(_3\)-R gating (5, 13, 15), which may play a role in both terminating Ca\(^{2+}\) release and preventing channel activation. Notably, InsP\(_3\) and ATP affect channel activity by allosteric modulation of the Ca\(^{2+}\) regulation of the channel (8, 9). Thus, the mechanisms underlying [Ca\(^{2+}\)] regulation of InsP\(_3\)-R activity are of central importance in determining the properties of [Ca\(^{2+}\)] signals in cells. Nevertheless, the molecular details of this regulation are still unknown. The relative roles of Ca\(^{2+}\) binding to the InsP\(_3\)-R and indirect regulation by Ca\(^{2+}\) binding to other proteins have been debated. Thus, it has been suggested that high [Ca\(^{2+}\)] inhibition of the InsP\(_3\)-R is mediated by calmodulin (16) or calmodulin (CaM) (17, 18). Nevertheless, the molecular identity of calmodin has not been established, and recent results strongly suggest that high [Ca\(^{2+}\)] inhibition of the InsP\(_3\)-R is not mediated by CaM (19—21).

We previously identified a biochemical and functional interaction between the InsP\(_3\)-R and a family of CaM-related Ca\(^{2+}\)-binding proteins (CaBPs) (22), a subset of the neuronal Ca\(^{2+}\) sensor (NCS) family of EF-hand-containing proteins that contains eight genes (CaBP1—8) with alternatively spliced forms (23, 24). CaBP1 bound with high affinity (apparent \(K_D < 50 \text{nM}\)) in a Ca\(^{2+}\)-dependent manner within the NH\(_2\)-terminal 600 residues of all three mammalian InsP\(_3\)-R isoforms, a region that encompasses the InsP\(_3\)-binding domain. Of note, in single-channel electrophysiological studies, acute application of recombinant CaBP1, CaBP2, and CaBP5 activated channel gating in the absence of InsP\(_3\). In contrast, CaM neither bound with high affinity nor activated channel gating (22). These studies therefore identified CaBPs as specific protein ligands of the InsP\(_3\)-R channel.

The Ca\(^{2+}\) dependence of the interaction with CaBP1 suggested that the InsP\(_3\)-R could possibly become engaged in vivo as

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* This work was supported by Grant R01-GM056328 from the National Institutes of Health (to J. K. F.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** The on-line version of this article (available at http://www.jbc.org) contains two supplemental figures.

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‡ The abbreviations used are: ER, endoplasmic reticulum; InsP\(_3\), 1,4,5-trisphosphate; InsP\(_3\)-R, inositol 1,4,5-trisphosphate receptor; CaM, calmodulin; CIB1, calcium- and integrin-binding protein; r-CIB1, recombinant CIB1; NCS, neuronal Ca\(^{2+}\) sensor; GST, glutathione S-transferase; CaBP, Ca\(^{2+}\)-binding protein.
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a Ca²⁺-induced Ca²⁺ release channel in the absence of InsP₃ generation and that the interaction might tune the sensitivity of the channel to InsP₃ (22). Subsequent studies revealed that overexpression of CaBP1 attenuated InsP₃R-dependent Ca²⁺ release in intact cells (25, 26). Taken together, the results appear to suggest that CaBPs can tune InsP₃R activity by both activation and inhibition. However, the mechanisms that enable these proteins to both activate and inhibit the channel are not clear.

CaBPs are exclusively expressed in the brain and retina (24). However, it is unknown whether non-neuronal proteins exist that function as protein ligands of the InsP₃R in peripheral tissues. We reasoned that more widely expressed, structurally similar EF-hand proteins might also interact with InsP₃Rs as channel ligands. To facilitate their identification, we have first characterized the structural determinants for CaBP1 binding to the InsP₃R. We found that disruption of any one of three functional EF-hands reduced binding to the InsP₃R, with EF3 and EF4 being particularly important. Based on these observations, we examined other ER-localized proteins with functional EF3 and EF4 for binding to the InsP₃R. We show that one of these, CIB1 (calcium- and integrin-binding protein; also called calmyrin or KIP), a widely expressed protein, bound strongly in a Ca²⁺-dependent manner within the InsP₃-binding region of the InsP₃R. In single channel experiments, CIB1 was found to act as a direct activating ligand of the channel in the absence of InsP₃. However, pre-exposure of the receptor to CIB1 reduced the number of channels available for subsequent activation by InsP₃, and overexpression of CIB1 decreased the amplitude of agonist-induced [Ca²⁺]ᵢ transients in intact cells. Our data identify CIB1 as a widely expressed novel protein ligand of the InsP₃R that gates the channel and inhibits subsequent InsP₃-dependent stimulation.

EXPERIMENTAL PROCEDURES

Cell Culture—COS-7 (Cercopithecus aethiops kidney) and HeLa cells were grown in Dulbecco’s modified Eagle’s medium/high glucose medium containing 10% (v/v) fetal bovine serum (Invitrogen), 100 units/ml-1 penicillin, and 100 μg ml⁻¹ streptomycin. PC12 cells were grown in F-12K medium (ATCC) supplemented with 15% (v/v) horse serum (Invitrogen), 2.5% (v/v) fetal bovine serum, and 100 μg ml⁻¹ penicillin and 100 μg ml⁻¹ streptomycin and maintained in a humidified 95, 5% air, CO₂ atmosphere. Spodoptera frugiperda (SF9) cells were maintained in suspension culture at 27 °C in serum-free SF-900 II SFM medium (Invitrogen).

Molecular Biology and Biochemistry—Cloning of CaBP1 short (GST-s-CaBP1) and NH₂-terminally truncated s-CaBP1 (GST-c-CaBP1) into pGex-6P-1 (Amersham Biosciences), and subsequent generation of GST fusion proteins has been described (22, 24). Using GST-c-CaBP1 as template, EF-hand mutants were generated with the QuikChange™ site-directed mutagenesis kit (Stratagene). Truncation mutants were generated from full-length s-CaBP1 in pGEX-6P-1 as template. CIB1 was subcloned from pBluescript KS(−) (27) into pGex-6P-1, which was then used as template to generate EF-hand mutants (primer sequences available upon request for all reagents described). The NH₂-terminal 521 residues of rat InsP₃R-2 were cloned into pcDNA/V5 (Invitrogen). Other InsP₃R constructs have been described (22). GST fusion proteins were expressed in Escherichia coli (BL-21; Stratagene), and pull-down assays were performed as described (22). Co-immunoprecipitation and Western blot analyses were performed according to standard protocols. To generate untagged CIB1, GST was cleaved from recombinant fusion protein by PreScission protease (Amersham Biosciences) and further purified according to the manufacturer’s instructions.

Electrophysiology of InsP₃R—Patch clamp experiments were performed on isolated Xenopus oocyte (5, 13) and SF9 cell (28) nuclei as described. SF9 cells were washed twice with phosphate-buffered saline and suspended in a nuclear isolation solution containing (in mM): 150 KCl, 250 sucrose, 1.5 β-mercaptoethanol, 10 Tris-HCl, 0.05 phenylmethylsulfonyl fluoride, protease inhibitor mixture (Complete, Roche Applied Science), pH 7.5. Nuclei were isolated using a Dounce glass homogenizer and plated onto a 1-ml glass-bottomed dish containing standard bath solution (in mM): 140 KCl, 10 HEPES, and 0.5 BAPTA (free [Ca²⁺] = 50–100 nM), pH 7.1. The pipette solution contained (in mM): 140 KCl, 0.5 ATP, 10 HEPES, pH 7.1. Free [Ca²⁺] was adjusted by the addition of appropriate Ca²⁺ chelators (5, 13) and routinely measured by indicator dye fluorescence. Experiments were performed at room temperature. Data were acquired using an Axopatch-1D amplifier (Axon Instruments) as described (5). Segments of current traces exhibiting one InsP₃R channel were used for open probability (Pₒ) determinations (29), and single channel analysis was performed using the QuB software (30). Particular consideration was given to the accurate determination of the number of active channels in nuclear membrane patches (Nₛ) from the experimental current records, using the set of criteria described in Ref. 28.

Calcium Imaging of Transfected Cells—CIB1, NCS-1, and s-CaBP1 cDNAs were cloned into pIRE2-EGFP (Clontech) and electroporated (Nucleofector device, Amaxa) according to the manufacturer’s instructions. Transfected cells were seeded onto glass coverslips, cultured for 48 h, transferred to a perfusion chamber on the stage of a microscope (Nikon TE2000), and incubated with fura-2 AM (Molecular Probes; 2 μM) for 60 min at room temperature in normal medium. Cells were continuously perfused with Hanks’ balanced salt solution (Sigma) containing 1.8 mm CaCl₂ and 0.8 mm MgCl₂, pH 7.4. Transfected cells were identified by green fluorescent protein fluorescence; fura-2 was alternately excited at 340 and 380 nm, and emitted fluorescence (510 nm) was collected and recorded using a CCD-based imaging system running Ultraview software (PerkinElmer Life Sciences). Dye calibration was achieved by applying experimentally determined constants to the equation: [Ca²⁺] = Kᵣ × [R]/(Rₘᵢₙ – R).

Analysis and Statistics—Data were summarized as mean ± S.E.; statistical significance of differences between means, assessed using unpaired t-tests, were accepted at the 95% level (p < 0.05).

RESULTS

Structural Determinants of CaBP1 Binding to InsP₃R—Both long and short NH₂-terminal splice variants of CaBP1 (l-CaBP1 and s-CaBP1) bind with high affinity within the InsP₃-binding domain.
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NH₂-terminal 600 residues of the InsP₃R. The interaction was strongly potentiated by Ca²⁺ (Refs. 22 and 25, although see Ref. 26), due in part to Ca²⁺ binding to CaBP1, because a CaBP1 mutant protein with all three functional EF-hands disabled failed to interact. A construct (c-CaBP1) encompassing the COOH-terminal region containing all four functional EF-hands that is shared among the splice variants bound nearly as well as s-CaBP1 (Fig. 1A). Deletion of EF-hands 1 and 2 (EF1, EF2), creating a truncated protein identified as calbrain (31), decreased binding efficiency by ∼50% (Fig. 1A). The substantial binding of this construct suggested that functional EF-hands 3 and 4 are important for InsP₃R binding. Disabling EF1, by replacements with alanines at positions one and three, reduced InsP₃R binding by ∼25% (Fig. 1B), whereas mutating either EF4 or EF3 reduced binding by ∼46 and ∼75%, respectively (Fig. 1B). Although some binding was observed to c-CaBP1 with only EF3 functional, mutation of EF3 and either EF1 or EF4 or all three EF-hands together eliminated binding (Fig. 1B). These data suggest that there is no strict requirement for specific EF-hands but that at least two are necessary, with Ca²⁺-binding EF3 and EF4 of particular importance.

Identification and Characterization of CIB1 as an InsP₃R Ligand-binding Domain-interacting Protein—We hypothesized that other ER-localized proteins with functional EF-hands 3 and 4 might also interact with the InsP₃R. We examined four candidates: sorcin, a penta-EF-hand protein that interacts with the ryanodine receptor Ca²⁺ release channel (32, 33); CIB1 (calmyrin, KIP), a ubiquitously expressed protein with homology to calcneurin B (57%) and calmodulin (54%) (34) that has only EF3 and EF4 of its four EF-hands functional for Ca²⁺ binding; and calsenilin (KChIP3, DREAM) and NCS-1 (frequin), two members of the NCS family that have EF1 non-functional for Ca²⁺ binding (35) (Fig. 2A). CaM, which contains four functional EF-hands, interacts only weakly with the InsP₃R ligand-binding domain, with an apparent affinity over an order of magnitude weaker than that of CaBP1 (22). Similarly, neither sorcin, calsenilin, nor NCS-1 interacted with the channel (Fig. 2B). In contrast, CIB1 was very efficient in pulling down the InsP₃R-1 NH₂-terminal domain from Sf9 cell lysates (Fig. 2B), although its affinity appears to be somewhat weaker than that of CaBP1. These results demonstrate specificity in the interactions of the InsP₃R with EF-hand-containing proteins and establish CIB1 as a protein interactor of the ligand-binding region of the InsP₃R.

GST-CIB1 effectively pulled down endogenously expressed InsP₃R-3 from lysates of COS-7 cells and whole rat lungs (Fig. 3A), as well as an expressed rat InsP₃R-2 InsP₃-binding region from COS-7 cell lysates (Fig. 3A). Thus, CIB1 interacts with all three InsP₃R isoforms. Immunoprecipitation of endogenous InsP₃R-3 co-precipitated endogenous CIB1 from COS-7 and HeLa cells (Fig. 3B), suggesting that the endogenous proteins can interact in vivo.

Ca²⁺ binding to EF-hand-containing proteins, including CaBP1 (36) and CIB1 (37), induces conformational changes that modulate target protein interactions (35, 38). The [Ca²⁺] dependence of CaBP1 binding to InsP₃R was examined previously by quantifying binding of GST-CaBP1 to endogenous COS-7 cell InsP₃R-3 in lysates with [Ca²⁺] fixed...
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![Diagram of InsP₃ Receptor Interaction]

FIGURE 2. Interaction of InsP₃R-1 with EF-hand-containing proteins. A, domain structures of EF-hand-containing proteins showing functional Ca²⁺-binding (filled rectangles) and non-functional (open rectangles) EF-hands. B, lysates of SF9 cells expressing V5-tagged NH₂-terminal 600 residues of InsP₃R-1 (1–600-InsP₃R-1) were incubated with various EF-hand-containing GST fusion proteins. Only s-CaBP1 and CIB1 pulled down the InsP₃R (upper gel). Coomassie Blue staining of GST fusion proteins (lower gel) was used to equalize the amount of GST fusion protein used in each in vitro binding reaction. Results are representative of three experiments.

Increasing [Ca²⁺] from 10 nM to 100 μM strongly enhanced binding by over 10-fold with apparent half-maximal activity at ~1–5 μM (Fig. 4A). The similar [Ca²⁺] dependences of the interactions of CIB1 and CaBP1 with the InsP₃R and the dependence on functional EF-hands in CaBP1 for the interaction suggested that the Ca²⁺-binding EF-hands in CIB1 are important for the interaction (Fig. 1). Disruption of either EF-hand profoundly reduced the interaction between CIB1 and InsP₃R-3 (Fig. 4B), confirming their functional importance. In summary, CIB1 interacts with the ligand-binding region of all three isoforms of the InsP₃R in a Ca²⁺-dependent manner that depends on Ca²⁺ binding to both functional EF-hands, features all highly reminiscent of the interaction of CaBP1 with the InsP₃R.

CIB1 Is a Protein Ligand of the InsP₃R Channel—We showed previously that in vitro interaction of CaBP1 with the InsP₃R activated single channel gating in the absence of added InsP₃ (22). The analogous biochemical interactions of CaBP1 and CIB1 with the InsP₃R suggested that CIB1 might also be functionally active. Single-channel activity of endogenous Xenopus InsP₃R-1 was recorded by patch clamp electrophysiology of outer membranes of nuclei isolated from Xenopus oocytes (5, 13). Robust channel activity (open probability, Pₒ ≈ 0.75) was observed with pipette solutions containing 10 μM InsP₃ and 20 μM Ca²⁺ to optimally activate gating (Fig. 5A) (13). Active channels were detected in 75% of patches, with mean number of active channels per patch (Nₒ) ≈ 2.3. Inclusion of 1 μM recombinant CIB1 (r-CIB1) in the pipette solution in lieu of InsP₃ activated InsP₃R channels with Pₒ ≈ 0.5 and Nₒ ≈ 0.5. A higher concentration (10 μM) did not elicit further increases (Fig. 5B). Channel activation required CIB1 binding since neither mutant CIB1 with either EF-hand disrupted activated gating (Fig. 5A).

These data establish CIB1 as a protein ligand of the channel, although CIB1 seems less efficacious than InsP₃ since it activated fewer channels (Nₒ) with lower activity (Pₒ) when compared with InsP₃ (Fig. 5B). The total InsP₃R-mediated ion flux across the ER membrane is the product of single-channel conductance, Pₒ and Nₒ. Because single-channel conductance remained constant under the present experimental conditions, NₒPₒ provides a measure of the total ion flux associated with InsP₃R activation. NₒPₒ, activated by CIB1 was less than 20% of...
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that achieved by saturating [InsP₃] (Fig. 5B), suggesting that CIB1 functions as a weak or partial agonist of the channel. CIB1 Inhibits InsP₃R-dependent Ca²⁺ Signaling in Intact Cells—The above results demonstrated that, at the molecular level in vitro, Ca²⁺-dependent binding of CIB1 to the InsP₃R results in channel activation, reminiscent of the effects of CaBP1 (22). Activation of InsP₃R by CaBP1 was subsequently questioned in two studies reporting that CaBP1 overexpression attenuated, rather than stimulated, InsP₃R-dependent [Ca²⁺], signaling in intact cells (25, 26). Nevertheless, we show that another EF-hand-containing protein similarly binds to and activates InsP₃R channel gating. The reasons for the seemingly conflicting results obtained in in vitro electrophysiological experiments and intact cells are not obvious. We therefore examined the effects of CaBP1 and CIB1 expression on [Ca²⁺], signaling to determine whether inhibitory effects observed in the other studies could be reproduced and whether CaBP1 and CIB1 have similar effects.

We first examined the PC12 cell neuroendocrine cell line, in which it was reported that CaBP1 expression inhibited InsP₃R-dependent [Ca²⁺].[Ca²⁺], signaling (25). Transient expression of either s-CaBP1 or CIB1 reduced the peak amplitude of the [Ca²⁺], transients elicited by 10 and 30 μM ATP by ~50% when compared with cells expressing the empty vector (Fig. 6, A–D). In contrast, there were no differences in cells expressing NCS-1 (Fig. 6, B and D), which does not bind to the InsP₃R (Fig. 2B) (25) but has similar Ca²⁺ binding properties to CaBP1 (25), indicating that changes in cellular Ca²⁺-buffering capacity cannot account for the observed inhibition. Caffeine-induced [Ca²⁺], transients mediated by ryanodine receptor activation were unaffected by expression of either CaBP1 or CIB1 (Fig. 6E), suggesting that the diminished ATP responses were not related to reduced stores of intracellular Ca²⁺ and were specific for the InsP₃R pathway. The store content was independently evaluated by measuring peak [Ca²⁺], responses in cells exposed to the Ca²⁺ ionophore ionomycin in Ca²⁺-free medium. Neither s-CaBP1 nor CIB1 expression reduced the total availability of stored Ca²⁺, as assessed by this protocol (Fig. 6F). The total InsP₃R protein content of the cells was unaffected by overexpression of s-CaBP1, CIB1, or NCS-1, indicating that the observed inhibition of [Ca²⁺], signaling could also not be accounted for by reduced InsP₃R expression levels (Fig. 6G).

Similar results were obtained when s-CaBP1 and CIB1 were transiently expressed in HeLa cells, the other cell type employed in the Haynes et al. study (25) (Supplementary Fig. 1).

The inhibitory effects we have observed are reminiscent of the reported effects of CaBP1 expression on [Ca²⁺], signals in PC12 and HeLa cells (25) and in COS-7 cells (26). Furthermore,
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we have now extended those observations by demonstrating that CIB1 impinges on InsP₃R-dependent signaling in a similar manner. We conclude that although CaBP1 and CIB1 behave as activating protein ligands of the InsP₃R in in vitro single channel experiments, they behave as antagonists of the InsP₃R-dependent [Ca²⁺] signaling pathway when overexpressed in intact cells.

CIB1 Decreases the Number of Channels Available for InsP₃ Activation—The effects of CaBP1 and CIB1 are paradoxical. On the one hand, their overexpression in intact cells inhibits InsP₃R-mediated Ca²⁺ release, whereas both proteins activate Ca²⁺ release in vitro at the single channel level. How can CaBP1 and CIB1 be inhibitory in vivo yet activating in vitro?

One fundamental difference in the two experimental approaches is the kinetic resolution of the assays with respect to the initial interaction between the Ca²⁺-binding proteins and the InsP₃R. The electrophysiology experiments measure the earliest response of the channel to interaction with the protein in the absence of InsP₃, whereas in vivo imaging of transfected cells examines the responses of the InsP₃R to InsP₃ at unknown and variable amounts of time after, or during, the interaction of CaBP1/CIB1 with the InsP₃R. Following initial activation of the channel by InsP₃, a process of ligand-dependent inactivation has been proposed to explain Ca²⁺ release termination in the presence of constant ligand concentrations (6, 39–41). InsP₃-dependent single-channel inactivation has been reported for Xenopus InsP₃R-1 (29) and SF9 cell InsP₃R (28) channels. Furthermore, we observed abrupt InsP₃R channel activity termination following activation by either CaBP1 or CIB1 (not shown). We therefore considered the possibility that binding of CaBP or CIB1 initially activates InsP₃R gating (as observed in single channel experiments), but the channel then undergoes ligand-dependent inactivation, rendering it refractory to subsequent InsP₃ stimulation (as observed in the intact cell studies). A mechanism of protein ligand-induced InsP₃R channel inactivation could therefore reconcile the seemingly disparate results obtained in electrophysiological and intact cell experiments.

To test this hypothesis, we attempted to recreate the long term interaction of the protein ligands with the InsP₃R in the intact cell experiments in vitro by exposing the InsP₃R to CIB1 prior to InsP₃ application. For these experiments, InsP₃R channel activity was studied by patch clamp electrophysiology of nuclei isolated from insect SF9 cells, believed to express only one InsP₃R isofrom. The SF9 system is ideal for studies of InsP₃R gating kinetics, including inactivation, because channels are consistently detected and their mean activity duration is much longer than in Xenopus oocyte nuclei (28). In control experiments, multiple channels (N₀ ≈ 4) with high Pₒ (~0.6) were observed with pipettes containing 100 nM InsP₃ and 1 μM Ca²⁺ (Fig. 7A and B), whereas less robust activity was recorded with r-CIB1 (1 μM) included in the pipette in lieu of InsP₃ (Pₒ ≈ 0.35, N₀ ≈ 0.9; Fig. 7A and B), consistent with the less robust responses to CIB1 of the Xenopus InsP₃R channel (Fig. 5). To mimic in vitro the effect of CIB1 expression in intact cells, isolated nuclei were preincubated with 5 μM CIB1 for 20–30 min in a bath solution containing 50–100 nM Ca²⁺, and subsequently patched with pipettes containing 100 nM InsP₃ and 1 μM Ca²⁺ (Fig. 7A). Channel Pₒ was not altered when compared with controls, whereas N₀ was decreased by nearly half (Fig. 7B). The product N₀Pₒ was correspondingly reduced (Fig. 7B), indicating that the ER ion flux was reduced ~50% by preincubation with CIB1. Simultaneous exposure of the channel to InsP₃ and CIB1 did not reduce N₀ or Pₒ (not shown), indicating that the pre-exposure was required.

These data suggest that pre-exposure to CIB1 renders some channels unavailable for activation by InsP₃, consistent with the hypothesis that prolonged exposure in vivo induces channel inactivation, causing the subsequent responses to InsP₃-generating agonists to be muted. The extent of channel inhibition we observed in these in vitro experiments is similar to the magnitude of the inhibition of [Ca²⁺], signaling observed in this work (Fig. 6) and previously in response to CaBP1 (25). The fact that it was still possible to activate some channels with InsP₃ after CIB1 pre-exposure suggests that not all channels had been driven into an inactivated state, consistent with the lower efficacy of CIB1 to recruit channels into activity (Figs. 5B and 7B).

DISCUSSION

InsP₃R-mediated Ca²⁺ signals are shaped in part by messenger generation, diffusion, degradation and removal, and kinetics of interaction with the channel, processes that are expected to vary greatly among different modulators of the release channel. Thus, the discovery of CaBPs as direct protein ligands of the

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InsP₃R extended existing concepts of the dimensions and versatility of the InsP₃R-dependent signaling system. However, the neuronally restricted expression of CaBP proteins raised the question of whether this new mode of regulation and [Ca²⁺], signaling extended more widely to non-neuronal cell types as well. We used structure-function analysis of the sequence determinants in CaBP1 that mediate its biochemical interaction with the InsP₃R to guide a directed search for other EF-hand-containing protein interactors of the channel. We identified CIB1 as a widely expressed CaM-related protein that shares many of the properties of CaBP1 with respect to its interactions with the InsP₃R. Thus, CIB1 binds to the first 600-residue ligand-binding region of all isoforms of the InsP₃R in a Ca²⁺-sensitive fashion dependent on functional Ca²⁺-binding EF-hands. Furthermore, purified CIB1 activates gating of the InsP₃R in the absence of InsP₃. These results extend the concept of protein ligands of the InsP₃R to peripheral tissues and suggest that regulation of the InsP₃R-mediated [Ca²⁺]

Previous studies revealed the interaction of CaBP1 with the InsP₃R to be strongly potentiated by Ca²⁺, with apparent Ca²⁺ affinity between 0.1 and 1 μM (Refs. 22 and 25, although see Ref. 26), dependent on functional EF-hands in CaBP1 (22). Using site-directed mutagenesis and protein truncations, we found that functional Ca²⁺-binding EF-hands 3 and 4 are particularly important in mediating the Ca²⁺ dependence. Recent structural analysis of CaBP1 confirmed that EF2 does not bind divalent metal ions and showed that EF1 has a low Ca²⁺ affinity (apparent Kᵦ of 300 μM) with little selectivity between Ca²⁺ and Mg²⁺, whereas Ca²⁺ binds cooperatively to EF3 and EF4 with an apparent affinity of 2.5 μM (36). These structural results are in good accord with binding data in the present and previous studies (22). Ca²⁺ binding to CaBP1 induces a conformational change specifically in the COOH-terminal region containing EF3 and EF4 (36). Together, these results suggest that the Ca²⁺ dependence of the interaction of CaBP1 with the InsP₃R is mediated by cooperative Ca²⁺ binding to EF3 and EF4 that induces localized conformational changes in the COOH-region of CaBP1 that facilitates its binding to the channel.

Based on these insights, we analyzed other EF-hand-containing proteins to discover whether additional ones could also interact with the InsP₃R. Although many proteins contain COOH-terminal pairs of EF-hands, we restricted our analysis to those previously shown to interact with ER- or Golgi-localized proteins. Of the relatively few proteins examined, only CIB1 interacted with the InsP₃R. The lack of interaction of the channel with NCS-1 confirms previous results (25), and the specific interaction with CIB1 demonstrates specificity in the interactions of EF-hand-containing proteins with the ligand-binding region of the InsP₃R.

Structural analyses have placed CIB1 and CaBP1 in distinct subfamilies of NCS proteins (34). CIB1 is myristoylated at its NH₂ terminus, which localizes it to cell membranes (27, 42), and both EF-hand-containing lobes are positioned in close contact to each other and bind to the same side of the substrate (34). Unlike CaBPs, which occur almost exclusively in the nervous system, CIB1 is ubiquitously expressed (42), where it has been shown to interact with a host of diverse target proteins in peripheral tissues (see Ref. 34 for references). We found that CIB1 binds to all three InsP₃R isoforms within the same ligand-binding domain as CaBP1 and can be co-immunoprecipitated with InsP₃R from cell lysates. Like CaBP1, CIB1 binding to InsP₃R has a similar dependence on functional EF-hands 3 and 4. CIB1 EF-hands 3 and 4 bind Ca²⁺ with Kᵦ of 0.5–2 μM Ca²⁺ (36, 43), which correlates well with the Ca²⁺ dependence of its interaction with the InsP₃R observed in this work.

The crystal structure of human CIB1 (34, 44) shows it to be a compact α-helical protein with two Ca²⁺ ions bound in a canonical fashion by the last two EF-hands. It is structurally similar to calcineurin B, calcineurin B homologous protein 1 (CHP1), and KChIP1 (34), which are in turn believed to be representative of the entire family of NCS proteins, with folds distinctly different from those of CaM. Like NCS proteins, CIB1 contains a hydrophobic pocket on one surface of the protein opposite from that of the Ca²⁺-binding sites. This pocket interacts with hydrophobic residues in amphipathic α-helices of interacting partners (45, 46). It is likely that CaBP proteins adopt similar folds and interact with target ligands in a similar fashion. These considerations suggest that amphipathic helices in the NH₂ terminus of the InsP₃R likely mediate its binding to CaBP1 and CIB1. Within the 600 NH₂-terminal residues of the InsP₃R, an NH₂-terminal β-sheet-rich β-trefoil domain contains an unusual helix-loop-helix insertion (H2 and H3) (47). Based on in vitro binding to synthesized small peptides, it was proposed that CaBP1 interacted with residues Pro-49–Asn-81 (26) within this region. In the crystal structure, the distal end of this region contains H2, possibly implicating it in the interaction with CaBP1/CIB1. However, the interaction with the peptide was Ca²⁺-independent, whereas the interaction of both CaBP1 (22, 25) and CIB1 (this study) are strongly Ca²⁺-sensitive. Furthermore, the peptide interacted with comparable affinity as CaM, whereas the interaction of CaBP1 with the InsP₃R has over an order of magnitude higher affinity than that of CaM (22). Thus, the relevance of this region for the Ca²⁺-dependent, high affinity interaction of CaBP1/CIB1 with the InsP₃R is unclear. The region encompassing residues 225–604, which includes the core InsP₃-binding domain, contains a distal armadillo-repeat domain (48). It is possible that CaBP1/CIB1 interact with the channel by binding to an α-helix in this region.

The functional consequences of the CIB1-InsP₃R interaction were determined by recording single InsP₃R channels in nuclei isolated from either Xenopus oocytes or Sf9 cells. With optimal [Ca²⁺] and in the absence of InsP₃, CIB1 activated channel gating from both species, establishing it as a novel protein agonist. When compared with optimal [InsP₃], however, CIB1 activated fewer channels to a lesser extent in both the oocyte and the Sf9 systems. Thus, CIB1 appears to behave as a partial agonist of the channel.

Despite the ability of CaBP1 to act as an activating ligand of the InsP₃R channel when applied acutely in patch clamp studies (22), it was subsequently shown that its overexpression inhibited InsP₃R-dependent Ca²⁺ release in cells (25, 26). Nevertheless, the discovery that another protein, CIB1, behaves in patch clamp studies similarly to CaBP1 reinforces the validity of those previous studies. The apparent discrepancy between results
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from electrophysiological studies and cell expression studies prompted us to explore the possible reasons. We found that overexpression of either s-CaBP1 or CIB1 attenuated agonist-mediated, InsP₃-dependent Ca²⁺ release, which could not be accounted for by reduced store content, diminished InsP₃R expression levels, or increased cytoplasmic Ca²⁺ buffering. Our results are therefore in accord with the previous reports (25, 26) of the effects of CaBP1 on [Ca²⁺], signaling.

Several possible mechanisms might reconcile that, on the one hand, acute exposure of the channel to purified recombinant CaBP/CIB1 proteins activates channel gating, whereas their overexpression mutes InsP₃R-dependent [Ca²⁺] signals in cells. First, in intact cells, CaBP1 or CIB1 might bind to the InsP₃R even under conditions of resting [Ca²⁺], by avidity if the proteins are in close proximity or because of the finite ability of the proteins to bind even in low [Ca²⁺]. Notably, both effects will be magnified in overexpression studies. Despite being liganded by these proteins, the channel may not be activated if the Ca²⁺ requirement for channel gating is not satisfied, whereas the protein-bound InsP₃R might be less sensitive to InsP₃. Consequently, agonist-induced [Ca²⁺] signals would be inhibited, as observed (present results and Refs. 25 and 26). Second, because InsP₃ binding is believed to drive the channel into an inactive state from which it recovers only after ligand dissociation (6, 28, 39–41), we considered the possibility that CaBP/CIB1 binding to the channel initially activates it but then subsequently drives it into an inactivated state that cannot be activated by InsP₃. We tested this model explicitly by pre-exposing the InsP₃R channels to CIB1 and then subsequently assaying the ability of the channels to become activated by InsP₃. CIB1 pre-exposure reduced the number of channels that could be activated by InsP₃, although channels that were activated had normal 
P. These results are consistent with CIB1-induced inactivation of a subset of the total channel population, with the remaining channels that had not been activated by the pre-exposure able to respond normally to InsP₃. In intact cells, the inactivated channels would remain inactivated as long as they were liganded by CIB1. Two factors might retain much of the InsP₃R channel population in an inactivated state in vivo. First, high [CaBP1] as a consequence of overexpression would ensure a high probability of binding to the InsP₃R. Second, slow unbinding of the NCS proteins from the channel may keep many channels inactivated even in the absence of overspereaction. The data suggest that exposure to CIB1 can effectively remove functional channels from the total InsP₃R population by a process of ligand-induced channel inactivation. In vivo, this would result in fewer channels available to respond to agonist-induced increases in [InsP₃], with consequent muted [Ca²⁺] signals, as observed in this study and previously (25, 26).

Our results indicate that CaBP and CIB1 can function in dual roles, as both activators and inhibitors of the InsP₃R. Cellular mechanisms might exist that regulate protein ligand dissociation from the channel and enable it to escape from inactivation. Phosphorylation of CaM decreases its affinity for target substrates (49), and mutation of the conserved phosphorylation site in CaBP1 modulated [Ca²⁺], signaling inhibition (26). Although this site is not conserved in CIB1, it is possible that covalent modifications or protein interactions could regulate the interactions of CaBP1 and CIB1 with the InsP₃R. Escape from inactivation may then enable the channel to become activated by protein ligand re-binding.

In conclusion, we have identified a novel interaction between the widely expressed Ca²⁺-binding protein CIB1 and the InsP₃R. Taken together, our data support a model in which CaBP1 or CIB1 serve as both positive and negative regulators of InsP₃R function and extend the concept of protein ligand regulation of InsP₃R function from neuronal tissues to peripheral ones as well. Future studies will be required to determine roles of these interactions in modulating the InsP₃R-dependent [Ca²⁺], signaling system in different cell types under a variety of physiological conditions.

Acknowledgments—For providing reagents, we thank Dr. A. Jeromin (NCS-1 cDNA), Dr. J. Buxbaum (calsenilin cDNA), Dr. T.-W. Kim (sorcin cDNA), Dr. J. F. DuFour (InsP₃R-2 cDNA), and Dr. S. Joseph (InsP₃R antibody). We thank Drs. D.-O. D., Mak and K.-H. Cheung for comments on the manuscript.

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JULY 28, 2006•VOLUME 281 • NUMBER 30
JOURNAL OF BIOLOGICAL CHEMISTRY

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