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Calcium-binding Protein 1 Is an Inhibitor of Agonist-evoked, Inositol 1,4,5-Trisphosphate-mediated Calcium Signaling*[S]

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Intracellular calcium signals are responsible for initiating a spectrum of physiological responses. The caldendrins/calcium-binding proteins (CaBPs) represent mammal-specific members of the CaM superfamily. CaBPs display a restricted pattern of expression in neuronal/retinal tissues, suggesting a specialized role in Ca^{2+} signaling in these cell types. Recently, it was reported that a splice variant of CaBP1 functionally interacts with inositol 1,4,5-trisphosphate ($InsP_3$) receptors to elicit channel activation in the absence of $InsP_3$ (Yang, J., McBride, S., Mak, D.-O. D., Vardi, N., Palczewski, K., Haeseleer, F., and Foskett, J. K. (2002) *Proc. Natl. Acad. Sci. U. S. A.* 99, 7711–7716). These data indicate a new mode of $InsP_3$ receptor modulation and hence control of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in neuronal tissues. We have analyzed the biochemistry of the long form splice variant of CaBP1 (L-CaBP1) and show that, *in vitro*, a recombinant form of the protein is able to bind Ca^{2+} with high affinity and undergo a conformational change. We also describe the localization of endogenous and overexpressed L-CaBP1 in the model neuroendocrine PC12 cell system, where it was associated with the plasma membrane and Golgi complex in a myristoylation-dependent manner. Furthermore, we show that overexpressed L-CaBP1 is able to substantially suppress rises in $[Ca^{2+}]_i$ in response to physiological agonists acting on purinergic receptors and that this inhibition is due in large part to blockade of release from intracellular Ca^{2+} stores. The related protein neuronal calcium sensor-1 was without effect on the $[Ca^{2+}]_i$ responses to agonist stimulation. Measurement of $[Ca^{2+}]$ within the ER of permeabilized PC12 cells demonstrated that L-CaBP1 directly inhibited $InsP_3$-mediated Ca^{2+} release. Expression of L-CaBP1 also inhibited histamine-induced $[Ca^{2+}]_i$ oscillations in HeLa cells. Together, these data suggest that L-CaBP1 is able to specifically regulate $InsP_3$ receptor-mediated alterations in $[Ca^{2+}]_i$ during agonist stimulation.

Calcium is a ubiquitous intracellular second messenger in all vertebrate cells (1). Intracellular resting free Ca^{2+} concentration ($[Ca^{2+}]_i$) is acutely maintained at around 100 nM via a variety of mechanisms, and even small local rises above this base line are able to elicit dramatic cellular responses. Rises in $[Ca^{2+}]_i$ are generated through two primary routes (2). First, many physiological agonists activate Ca^{2+} channels that mediate the direct entry of extracellular Ca^{2+}. Second, $[Ca^{2+}]_i$ can be elevated through release of sequestered Ca^{2+} from intracellular stores (3), most notably the endoplasmic reticulum (ER).[1] Ca^{2+} release from intracellular stores is controlled predominantly by two related Ca^{2+} channel families, the inositol 1,4,5-trisphosphate ($InsP_3$) receptor ($InsP_3R$) and the ryanodine receptor (RyR). $InsP_3Rs$ couple to extracellular stimuli that drive the activation of phospholipase C and subsequent liberation of the soluble second messenger $InsP_3$. Activation of ER-localized $InsP_3Rs$ by $InsP_3$ in turn elicits Ca^{2+} release into the cytosol. $InsP_3R$ activity can be further modulated by direct binding of Ca^{2+} itself, and this regulation explains a form of calcium-induced calcium release that is responsible for the establishment of complex regenerative Ca^{2+} signals within cells (2). In neurons, such Ca^{2+} signals have been intimately linked to processes as divergent as neurodegeneration, neurite outgrowth, alterations in neuronal gene expression, and neurotransmitter release (4–10). Factors affecting the generation and propagation of intracellular Ca^{2+} signals therefore represent potential critical integrators of neuronal responses to complex and varied stimuli.

Although Ca^{2+} is capable of eliciting direct cellular responses such as the aforementioned gating of $InsP_3Rs$, many of its effects are registered indirectly through specific Ca^{2+}-binding proteins. Calmodulin (CaM) is a universal Ca^{2+}-binding protein, homologues of which are present in all eukaryotes (11), and a large number of Ca^{2+} driven alterations in cell physiology can be mapped to its Ca^{2+} sensing activity. CaM coordinates Ca^{2+} ions through specialized EF-hand domains, and a large number of related proteins have now been characterized. In neurons, these include the neuronal calcium sensor (NCS) family of Ca^{2+}-binding proteins (12). More recently, a group of CaM-like proteins, termed caldendrins (13, 14) or calcium-binding proteins (CaBPs) (15) have been identified that are vertebrate-specific and display a predominantly neuronal/retinal pattern of expression (14–16). It has since been reported that members of the CaBP family are able to interact with and modulate the activity of $InsP_3Rs$ (17). Uniquely, it has been suggested that CaBPs may be capable of activating the $InsP_3R$ independently of the natural ligand $InsP_3$. The ability to release stored Ca^{2+} in the absence of $InsP_3$ production represents a potentially important new mechanism for the generation of intracellular Ca^{2+} signals in neuronal cell types. It has also

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[S] The on-line version of this article (available at http://www.jbc.org) contains two additional figures.

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[1] The abbreviations used are: ER, endoplasmic reticulum; CaBP, calcium-binding protein; L-CaBP1, long splice isoform of CaBP1; Tr-CaBP1, truncated form of CaBP1; CaM, calmodulin; ECFP, enhanced cyan fluorescent protein; EYFP, enhanced yellow fluorescent protein; GST, glutathione *S*-transferase; $InsP_3$, inositol 1,4,5-trisphosphate; $InsP_3R$, $InsP_3$ receptor; $InsP_3R1$, type I $InsP_3R$; IP_3R3, type III $InsP_3R$; NCS, neuronal calcium sensor; RyR, ryanodine receptor; PIPES, 1,4-piperazinediethanesulfonic acid.

been proposed that CaBPs may additionally harbor the capacity to negatively regulate influx of extracellular Ca^{2+} through a direct inhibitory interaction with plasma membrane P/Q type Ca^{2+} channels (18). Preliminary results therefore seem to indicate that members of the CaBP subfamily represent important new neuronal Ca^{2+}-sensing proteins that can control multiple Ca^{2+} fluxes in the modulation of intracellular Ca^{2+} signaling.

No data are available concerning the effect of CaBPs on agonist-induced Ca^{2+} signals in intact cells. Several splice variants of CaBP1 and caldendrin have been identified. In this study, we have attempted to analyze the effect of long form CaBP1 (L-CaBP1) protein on intracellular Ca^{2+} signals in the model neuroendocrine PC12 cell system. We show by Western blotting and immunofluorescence that endogenous L-CaBP1 is detectable in PC12 cells and that a recombinantly expressed form of the protein is able to bind Ca^{2+} *in vitro* with high affinity. An overexpressed, EYFP-tagged, version of L-CaBP1 displays a dual plasma membrane/perinuclear localization in PC12 cells, which mirrors the localization of the endogenous protein. It seems likely that the wild-type protein is myristoylated *in vivo*, since mutation of its putative N-terminal myristoylation motif causes a complete abolition of membrane association. In addition, the localization of both $InsP_3R1$ and $InsP_3R3$ with L-CaBP1-EYFP has been studied. Using pull-down type *in vitro* experiments, we also show that recombinant GST-L-CaBP1 is able to specifically interact with the type I $InsP_3R$ and the $Ca_v2.1$-α_{1A} channel subunit from rat brain extracts in a Ca^{2+}-dependent manner. Furthermore, we have applied confocal imaging to monitor intracellular Ca^{2+} responses in cells overexpressing L-CaBP1, a truncated version of this protein (Tr-CaBP1), and the related neuronal Ca^{2+}-sensing protein, NCS-1. The data presented show that, *in vivo*, in neuroendocrine cells that endogenously express L-CaBP1, overexpression of the protein dramatically inhibits intracellular Ca^{2+} rises due to release from $InsP_3R$-sensitive intracellular stores.

MATERIALS AND METHODS

Cloning of DNA Constructs—The long form of human CaBP1 (L-CaBP1; GenBank™ accession number AF169149) was amplified by PCR from human brain cDNA (Clontech, Palo Alto, CA) using an Omn-E dry block thermocycler (Hybaid, Middlesex, UK) and Bio-X-Act DNA polymerase (Bioline, London, UK) with primers containing restriction endonuclease sites (underlined) for subsequent cloning: sense (HindIII), 5′-GCCGAAGCTTATGGGCAACTGTGTCAAGTATCC-3′; antisense (SacII), 5′-GCCGCCGCGGGCGGGACATCATCCGGAC-3′; sense (BamHI), 5′-GCCGGGATCCATGGGCAACTGTGTCAAGTATCC-3′; antisense (XhoI), 5′-GCCGCTCGAGTCAGCGGGACATCATCCGGAC-3′.

Tr-CaBP1 was amplified by PCR from the wild-type template using the following primers containing restriction endonuclease sites (underlined) for subsequent cloning: sense (HindIII): 5′-GCCGAAGCTTCTGCGACCAGAGGAAATTG-3′; antisense (SacII), 5′-GCCGCCGCGGGCGGGACATCATCCGGAC-3′; sense (BamHI), 5′-GCCGGGATCCCTGCGACCAGAGGAAATTG-3′; antisense (XhoI), 5′-GCCGCTCGAGTCAGCGGGACATCATCCGGAC-3′.

HindIII/SacII-digested PCR products were ligated into EYFP-N1 (Clontech) for imaging and immunofluorescence studies. BamHI/XhoI-digested PCR products were ligated into pGex-6P-1 (Amersham Biosciences) for recombinant protein expression. BamHI/XhoI-digested L-CaBP1 PCR product was additionally ligated into pcDNA3.1(+) (Invitrogen) for expression of untagged protein.

NCS-1 was amplified by PCR from an existing expression construct (19) using the following primers for subsequent subcloning into the pGex-6P-1 vector: sense (BamHI), 5′-GCCGGGATCCATGGGGAAATCCAACAGCAAG-3′; antisense (XhoI), 5′-GCCGCTCGAGCTATACCAGCCCGTCGTAGAG-3′.

The CaBP1-EYFP(G2A) mutant was generated from wild-type CaBP1-EYFP template by site-directed mutagenesis using the QuikChange™ system (Stratagene, La Jolla, CA). The primers were as follows: sense, 5′-CTCGAGCTCAAGCTTATGG(G/C)CAACTGTGTCAAGTATC-3′; antisense, 5′-GATACTTGACACAGTTG(C/G)CCATAAGCTTGAGCTCGAG-3′.

Nucleotides in parentheses indicate bases that were changed to generate the appropriate amino acid substitution. All DNA constructs were checked by automated sequencing (Oswel, Southampton, UK).

Expression and Purification of GST-tagged Proteins—pGex-L-CaBP1, pGex-Tr-CaBP1, and pGex-NCS-1 plasmids were transformed into BL-21 (DE3) *Escherichia coli* (Stratagene), and protein expression was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h at 37 °C. Proteins were purified from cytosolic fractions on glutathione-Sepharose (Amersham Biosciences). For proteolytic removal of the GST moiety, glutathione-Sepharose beads with bound GST fusion proteins were washed extensively in prescission buffer (50 mM Tris-HCl (pH 7.0), 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol) and then incubated with 40 units of prescission protease (Amersham Biosciences) overnight at 4 °C with constant agitation. Supernatants containing GST-free protein were collected following centrifugation.

Fluorescence Measurements of Ca^{2+} Binding to L-CaBP1 and NCS-1—GST-free NCS-1 and L-CaBP1 (both at 1 μM) were incubated in Ca^{2+}-free buffer (20 mM HEPES (pH 7.4), 139 mM NaCl, 5 mM EGTA, 5 mM nitrilotriacetic acid). An emission spectrum was obtained by excitation of protein samples at 280 nm and collection of emission fluorescence between 290 and 410 nm using an LS-5 luminescence spectrometer (PerkinElmer Life Sciences). $CaCl_2$ was then added stepwise to samples, with mixing, to give calculated free Ca^{2+} concentrations ranging from 0.1 to 10 μM (19). After each addition of Ca^{2+}, a new spectrum was obtained as described above. Buffer alone and buffer plus 10 μM free Ca^{2+} control spectra were obtained using the same protocol. Percentages of Ca^{2+}-dependent changes in peak fluorescence intensity were then calculated and plotted as a function of free Ca^{2+} concentration.

Binding of GST-L-CaBP1 to Rat Brain Membrane Protein Extracts—Rat brain membrane protein fractions were prepared as previously described (20). GST-L-CaBP1, GST-NCS-1, or GST (all at 1 μM) were immobilized onto 50 μl of glutathione-Sepharose that had been prewashed in membrane buffer (25 mM Tris-HCl (pH 7.8), 50 mM KCl, 5 mM EGTA, 5 mM nitrilotriacetic acid, 1 mM dithiothreitol, 1% (v/v) Triton X-100) with or without 1 μM free Ca^{2+} by incubation for 30 min at 4 °C. 500 μl of rat brain membrane protein extract containing 0 or 1 μM free Ca^{2+} was then added to the appropriate samples, and binding was allowed to proceed for 2 h at 4 °C with constant agitation. Sepharose pellets were collected by centrifugation and washed extensively in the binding buffer. Final pellets were extracted in 50 μl of SDS dissociation buffer (125 mM HEPES (pH 6.8), 10% (w/v) sucrose, 10% (v/v) glycerol, 4% (w/v) SDS, 1% (v/v) β-mercaptoethanol, 2 mM EDTA), and samples were boiled for 5 min. Bound proteins were analyzed on SDS-PAGE (12.5% gel), and proteins were transferred to nitrocellulose membranes for immunoblotting via transverse electrophoresis. Proteins were detected with anti-$InsP_3R1$ antibody (1:500; Affinity Bioreagents, Golden, CO) or anti-$Ca_v2.1$-α_{1A} (1:500; Sigma) and visualized using ECL-Plus reagents (Amersham Biosciences).

Production of Anti-CaBP1 Antiserum—Anti-CaBP1 was raised against the prescission-cleaved, GST-free form of recombinant L-CaBP1 by immunization of rabbits with 100 μg of protein/animal (CovalAb, Cambridge, UK) and affinity-purified by binding to recombinant protein and elution before use.

Localization of EYFP and ECFP Constructs and Immunofluorescence—PC12 cells (0.25×10^6 cells/well) were plated onto collagen-coated glass coverslips and transiently transfected with 1 μg of either EYFP-N1 control plasmid or 1 μg of CaBP1-EYFP, Tr-CaBP1-EYFP, or L-CaBP1 (G2A)-EYFP constructs using LipofectAMINE 2000 reagent (Invitrogen) according to the manufacturer's protocol. For co-localization of CaBP1-EYFP with NCS-1-ECFP (21) or ECFP-ER (Clontech), cells were transfected with 1 μg of each construct. 24 h following transfection, cells were fixed in 4% formaldehyde in PBS for 30 min. For staining of endogenous L-CaBP1 or co-staining experiments with $InsP_3R1$ or $InsP_3R3$ antibodies, cells were then incubated in PBT (phosphate-buffered saline plus 0.1% (v/v) Triton X-100 and 0.3% (w/v) bovine serum albumin) for 30 min. Cells were subsequently incubated with anti-CaBP1 (1:50), anti-$InsP_3R1$ (1:50), or anti-$InsP_3R3$ (1:50, BD Biosciences, Oxford, UK) followed by incubation with an appropriate species-specific biotin-conjugated secondary antibody (1:100; Amersham Biosciences). Cells were then washed and incubated with a streptavidin-Texas Red-conjugated tertiary antibody (1:50; Amersham Biosciences) prior to mounting of coverslips onto glass slides. All images were captured using laser-scanning confocal microscopy with a Leica TCS-SP-MP microscope (Leica Microsystems, Heidelberg, Germany) using a 22-μm pin hole and a ×63 water immersion objective with a 1.2 numerical aperture. For optimal imaging of the spatial distribution of

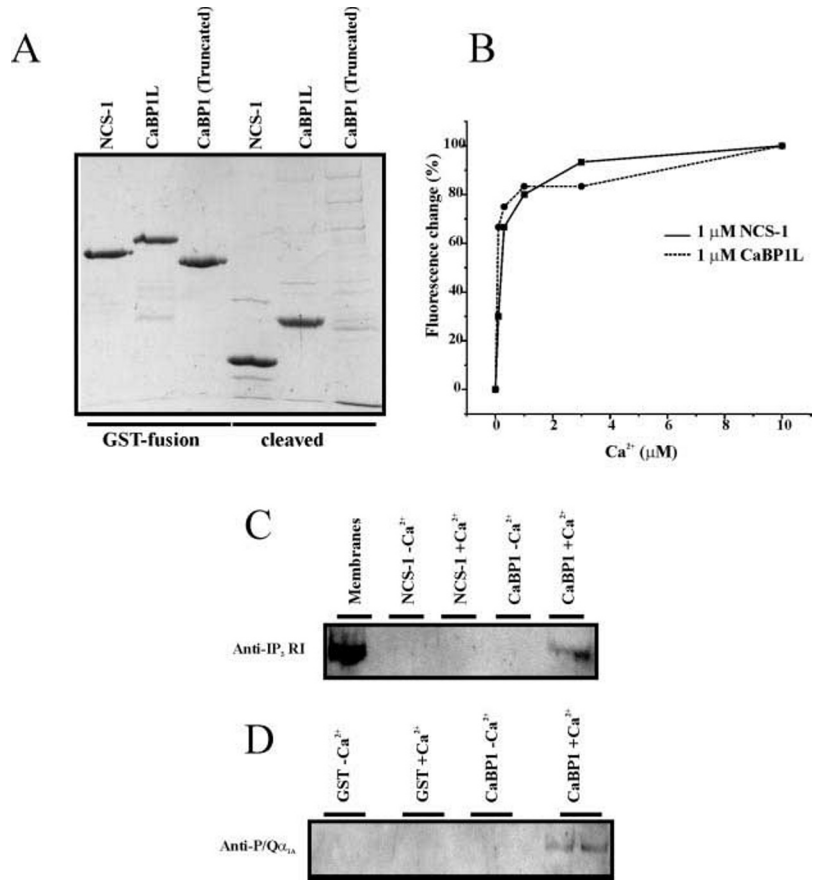

FIG. 1. **Expression of recombinant proteins, analysis of Ca^{2+}-induced conformational change in L-CaBP1, and binding of GST-L-CaBP1 to IP_3R1 and $Ca_v2.1$-α_{1A}.** *A*, SDS-PAGE analysis of GST fusion proteins and their GST-free forms. Recombinantly expressed GST-NCS-1, GST-L-CaBP1, and GST-Tr-CaBP1 (1 μg of each) were separated on a 12.5% gel and visualized via Coomassie Blue staining (*lanes 1–3*). Prescission-cleaved, GST-free forms of recombinant NCS-1, L-CaBP1, and Tr-CaBP1 were analyzed on the same gel (*lanes 4–6*). *B*, changes in fluorescence emission on binding of Ca^{2+} to prescission-cleaved, GST-free, NCS-1, and L-CaBP1. 1 μM NCS-1 or 1 μM L-CaBP1 were excited at 280 nm, and emission spectra were collected between 290 and 410 nm. Spectra were collected over a range of free Ca^{2+} concentrations, and Ca^{2+}-dependent changes in peak fluorescence (a rise for NCS-1 and a decline for L-CaBP1) were plotted as a function of free Ca^{2+} concentration. *C* and *D*, binding of GST-L-CaBP1 to $InsP_3R1$ (*Anti-IP_3RI*) and the $Ca_v2.1$-α_{1A} (*Anti-$P/Q\alpha_{1A}$*) channel subunit from rat brain membrane protein extract. 1 μM GST-L-CaBP1, GST-NCS-1, or GST was incubated with rat brain membrane protein extract in the presence or absence of 1 μM free Ca^{2+} and bound $InsP_3R1/Ca_v2.1$-α_{1A} subunit detected by immunoblotting. *D*, duplicated samples were separated and probed.

EYFP, the cells were excited at 514 nm and light was collected at 545–625 nm. Texas Red was imaged using excitation at 543 nm and light collection at 600–650 nm. For dual imaging of EYFP and ECFP, the cells were excited at 514 nm with light collected at 560–600 nm for EYFP or excited at 458 nm with light collected at 465–500 nm for ECFP detection. Images were exported as Tiff files and compiled in CorelDraw.

Imaging of Intracellular [Ca^{2+}] in PC12 Cells—PC12 cells (0.75×10^6 cells/well) were plated onto collagen-coated glass coverslips and transiently transfected with 2 μg of either EYFP-N1 control plasmid or 2 μg of CaBP1-EYFP, Tr-CaBP1-EYFP, L-CaBP1(G2A)-EYFP, or NCS-1-EYFP constructs using LipofectAMINE 2000 reagent (Invitrogen) according to the manufacturer's protocol. 24–48 h post-transfection, cells were washed in Krebs buffer (20 mM HEPES (pH 7.4), 145 mM NaCl, 10 mM glucose, 5 mM KCl, 2 mM $CaCl_2$, 1.3 mM $MgCl_2$, 1.2 mM NaH_2PO_4) and then incubated with 1 μM X-rhod-1 AM (Molecular Probes Europe BV, Leiden, The Netherlands) in Krebs buffer for 30 min at room temperature. Cells were washed extensively in Krebs buffer before imaging using laser-scanning confocal microscopy. An EYFP image was taken prior to each experiment to permit distinction between transfected/untransfected cells. X-rhod-1 was imaged using the Leica TCS-SP-MP microscope with excitation at 543 nm and light collection at 600–650 nm. X-rhod-1 was used in the study of PC12 cells rather than Fura Red (22), since the latter dye could not be successfully loaded into PC12 cells, and other dyes were not compatible with measurements in EYFP-expressing cells. The X-rhod-1 fluorescence was diffusely and uniformly distributed, suggesting cytosolic localization of the probe.

Imaging of Intracellular Ca^{2+} Changes in HeLa Cells—HeLa cells (0.75×10^6 cells/well) were plated onto glass coverslips and transiently transfected with 2 μg of L-CaBP1-EYFP using FuGene6 reagent (Roche Applied Science) according to the manufacturer's protocol. 24–48 h post-transfection, cells were washed in Krebs buffer and then incubated with 1 μM Fura Red AM (Molecular Probes) in Krebs buffer for 30 min at room temperature. Cells were washed extensively in Krebs buffer before imaging of intracellular Ca^{2+} using the Leica TCS-SP-MP microscope. An EYFP image was taken prior to each experiment to permit distinction between transfected and nontransfected cells. For the monitoring of Fura Red fluorescence, cells were excited at 488 nm, and light was collected at 625–725 nm.

Imaging of $InsP_3$-induced Ca^{2+} Release from Permeabilized PC12 Cells—PC12 cells (0.75×10^6 cells/well) were plated onto collagen-coated glass coverslips and transiently transfected with 1 μg of ECFP-N1 plasmid and 1 μg of pcDNA-L-CaBP1 construct using LipofectAMINE 2000 reagent (Invitrogen) according to the manufacturer's protocol. 24–48 h post-transfection, cells were washed in Krebs buffer and then incubated with Mag-fluo4 AM (Molecular Probes) in Krebs buffer for 45 min at 37 °C to allow dye loading into the ER. Cells were washed extensively in Krebs and then perfused for 5 min with KGEP-MgATP buffer (20 mM PIPES (pH 6.5), 139 mM potassium glutamate, 5 mM EGTA, 2 mM ATP, 2 mM $MgCl_2$). Cells were then perfused with KGEP-MgATP buffer containing 20 μM digitonin (Calbiochem) for 6 min (23) and subsequently stimulated with 10 μM $InsP_3$ in KGEP-MgATP for 1 min. All images were collected using the Leica TCS-SP-MP microscope with excitation at 488 nm with collection of emission fluorescence between 500 and 560 nm. The data on Mag-fluo4 fluorescence were corrected for photobleaching during the time course of the experiment. An ECFP image was taken prior to each experiment to permit distinction between transfected and nontransfected cells.

RESULTS

Expression of Recombinant Proteins, Analysis of Ca^{2+} Binding, and Interactions with $InsP_3R1$ and $Ca_v2.1\alpha_{1A}$—In order to examine the *in vitro* biochemical properties of L-CaBP1, we generated a series of GST fusion protein constructs that were purified from *E. coli* lysates. Fig. 1*A* depicts SDS-PAGE analysis of the purified versions of GST-NCS-1, GST-L-CaBP1, and GST-Tr-CaBP1 (*lanes 1–3*). Tr-CaBP1 corresponds to c-CaBP1 as used in the study by Yang *et al.* (17). This construct represents an N-terminally truncated version of the short form splice variant of CaBP1 (15) that lacks the amino-terminal 18 residues of this protein. This truncation eliminates a putative glycine myristoylation site at position 2 in the full-length protein. We have included this construct in our analysis, since in the original report the recombinant version of this protein was able to dramatically stimulate $InsP_3R$ activity. The related Ca^{2+}-binding protein NCS-1 (19, 24, 25) was also included for comparative purposes. Studies by Yang *et al.* (17) utilized the

GST-free forms of the recombinantly expressed proteins, which were obtained by proteolytic cleavage of the GST fusion constructs. We used the same proteolytic treatment to generate GST-free forms of L-CaBP1, Tr-CaBP1, and NCS-1 (Fig. 1*A*, *lanes 4–6*). It is apparent from PAGE analysis that proteolytic removal of GST was efficient for both NCS-1 and L-CaBP1 (Fig. 1*A*, *lanes 4* and *5*). In contrast, similar treatment of Tr-CaBP1 resulted in severe degradation of the protein, formation of high molecular weight aggregates, and almost no recovery of the GST-free form (Fig. 1*A*, *lane 6*).

Binding of Ca^{2+} to the GST-free forms of both L-CaBP1 and NCS-1 was examined *in vitro* by monitoring changes in protein conformation upon Ca^{2+} binding (24) as determined by alterations in tryptophan (NCS-1) or tyrosine (L-CaBP1) fluorescence emission spectra (Fig. 1*B*). From these experiments, it is clear that both NCS-1 and L-CaBP1 are capable, *in vitro*, of binding Ca^{2+} with affinities in the submicromolar range (K_D for both L-CaBP1 and NCS-1 was between 0.1 and 0.3 μM free Ca^{2+}). This demonstrates that binding of Ca^{2+} to L-CaBP1 is of high affinity and drives a significant conformational change in the protein.

Recent reports have suggested that L-CaBP1 is able to interact with plasma membrane P/Q type Ca^{2+} channels (18) and that Tr-CaBP1 is able to interact with $InsP_3R$ types 1 and 3 (17). These reports are somewhat confusing in that the interaction with P/Q type channels appears to be independent of Ca^{2+}, and interactions of Tr-CaBP1 with $InsP_3Rs$ involve binding to a form of CaBP1 that is not expressed endogenously in cells. In an attempt to resolve these issues, we analyzed the binding of GST-L-CaBP1 to rat brain protein extracts in the absence or presence of 1 μM free Ca^{2+}. In our hands, GST-L-CaBP1 seemed to interact with both the $InsP_3R1$ and the α_{1A} subunit of the P/Q type Ca^{2+} channel in an acutely Ca^{2+}-dependent manner (Fig. 1, *C* and *D*). $InsP_3R1$ was present in rat brain membrane protein extracts (Fig. 1*C*, *lane 1*) (26) and bound to GST-L-CaBP1 only in the presence of free Ca^{2+} (Fig. 1*C*, *lane 4 versus lane 5*). This interaction was specific for L-CaBP1, since no binding was observed to GST-NCS-1 in either the presence or absence of free Ca^{2+} (Fig. 1*C*, *lanes 2* and *3*). Samples from the same binding experiments were also probed with an antibody specific for the α_{1A} subunit of the P/Q type Ca^{2+} channel (Fig. 1*D*). Binding of GST-L-CaBP1 to the α_{1A} subunit was observable only in the presence of free Ca^{2+} (Fig. 1*D*, *lanes 5* and *6 versus lanes 7* and *8*). This association was specific for GST-L-CaBP1, since no binding was observable to control GST protein in either the presence or absence of free Ca^{2+} (Fig. 1*D*, *lanes 1–4*).

Localization of Endogenous and Overexpressed CaBP1 in PC12 Cells—Initial localization studies concerning CaBP1 (15) indicated that L-CaBP1 was most likely associated with cytoskeletal structures and that the splice variant S-CaBP1 was most likely with the plasma membrane. These experiments employed overexpression in CHO cells, and it is unlikely that L-CaBP1 is an endogenously occurring protein in this cell line. PC12 cells represent a neuroendocrine model cell line, and with the knowledge that L-CaBP1 displays a predominantly neuronal/retinal pattern of expression, we aimed to examine its expression/localization in this system. To confirm that PC12 cells represent a legitimate model system in which to study CaBP1 function, we first tested for the presence of endogenously expressed protein. GST-free L-CaBP1 was used to generate an anti-CaBP1 antibody for use in these studies. In immunofluorescence experiments on untransfected PC12 cells, anti-CaBP1 immunoreactivity was detected most strongly at the plasma membrane but was also present intracellularly (Fig. 2*B*, anti-CaBP1). Western blotting of rat brain and PC12 cell lysates with anti-CaBP1 led to the detection of two species (Fig. 2*A*) with molecular masses of ~33 and 28 kDa, respectively. L-CaBP1 is itself a splice variant of the larger neuronal Ca^{2+}-binding protein caldendrin (13). Antisera raised against L-CaBP1 will therefore almost certainly detect caldendrin, if present, and previous studies examining the distribution of these proteins have indicated that the 33-kDa species is likely to represent caldendrin, with the smaller 28-kDa protein corresponding to L-CaBP1 (27). Our data confirm these findings and show that both caldendrin and L-CaBP1 are present at similar levels in PC12 cells.

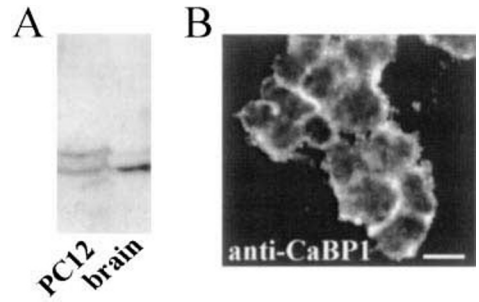

FIG. 2. **Expression and localization of endogenous CaBP1 proteins in PC12 cells.** *A*, L-CaBP1 is expressed endogenously in PC12 cells and detected in rat brain extract as determined by Western blotting. Western blotting with an anti-CaBP1 antibody detects two reactive species in PC12 cell lysates at ~28 and ~33 kDa. The same species are identified from a rat brain protein lysate with anti-CaBP1. *B*, confocal imaging of endogenous L-CaBP1 expression in untransfected PC12 cells as detected by the anti-L-CaBP1 antiserum.

Further localization studies were conducted using EYFP-tagged versions of L-CaBP1, Tr-CaBP1, and L-CaBP1 (G2A) and overexpression in PC12 cells. L-CaBP1 (G2A) represents a mutant version of L-CaBP1 where the glycine residue at position 2 of the protein sequence was mutated to alanine. In wild-type L-CaBP1, glycine 2 represents a putative site for myristoylation of the protein, and we therefore used this mutant to examine whether this modification was likely to occur under *in vivo* conditions. L-CaBP1-EYFP appeared to be localized mainly at the plasma membrane in PC12 cells and was also seen in a perinuclear region in favorable confocal sections (Fig. 3, *L-CaBP1*). This localization correlates well with that observed for endogenous L-CaBP1 expression (Fig. 2, anti-CaBP1). In contrast, control EYFP-transfected cells displayed a diffuse cytosolic signal (Fig. 3, *EYFP*). Tr-CaBP1-EYFP and L-CaBP1 (G2A)-EYFP both displayed a diffuse cytosolic distribution that was similar to EYFP control expression (Fig. 3, Tr-CaBP1 and L-CaBP1 (G2A)). As aforementioned, Tr-CaBP1 lacks the putative myristoylation motif of the short form splice variant of L-CaBP1; similarly, L-CaL-CaBP1 (G2A) lacks the putative myristoylation motif of L-CaBP1. The fact that both of these constructs showed a diffuse cytosolic distribution compared with the well defined plasma membrane/perinuclear localization of L-CaBP1 would tend to suggest that, *in vivo*, L-CaBP1 is indeed myristoylated and that this modification is involved in targeting of the protein to specific membrane domains. The localization of L-CaBP-1-EYFP to the plasma membrane and a perinuclear compartment was reminiscent of myristoylated NCS-1, which is localized to the plasma membrane and the trans-Golgi network in PC12 and other cell types (21, 22, 28, 29). In cells co-transfected to express L-CaBP1-EYFP (Fig. 4*A*, *L- CaBP1*) and NCS-1-ECFP (Fig. 4*A*, *NCS-1*), the two proteins showed essentially complete overlap (Fig. 4*A*, *overlay*).

The most intriguing report concerning CaBP1 function relates to its potential role as an $InsP_3$-independent $InsP_3R$ agonist (17). The two $InsP_3R$ subtypes expressed in PC12 cells, $InsP_3R1$ and $InsP_3R3$ (30), also happen to represent the forms

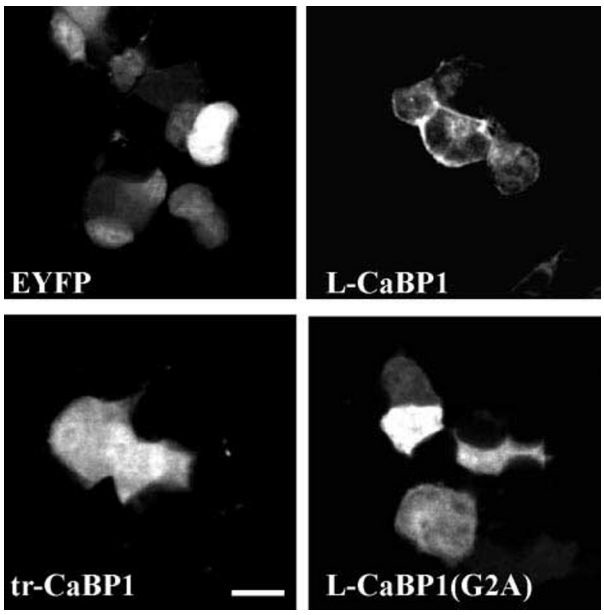

FIG. 3. **Localization of overexpressed CaBP1-EYFP constructs in PC12 cells.** Confocal images of PC12 cells transfected with either control EYFP empty vector (*EYFP*), CaBP1-EYFP (L-CaBP1), Tr-CaBP1-EYFP (*Tr-CaBP1*), or L-CaBP1(G2A)-EYFP (L-CaBP1 (G2A)) are shown as indicated. The *scale bar* represents 10 μm.

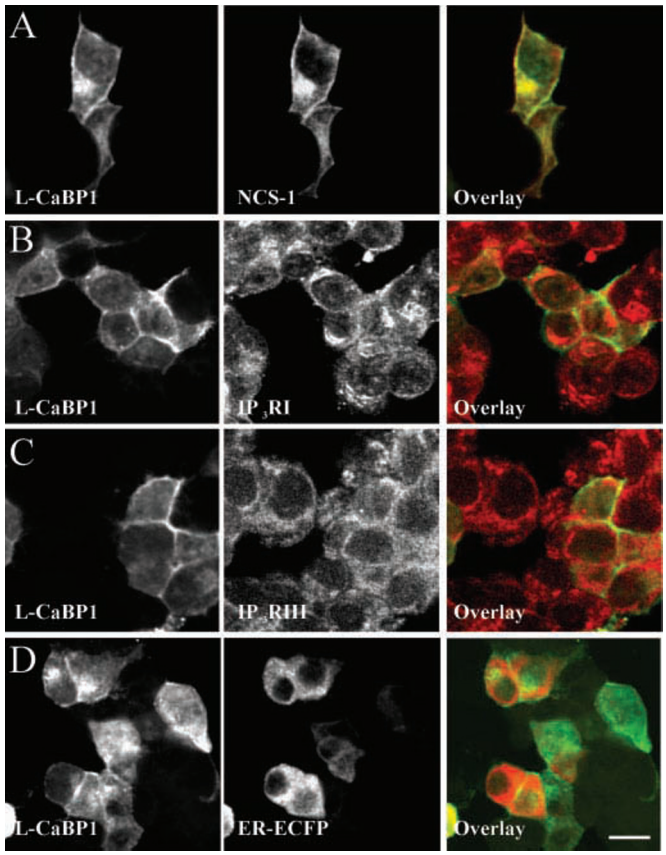

FIG. 4. **Examination of co-localization of L-CaBP1-EYFP with NCS-1-ECFP, InsP3R types 1 and 3, and an ER-specific marker.** *A*, confocal images of PC12 cells transfected to express L-CaBP-1-EYFP and NCS-1-ECFP. Co-localization is seen as *yellow* in the overlay image. *B* and *C*, confocal images of cells expressing L-CaBP1-EYFP (L-CaBP1) that were fixed and subsequently immunostained for the presence of $InsP_3R1$ (*$InsP_3RI$*) or $InsP_3R3$ (*$InsP_3RIII$*). The overlays of L-CaBP1 and $InsP_3R$ are shown to display any co-localization. *D*, confocal images of PC12 cells transfected with L-CaBP1-EYFP (L-CaBP1) and a specific ER marker construct (*ER-ECFP*) are shown as indicated. The *scale bar* represents 10 μm.

of the receptor that have previously been demonstrated to bind GST-Tr-CaBP1. We compared, therefore, the localization of L-CaBP1-EYFP with these two receptor subtypes in PC12 cells using receptor-specific antibodies. $InsP_3R1$ (Fig. 4*B*, *$InsP_3RI$*) and $InsP_3R3$ (Fig. 4*C*, *$InsP_3RIII$*) displayed a punctate distribution throughout the PC12 cells. The distribution of both $InsP_3R1$ and $InsP_3R3$ may reflect their presumed localization to regions of the ER; however, neither receptor showed obvious extensive co-localization with L-CaBP1 (Fig. 4, *B* and *C*, *overlap*). CaBP1 also failed to show any obvious extensive co-localization with a general ER content marker tagged with ECFP in live cells (Fig. 4*D*).

The Effect of L-CaBP1 Overexpression on ATP-stimulated $[Ca^{2+}]_i$ Elevation in PC12 Cells—Through the application of selective purinergic receptor agonists, it is possible, with PC12 cells, to activate simultaneous entry of extracellular Ca^{2+} and release from $InsP_3$-dependent intracellular stores (ATP as agonist) or to activate release solely from the $InsP_3$-dependent stores (UTP as agonist) (31, 32). We therefore attempted to dissect the likely mode of action of overexpressed L-CaBP1, Tr-CaBP1, and L-CaBP1 (G2A) with respect to $InsP_3R$ function, in PC12 cells, by examining alterations in $[Ca^{2+}]_i$ in response to stimulation by ATP or UTP. In cells overexpressing EYFP protein alone, robust $[Ca^{2+}]_i$ rises in response to application of 100 μM ATP were observed, which were almost identical to responses recorded from control, nontransfected cells monitored simultaneously (Fig. 5*A*). This result suggests that transfection and overexpression of EYFP does not adversely affect normal rises in $[Ca^{2+}]_i$. Averaged peak responses from a number of cells (Fig. 5*A*) confirmed that there was no statistically significant difference in $[Ca^{2+}]_i$ responses to ATP between EYFP-transfected and nontransfected cells. In contrast, overexpression of L-CaBP1-EYFP elicited a dramatic inhibitory effect on ATP-evoked elevation of $[Ca^{2+}]_i$ (Fig. 5*B*). Averaged responses from L-CaBP1-EYFP-transfected *versus* nontransfected control cells from the same experiment (Fig. 5*B*) indicated that L-CaBP1 reduced the peak rises in $[Ca^{2+}]_i$ by 66% compared with control cells.

Previous work has suggested a stimulatory rather than inhibitory role for CaBP1 (17). One possibility is that the inhibitory effect we observed was a consequence of the presence of the C-terminal EYFP tag on the expressed L-CaBP1 modifying the protein function. We therefore also tested the effects of untagged L-CaBP1 expressed from a pcDNA3-based construct. In this case, the PC12 cells were co-transfected with a second plasmid encoding EYFP for their identification. Such an approach with PC12 cells results in co-expression of both proteins in at least 95% of cells (33). In these experiments, L-CaBP1 produced a 46% inhibition (Supplemental Fig. 1) of $[Ca^{2+}]_i$ responses to ATP compared with nontransfected cells in the same fields ($p < 0.0001$, $n = 24$ for nontransfected and $n = 22$ for transfected cells), indicating that the untagged CaBP1 protein has the same effect on agonist-induced responses as the EYFP-tagged version.

The effect of Tr-CaBP1, L-CaBP1 (G2A), and NCS-1 Overexpression on ATP-stimulated $[Ca^{2+}]_i$ Elevation in PC12 Cells—It has been previously reported that a recombinant, GST-free form of Tr-CaBP1 is able to stimulate $InsP_3R$-dependent Ca^{2+} release in the absence of $InsP_3$ (17). Having observed that L-CaBP1 is able to exert a dramatic inhibitory effect on rises in $[Ca^{2+}]_i$ in intact PC12 cells, we decided to test whether Tr-CaBP1 would behave in a similar manner in this system. We also aimed to test whether myristoylation of L-CaBP1 is likely to be of importance to its inhibitory mode of action by analyzing the effect of L-CaBP1 (G2A) on ATP-driven rises in $[Ca^{2+}]_i$. When overexpressed, Tr-CaBP1-EYFP inhibited ATP-

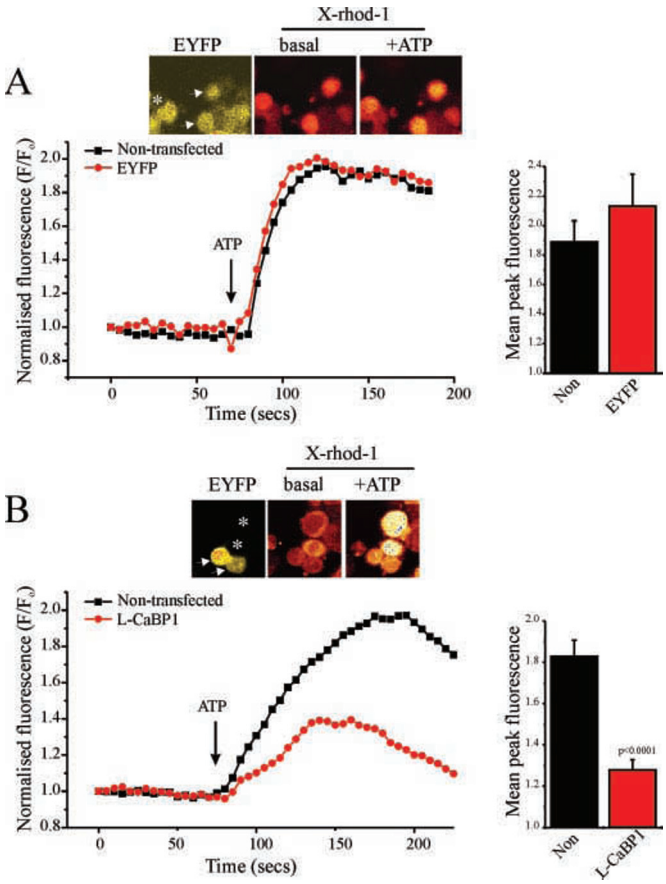

FIG. 5. **Effect of EYFP and L-CaBP1 overexpression on ATP-evoked intracellular Ca^{2+} transients in PC12 cells.** *A*, following transfection, PC12 cells were loaded with X-rhod-1, and fluorescence was monitored. The *images* at the *top* are of EYFP or X-rhod-1 fluorescence before and after the addition of 100 μM ATP and show responses from two transfected cells (*arrowheads*) and one nontransfected cell (*asterisk*). Representative normalized (F/F_0) fluorescence changes *versus* time are shown from EYFP-transfected and nontransfected control cells. The *bar graph* shows averaged peak fluorescence responses from EYFP-transfected *versus* control, nontransfected (*Non*), PC12 cells in response to 100 μM ATP ($n = 7$ for EYFP-transfected; $n = 7$ for nontransfected controls; mean ± S.E.). *B*, the *images* at the *top* are of EYFP or X-rhod-1 fluorescence before and after the addition of 100 μM ATP and show responses from two transfected cells (*arrowheads*) and two nontransfected cells (*asterisks*). Representative normalized (F/F_0) fluorescence changes *versus* time from L-CaBP1-EYFP-transfected and nontransfected control PC12 cells are shown. The *bar graph* shows averaged peak fluorescence responses from L-CaBP1-EYFP- transfected *versus* control, nontransfected, PC12 cells in response to 100 μM ATP ($n = 13$ for L-CaBP1-transfected; $n = 13$ for nontransfected controls; mean ± S.E.; $p < 0.0001$ from Student's unpaired t test).

stimulated rises in $[Ca^{2+}]_i$ in a manner similar to L-CaBP1-EYFP (Fig. 6*A*). Averaged peak responses to ATP for Tr-CaBP1-EYFP-transfected *versus* nontransfected, control cells from the same experiment (Fig. 6*A*) indicated that Tr-CaBP1 inhibited the rises in $[Ca^{2+}]_i$ to a similar magnitude as for L-CaBP1 (average inhibition was 64%). Similarly, overexpression of L-CaBP1 (G2A)-EYFP also led to an inhibition of ATP-evoked $[Ca^{2+}]_i$ rises compared with control, nontransfected cells (Fig. 6*B*). Average peak responses indicated that this mutant induced a 44% suppression of the ATP-induced rise in $[Ca^{2+}]_i$, which is less dramatic than for L-CaBP1 or Tr-CaBP1 but nonetheless represents a significant blockade of the Ca^{2+} signal.

The observed inhibitory effects of all CaBP1 constructs tested could simply be attributed to a Ca^{2+} buffering artifact resulting from overexpression of a protein that has a high affinity for Ca^{2+}. In order to rule out this possibility, we also tested the effect of overexpression of the related Ca^{2+}-sensing protein, NCS-1 (12). It is clear that NCS-1, *in vitro*, exhibits an affinity for Ca^{2+} that is almost identical to L-CaBP1 (Fig. 1*B*), has a closely similar localization (Fig. 4*A*), and is also endogenously expressed in PC12 cells (19) and therefore represents an ideal control protein for these experiments. Overexpression of NCS-1-EYFP elicited no significant alteration in rises in $[Ca^{2+}]_i$ on stimulation with ATP (Fig. 6*C*). Averaged peak response data from a population of cells confirmed this observation (Fig. 6*C*). The finding that NCS-1 is unable to suppress rises in $[Ca^{2+}]_i$ in a similar fashion to the CaBP1 proteins suggests that the repression observed is due to a specific inhibitory function of the CaBPs and not a nonspecific Ca^{2+} buffering effect.

The Effect of L-CaBP1 Overexpression on UTP and ATP-stimulated $[Ca^{2+}]_i$ Elevation in PC12 Cells—In order to delineate more precisely the likely targets for CaBP1-mediated inhibition of $[Ca^{2+}]_i$ elevation, we utilized both ATP and UTP stimulation of PC12 cells to distinguish between entry through plasma membrane Ca^{2+} channels and release, via $InsP_3Rs$, from intracellular stores. Application of 1 mM UTP to control, nontransfected cells (Fig. 6*D*), generated an intracellular Ca^{2+} transient that represents release of Ca^{2+} from $InsP_3$-gated intracellular stores (31, 32). In contrast, overexpression of L-CaBP1-EYFP led to an almost complete abolition of the UTP-evoked transient (Fig. 6*D*). Averaged peak responses to UTP confirmed that CaBP1-EYFP was able to significantly inhibit responses to this agonist (Fig. 6*D*, the averaged inhibition was 70%). Application of ATP following initial stimulation with UTP generated a further $[Ca^{2+}]_i$ rise that is still inhibited in cells overexpressing L-CaBP1-EYFP (Fig. 6*D*). Averaged responses to ATP stimulation indicate that, following UTP treatment, L-CaBP1-EYFP remained capable of inhibiting rises in $[Ca^{2+}]_i$ (Fig. 6*D*). The percentage inhibition was lower than seen with UTP stimulation in the same cells, suggesting that the major target through which the protein is able to suppress rises in $[Ca^{2+}]_i$ is an $InsP_3R$-dependent release from intracellular stores. One potential explanation for the observed inhibition of intracellular Ca^{2+} release by L-CaBP1 would be if overexpression of the protein were somehow responsible for depleting Ca^{2+} from the ER, which would lead to little or no release from stores on application of UTP. To address this question, we determined ER Ca^{2+} loading in untransfected *versus* L-CaBP1-expressing cells through the application of the ER Ca^{2+} ATPase inhibitor thapsigargin in the absence of external Ca^{2+}. Application of thapsigargin led to depletion of ER Ca^{2+} and a concomitant rise in $[Ca^{2+}]_i$. The magnitude of the induced rise in $[Ca^{2+}]_i$ was similar for both nontransfected and L-CaBP1-expressing PC12 cells (Supplemental Fig. 2). This observation indicates that L-CaBP1 overexpression does not affect ER Ca^{2+} loading and suggests that the observed suppression of $[Ca^{2+}]_i$ elevation in response to UTP application in L-CaBP1-transfected cells arises as a consequence of a direct inhibition of $InsP_3R$-mediated Ca^{2+} release.

The Effect of L-CaBP1 Overexpression on $InsP_3$-induced Release of Ca^{2+} from Intracellular Stores in PC12 Cells—Observations from intact cells suggested a direct inhibitory function for L-CaBP1 on Ca^{2+} release from $InsP_3$-gated intracellular stores. It was not possible, however, to demonstrate this with absolute certainty from intact cell experiments utilizing agonists such as ATP and UTP. We therefore aimed to provide further evidence of a direct inhibitory role for L-CaBP1 on $InsP_3$-evoked intracellular Ca^{2+} release by employing a permeabilization strategy that permitted acute triggering of release from intracellular stores. A standard protocol has been established for selectively permeabilizing only the plasma mem-

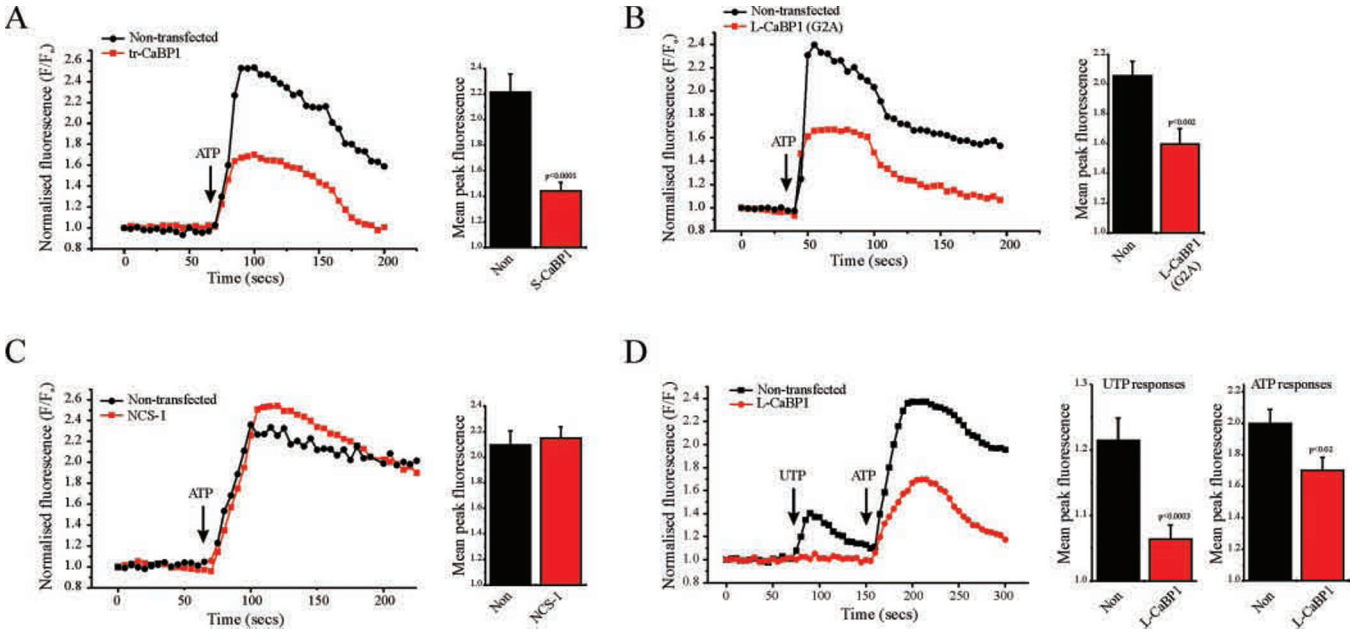

FIG. 6. **Effects of Tr-CaBP1, L-CaBP1(G2A), and NCS-1 overexpression on evoked intracellular Ca^{2+} transients in PC12 cells.** *A*, representative normalized (F/F_0) fluorescence changes *versus* time from Tr-CaBP1-EYFP transfected and nontransfected PC12 cells. The *bar graph* shows averaged peak fluorescence responses from Tr-CaBP1-EYFP-transfected *versus* control, nontransfected PC12 cells in response to 100 μM ATP ($n = 7$ for Tr-CaBP1-transfected; $n = 11$ for nontransfected controls; mean ± S.E.; $p < 0.001$). *B*, representative normalized (F/F_0) fluorescence changes *versus* time from L-CaBP1 (G2A)-EYFP transfected and nontransfected PC12 cells. The *bar graph* shows averaged peak fluorescence responses from L-CaBP1 (G2A) EYFP-transfected *versus* control, nontransfected PC12 cells in response to 100 μM ATP ($n = 16$ for L-CaBP1 (G2A)-transfected; $n = 17$ for nontransfected cells; mean ± S.E.; $p < 0.002$). *C*, representative normalized (F/F_0) fluorescence changes *versus* time from NCS-1-EYFP-transfected and nontransfected PC12 cells. The *bar graph* shows averaged peak fluorescence responses from NCS-1-EYFP-transfected *versus* nontransfected, PC12 cells in response to 100 μM ATP ($n = 18$ for NCS-1 transfected; $n = 24$ for nontransfected controls; mean ± S.E.). *D*, representative normalized (F/F_0) fluorescence changes *versus* time from L-CaBP1-EYFP-transfected and nontransfected control PC12 cells in response to sequential application of 1 mM UTP and 100 μM ATP. The *bar graphs* show the averaged peak fluorescence responses from L-CaBP1-EYFP-transfected *versus* nontransfected PC12 cells in response to 1 mM UTP or subsequent challenge with 100 μM ATP ($n = 26$ for L-CaBP1-transfected; $n = 20$ for untransfected controls; mean ± S.E.).

brane of PC12 cells through application of the agent digitonin for a short time (23). We employed an identical approach in experiments where cells overexpressing untagged L-CaBP1 and a marker for transfection (ECFP) were loaded with a low affinity Ca^{2+}-sensitive fluorophore (Mag-fluo4) that partitions into intracellular compartments including the ER (34). Cells were subsequently treated with digitonin to release cytosolic dye and then perfused with buffer containing $InsP_3$. The initial fluorescence intensities for Mag-fluo4 were the same in control and transfected cells (61.12 ± 2.95 arbitrary fluorescence units in nontransfected cells ($n = 17$) and 56.12 ± 3.98 units in transfected cells ($n = 26$)), suggesting that the luminal ER [Ca^{2+}] did not differ. Release of Ca^{2+} from stores was then monitored as a decrease in fluorescence signal following application of $InsP_3$. Application of low concentrations of $InsP_3$ to either control or transfected cells did not release more than minor amounts of ER Ca^{2+}, suggesting that CaBP1 did not act as an activating ligand for $InsP_3$ receptors. (A lack of effect of low doses of $InsP_3$ in control cells may be due to a limitation in access to the cytosol in these mildly permeabilized cells or conversion of cytosolic $InsP_3$ to inactive forms.) The addition of 10 μM $InsP_3$ to control, untransfected cells led to significant Ca^{2+} release from the ER, but this effect was abolished or reduced in all L-CaBP1-transfected cells analyzed (Fig. 7). Averaged responses to $InsP_3$ treatment from a population of cells confirmed that L-CaBP1 was able to significantly diminish release of intracellular Ca^{2+} in response to direct $InsP_3$ challenge with an average inhibition of 75% (Fig. 7).

The Effect of L-CaBP1 Overexpression on Histamine-evoked Intracellular Ca^{2+} Oscillations in HeLa Cells—To test the general applicability of L-CaBP1 function, we decided to further analyze the effect of its expression in a nonneuronal cell line.

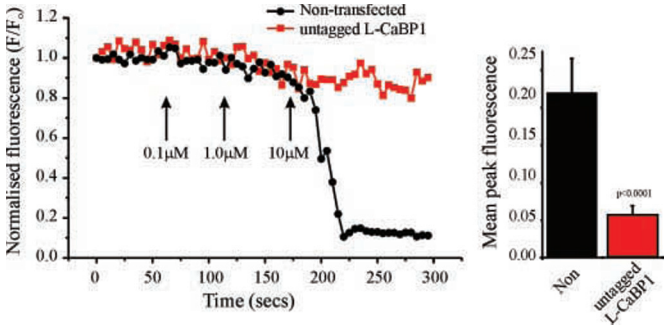

FIG. 7. **Effect of overexpression of untagged L-CaBP1 on $InsP_3$-evoked intracellular Ca^{2+} release in permeabilized PC12 cells.** PC12 cells were co-transfected with plasmids to express untagged L-CaBP1 and ECFP to mark the transfected cells. Following transfection, cells were loaded with Mag-fluo4, and fluorescence was monitored. The cells were permeabilized by perfusion with 20 mM digitonin for 6 min and then perfused with buffer containing 10 mM $InsP_3$. Representative normalized (F/F_0) fluorescence changes *versus* time are shown from EYFP/CaBP1-transfected and nontransfected control cells. The *bar graph* shows averaged peak changes in fluorescence from EYFP/CaBP1-transfected *versus* control, nontransfected, PC12 cells ($n = 26$ for transfected; $n = 17$ for nontransfected controls; mean ± S.E.; $p < 0.0001$).

HeLa cells are unlikely to express endogenous L-CaBP1 and display well characterized $[Ca^{2+}]_i$ oscillations in response the agonist histamine (35, 36). We therefore examined the consequence of L-CaBP1 expression on $[Ca^{2+}]_i$ rises in this system. In control, untransfected, cells, application of histamine induced a prolonged series of oscillatory fluctuations in $[Ca^{2+}]_i$ (Fig. 8). In contrast, in cells expressing L-CaBP1-EYFP, histamine-evoked $Ca^{2+}{}_i$ oscillations were almost completely abol-

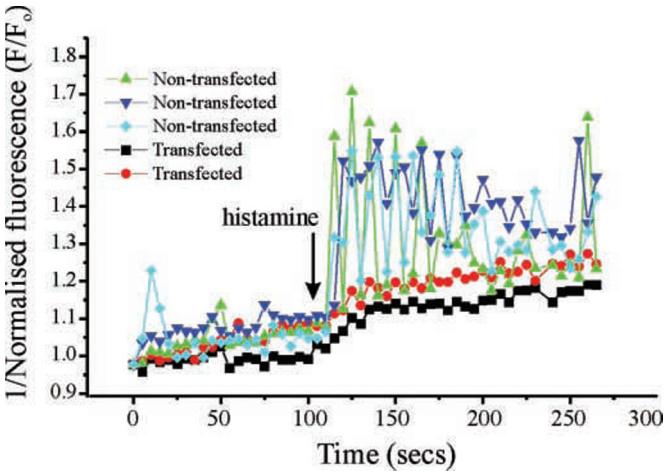

FIG. 8. **Effect of L-CaBP1 overexpression on histamine-induced intracellular Ca^{2+} oscillations in HeLa cells.** Representative normalized (F/F_0) fluorescence changes *versus* time from L-CaBP1-EYFP transfected and nontransfected control HeLa cells in response to application of 100 μM histamine are shown for three nontransfected control and two transfected cells in the same microscope field.

ished (Fig. 8). This result suggests that L-CaBP1 is capable of suppressing $InsP_3R$-mediated $[Ca^{2+}]_i$ rises in a generalized manner and that its restricted pattern of expression lends functional specificity in neuronal/retinal tissues.

DISCUSSION

Many physiological processes driven by Ca^{2+} are transduced by specific Ca^{2+}-binding proteins (11, 37–39). In recent years, new members of the CaM superfamily have been identified that appear to mediate particular signaling pathways in specific tissue and cell types (2, 12, 38). One recently discovered CaM subfamily, the CaBPs (15), appear to display a predominantly neuronal pattern of expression, and early functional reports have inferred roles in the regulation of $[Ca^{2+}]_i$ (17, 18). Forms of CaBP1 have been identified as $InsP_3$-independent agonists of the $InsP_3R$ (17). In this study, we have analyzed aspects of the basic biochemistry of L-CaBP1 in combination with functional studies employing the model neuroendocrine PC12 cell system to investigate the consequences of L-CaBP1 expression on intracellular Ca^{2+} signals.

Proteins that act as transducers of Ca^{2+} signals switch conformation upon binding Ca^{2+} (40). Conversely, many proteins whose function is simply to buffer excess free Ca^{2+} do not undergo significant changes in conformation upon Ca^{2+} binding (40, 41). We have monitored fluorescence emission spectra for both NCS-1 (24, 42) and L-CaBP1 in response to Ca^{2+} binding and show that both proteins exhibit submicromolar binding affinities that are coupled to significant conformational alterations, providing evidence that L-CaBP1 exhibits characteristics in common with established sensors of intracellular Ca^{2+} signals such as CaM and NCS-1.

Forms of CaBP1 have been suggested to bind to both $InsP_3Rs$ and P/Q type voltage-gated Ca^{2+} channels (17, 18). We have investigated the interaction of L-CaBP1 with both of these channels and have determined that it is capable of binding to both $InsP_3R1$ and the $Ca_v2.1$-α_{1A} channel subunit in a Ca^{2+}-dependent fashion. These binding data, in conjunction with the determination of submicromolar Ca^{2+} affinity, suggest that the recombinant L-CaBP1 construct used in these studies is fully functional in Ca^{2+} binding and that this binding is important for interaction with target proteins. Association with the $InsP_3R1$ receptor has already been reported to be Ca^{2+}-dependent (17); however, interaction with the P/Q $Ca_v2.1$ channel was determined to be insensitive to Ca^{2+} (18). The reason for the discrepancy between our observations and the earlier report is unclear but may be due to differences in experimental procedure.

Almost all current experimental data that have been generated concerning the localization and function of CaBP1 have either involved *in vitro* analyses or expression/application of the protein in systems where it does not endogenously occur. We therefore attempted to examine CaBP1 function in a more applicable *in vivo* system. PC12 cells represent a well characterized neuroendocrine cell line that has been applied to the study of various aspects of neuronal cell function, especially neurosecretion (43). We have shown that these cells do express L-CaBP1, along with its larger splice isoform caldendrin, and that anti-L-CaBP1 antiserum prominently stains the plasma membrane region of these cells and intracellular structures. Due to cross-reactivity of the anti-L-CaBP1 antibody, at present we are unable to determine whether this staining derives entirely from L-CaBP1 or a combination of L-CaBP1 and caldendrin. However, an EYFP-tagged version of L-CaBP1 displays a very similar localization to that observed for the endogenous protein and in favorable confocal sections could be seen to be associated with perinuclear structures, probably the Golgi complex as seen for endogenous and exogenous NCS-1 in PC12 cells (21, 29), which colocalizes with CaBP1.

Analysis of myristoylation-defective forms of L-CaBP1 (L-CaBP1 (G2A) and Tr-CaBP1) suggests that the protein is indeed acylated *in vivo* and that this modification is essential for productive targeting to specific cellular membranes. In related studies, we have also examined the co-localization of L-CaBP1 with an ER specific marker and with the type 1 and 3 $InsP_3Rs$. There was no obvious extensive co-localization of L-CaBP1 with any of these markers in PC12 cells. Previous work has demonstrated that endogenous CaBP1 can be immunoprecipitated with both types 1 and 3 IP_3Rs from whole brain extracts (17), providing strong evidence for a direct *in vivo* interaction between these proteins. It is likely, therefore, that the apparent discrepancy between the immunolocalization and binding assays is due to only a small proportion of expressed CaBP1 actually being associated with IP_3Rs. It should be noted that there is growing evidence suggesting that $InsP_3Rs$ may be present on the Golgi apparatus (44). The L-CaBP1 localizes to a perinuclear region in PC12 cells that is likely to correspond to the Golgi complex, and therefore this observation may warrant further investigation.

The most interesting feature of the CaBP1 protein relates to its reported ability to stimulate $InsP_3R$-mediated Ca^{2+} release in the absence of $InsP_3$ (17). Since L-CaBP1 is endogenously expressed in PC12 cells, we examined the effects of L-CaBP1 expression on intracellular Ca^{2+} release in these cells. Overexpression of L-CaBP1 dramatically inhibited both ATP- and UTP-evoked rises in $[Ca^{2+}]_i$. These observations are consistent with L-CaBP1 exerting a direct inhibitory function on $InsP_3$-gated Ca^{2+} release from intracellular stores, although we are unable to formally dismiss a small inhibitory effect on influx of extracellular Ca^{2+}. It seems likely that the effects exerted by L-CaBP1 are functionally relevant, since they were unaffected by removal of the EYFP tag, overexpression of the protein did not appear to alter loading of intracellular Ca^{2+} stores as determined by thapsigargin treatment of transfected *versus* untransfected cells, and overexpression of the related Ca^{2+} sensor protein NCS-1 did not inhibit ATP-evoked $[Ca^{2+}]_i$ rises. In addition, a direct inhibitory effect of L-CaBP1 on $InsP_3$-mediated Ca^{2+} release from the ER was shown by examination of changes in ER $[Ca^{2+}]$ in permeabilized PC12 cells after application of exogenous $InsP_3$. In parallel studies employing myristoylation-defective forms of L-CaBP1, we have also demonstrated that this modification, although essential for mem-

brane localization of the protein, is dispensable for the inhibitory effects observed in relation to agonist evoked $[Ca^{2+}]_i$ rises. The observation that largely nontargeted L-CaBP1 is able to almost abolish $InsP_3$-mediated intracellular Ca^{2+} release might be consistent with an overall lack of extensive co-localization of these proteins *in vivo*, suggestive instead of a small pool of IP_3R-associated protein.

We furthered our functional studies to determine whether L-CaBP1 was capable of exerting a similar inhibitory phenotype on intracellular Ca^{2+} signals in a cell line where the protein is not endogenously expressed. In HeLa cells, expression of L-CaBP1 almost completely abolished histamine-evoked intracellular Ca^{2+} transients, suggesting that this protein may be capable of a more generalized inhibition of $InsP_3R$-mediated intracellular Ca^{2+} release. It seems likely that the restricted pattern of expression of L-CaBP1 is responsible for localizing its inhibitory function on $InsP_3Rs$ to specialized regions of the central nervous system; however, the physiological relevance of this activity remains to be investigated.

Initial reports concerning CaBP1 function hinted at a potentially important new mode of control for the generation of intracellular Ca^{2+} signals mediated through $InsP_3R$-governed stores (17). Results from a model neuroendocrine cell system presented in this study confirm that L-CaBP1 is capable of binding to $InsP_3$ receptors and affecting Ca^{2+} release from intracellular stores as originally suggested. Our data indicate, however, that the protein exerts an inhibitory rather than stimulatory effect during agonist-induced intracellular Ca^{2+} signaling. The reasons for these converse results are not immediately clear. One possibility could relate to the stability of recombinant CaBP proteins, especially for truncated forms as found in the present study. Fragments derived from full-length CaBP1 could have altered functional effects. It should also be noted that the stimulatory effect of CaBP1 that was reported was not in an intact cell system and was seen with $InsP_3$ receptors of *Xenopus*, a species that does not appear to express CaBPs based on data base searches. CaBP1 has also been found to inhibit an alternative Ca^{2+}-induced Ca^{2+} release pathway (45), although NCS-1 had a similar effect. In addition, results recently presented by Roderick *et al.* (46) also suggest that CaBP1 has an inhibitory action in nonneuronal COS cells. In other studies we have been unable to observe any activation of Ca^{2+} responses following introduction of recombinant L-CaBP1 into pancreatic acinar cells.[2] Overall, the accumulating evidence would appear to indicate that CaBP proteins are likely to be important new negative regulators in the control of intracellular Ca^{2+} signals in neuronal tissues.

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[2] S. Voronina, unpublished observations.