Regulation of InsP₃ receptor activity by neuronal Ca²⁺-binding proteins

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Introduction

Changes in intracellular Ca²⁺ control a diverse array of cellular processes, including fertilisation, memory formation and gene transcription (Berridge et al., 2003). Increases in cytosolic Ca²⁺ levels may arise as a result of either its release from the endoplasmic reticulum (ER) intracellular Ca²⁺ store or influx across the plasma membrane. The principal Ca²⁺ release channels on the ER belong to the ryanodine receptor (RyR) and inositol 1,4,5-trisphosphate receptor (InsP₃R) families (Ehrlich, 1995; Iino, 1999).

Both families of channels are biphasically regulated by Ca²⁺ (Bezprozvanny et al., 1991). In addition, inositol 1,4,5-trisphosphate (InsP₃) is a co-agonist for InsP₃Rs (Bezprozvanny et al., 1991). The mechanism by which Ca²⁺ exerts its effect has been the subject of intense investigation resulting in the identification of several Ca²⁺ binding sites in the InsP₃R (Sienaert et al., 1997). In addition to a direct action of Ca²⁺ on the receptor, Ca²⁺ may function through Ca²⁺-sensing proteins that either bind directly to or modify channel activity by the addition/removal of secondary modifications such as phosphate groups (Patel et al., 1999; Thrower et al., 2001; Nadif Kasri et al., 2002; Taylor and Laude, 2002; Roderick and Bootman, 2003). The tetra EF-hand-containing Ca²⁺ sensor protein calmodulin (CaM) has been shown to modulate the activity of InsP₃Rs in both a Ca²⁺ dependent and independent manner (Reviewed in Nadif Kasri et al., 2002; Taylor and Laude, 2002). The Ca²⁺ independent CaM binding domain has been mapped to two noncontiguous sites in the NH₂-terminal 159 amino acids (aa) of the type 1 InsP₃R (Sienaert et al., 2002). The interaction of CaM with these sites was shown to decrease the affinity of the receptor for InsP₃ (Sienaert et al., 2002). The inhibition of InsP₃-induced Ca²⁺ release (IICR) by CaM-like proteins may have great significance in neurons where it has been shown to underlie processes including axonal guidance, growth cone formation as well as excitability, which govern long-term potentiation and depression (LTD) associated with memory formation (Nakamura et al., 1999; Nagase et al., 2003). The propagation of Ca²⁺ release following repetitive stimulation of the parallel fibre/Purkinje cell synapses is generally restricted to the postsynaptic spines. This is surprising since Purkinje cells abundantly express InsP₃Rs throughout the cell (Snyder and Supattapone, 1989; Volpe et al., 1991). Thus, it has been proposed that the high levels of CaM also present in these cells (~10μM; Kakiuchi et al., 1982) decrease IICR and restrict excitability (Cardy and Taylor, 1998).

A family of neuronal Ca²⁺-binding proteins (CaBPs), sharing significant homology in sequence and structure with CaM, has recently been described in the retina and brain (Haeseleer et al., 2000). This family of proteins belongs to the superfamily of neuronal Ca²⁺ sensor proteins that also include NCS1 (or frequenin) and KChIPs, which have been shown to be involved in neuronal signalling (Burgoyne and...
Unlike CaM, one or two of the EF hand Ca\(^{2+}\)-binding domains in CaBPs are nonfunctional (Haeseleer et al., 2000). CaBPs have been shown to bind to the NH\(_2\)-terminus of InsP\(_3\)Rs in a Ca\(^{2+}\)-dependent manner. Furthermore, it was reported that CaBP1 activated Ca\(^{2+}\) release through InsP\(_3\)Rs independent of InsP\(_3\) binding (Yang et al., 2002). Thus, if CaBPs act as endogenous surrogates for InsP\(_3\), it is possible that InsP\(_3\)Rs may be directly regulated by Ca\(^{2+}\), allowing them to act as true Ca\(^{2+}\)-induced Ca\(^{2+}\) release channels in a manner similar to RyRs (Bootman et al., 2002).

In this study, we rigorously tested the functional relationship between CaBP- and InsP\(_3\)-mediated Ca\(^{2+}\) release. We determined that CaBP1 interacted with InsP\(_3\)Rs in a Ca\(^{2+}\)-independent manner. This interaction was localised to a peptide sequence in the NH\(_2\)-terminal 159 aa of InsP\(_3\)R1 to which CaM has also been shown to bind. CaBP1 binding resulted in a decrease in the sensitivity of IICCR. This was demonstrated by measuring IICCR in COS cells overexpressing CaBP1, in Xenopus oocytes microinjected with recombinant CaBP1 and permeabilised COS cells exposed to recombinant CaBP1. In addition, CaBP1 by itself could not activate Ca\(^{2+}\) release. We also show that CaBP1 is phosphorylated at a casein kinase 2 consensus sequence, which regulates its efficacy to inhibit IICCR. The use of COS cells and Xenopus oocytes, which do not express endogenous CaBP1, provided an ideal null background to investigate the function of CaBP1. Our data suggest that CaBP1 can behave as an endogenous regulator of InsP\(_3\)R activity, and may serve to tune the sensitivity of InsP\(_3\)Rs to InsP\(_3\). Although structurally similar to CaM, CaBPs have distinct effects and provide an additional facet of InsP\(_3\)R regulation.

**Results**

**CaBP1 inhibits agonist-induced Ca\(^{2+}\) signals**

CaBP1 has previously been reported to increase the open probability of InsP\(_3\)Rs independently of InsP\(_3\) in nuclei isolated from Xenopus oocytes (Yang et al., 2002). To test whether this was also true in an intact cellular system, we first adopted a heterologous expression approach. Expression vectors for both the naturally occurring long and short CaBP1 isoforms were generated. In addition, to identify cells expressing these constructs, YFP was appended to the COOH-terminus of the CaBPs (Figure 1A). Figure 1B shows that when over-expressed, both SCaBP1-YFP and LCaBP1-YFP are targeted primarily to the plasma membrane, with some of the protein also residing on intracellular membranes, including the Golgi apparatus. A weak, but significant, fluorescence was also observed in the cytosol. Transiently expressed SCaBP1-YFP and LCaBP1-YFP, detected using an anti-GFP antibody, migrated at the expected molecular mass (Figure 1C, top panel).

Equivalent levels of expression of the endogenous type 3 InsP\(_3\)R (InsP\(_3\)R3) were detected (the major InsP\(_3\)R isoform in Xenopus oocytes microinjected with recombinant CaBP1 and permeabilised COS cells exposed to recombinant CaBP1. In addition, CaBP1 by itself could not activate Ca\(^{2+}\) release. We also show that CaBP1 is phosphorylated at a casein kinase 2 consensus sequence, which regulates its efficacy to inhibit IICCR. The use of COS cells and Xenopus oocytes, which do not express endogenous CaBP1, provided an ideal null background to investigate the function of CaBP1. Our data suggest that CaBP1 can behave as an endogenous regulator of InsP\(_3\)R activity, and may serve to tune the sensitivity of InsP\(_3\)Rs to InsP\(_3\). Although structurally similar to CaM, CaBPs have distinct effects and provide an additional facet of InsP\(_3\)R regulation.

![Figure 1](image-url)

**Figure 1** Design and expression of CaBP-YFP vectors. (A) Graphical representation of the LCaBP1-YFP and SCaBP1-YFP constructs used. The location of the additional aa in LCaBP1 compared to SCaBP1 is indicated by the red box. The central z-helix is indicated by the two triangles and the myristoylation sequence is represented by a circle. The four EF hand Ca\(^{2+}\)-binding domains are indicated and the nonfunctional EF hand is shown by the red cross. The yellow ellipse illustrates the COOH-terminal YFP. In the lower panel, aa boundaries for both S/LCaBP and truncated cCaBP (additional methionine at the start of cCaBP shown in grey) are shown. The myristoylated glycine is in bold. (B) COS-7 cells were transiently transfected with S/LCaBP-YFP constructs. At 48 h post-transfection, cells were imaged confocally through a ×100, 1.4 n.a. oil immersion objective (Perkin-Elmer, Cambridge UK). The 488 nm line of the krypton/argon laser was used for excitation and emission collected >505 nm. (C) Mobility of YFP, SCaBP1-YFP and LCaBP1-YFP was determined in transiently transfected COS-7 cells by Western blot using anti-GFP antibody. (Lower panel) Abundance of InsP\(_3\)R3 was determined in cells transfected with YFP, SCaBP1, and LCaBP1 isolated by FACS.

At 48 h post-transfection, ATP-evoked Ca\(^{2+}\) transients were monitored in Fura-2-loaded cells. Expression of either LCaBP1 or SCaBP1 resulted in a significant decrease in the number of responsive cells at both 0.5 and 1 μM ATP, and a decrease in the occurrence of agonist-evoked oscillations in those cells that responded (Figure 2A and B). In addition, the integrated Ca\(^{2+}\) response at these agonist concentrations was reduced (Figure 2D). The integrated Ca\(^{2+}\) response to maximal agonist (100 μM ATP) was not significantly affected by CaBP1 expression, indicating that the Ca\(^{2+}\) content of the agonist-sensitive store was unchanged (Figure 2D). Although the amplitude and integrated Ca\(^{2+}\) response to maximal ATP were not altered by CaBP expression, the latency between agonist addition and the peak response was significantly...
ATP. (expression of EYFP alone (data not shown). Since both

concentration applied are indicated in the bar graph. Statistical

red bar). The peak amplitudes of the Ca2+

D) Integrated Ca2+ signal observed with 0.5, 1 and 100 μM

ATP. (E) Caffeine-induced Ca2+ release in HEK293 cells stably

expressing RyR3 transiently transfected with ScaBP1-YFP. The

inhibition of IICR in CaBP-expressing cells was confirmed by

application of the InsP3-generating agonist carbachol (10 μM CCH,

red bar). The peak amplitudes of the Ca2+

release in HEK293 cells stably

expressed regions of these two isoforms (cCaBP) was expressed

(Figure 1A). cCaBP resulted in a significant decrease in the

percentage of responding cells at 0.5 μM ATP from 63.9 ± 2.9% in control cells to 26.4 ± 3.7% in cCaBP-expressing

cells (n = 11, P < 0.05), which is comparable to that observed for long and short CaBP (Figure 2B). The specificity

of the effect of CaBP1 upon IICR was further demonstrated by

the absence of any inhibition of caffeine-induced Ca2+

release through RyRs in HEK293 cells in which they are stably

expressed (Rossi et al, 2002) (Figure 2E). Carbachol-

induced Ca2+ release measured in the same cells overexpressing

CaBP1 was however inhibited, suggesting that InsP3Rs

were specifically targeted.

To rule out that the effect of CaBP1 on IICR was mediated by

Ca2+ buffering, the EF-hand-containing Ca2+ buffer protein calbindin was overexpressed and its effects on Ca2+

signals were examined. Unlike CaBP, calbindin overexpression
did not significantly reduce the number of cells responding
to ATP, even at the lowest agonist concentrations used
(Supplementary Figure 1). Furthermore, overexpression of

CaBP1 in which the three EF hands were disabled (CaBP1134)

(Yang et al, 2002) resulted in a similar level of inhibition of

IICR as cells expressing wild-type CaBP1 (13 ± 6% of cells

expressing CaBP1134 versus 17.6 ± 4.5% of cells for SCaBP1

responded to 0.5 μM ATP, P > 0.05). Thus, we conclude that

the inhibition of IICR by CaBP1 is Ca2+ independent.

CaBP1 directly targets InsP3Rs to inhibit IICR

To test that the inhibition of IICR was due to a direct effect on

InsP3Rs, we employed a number of strategies. Application of

a cell-permeant InsP3 ester (InsP3BM) (Thomas et al, 2000)

induced a robust Ca2+ transient preceded by Ca2+ oscillations in control COS-7 cells, which was not observed in

CaBP1-overexpressing cells (Figure 3A). Confocal microscopy

was also used to monitor the effect of CaBP on IICR in Fluo-4-

loaded Xenopus oocytes. The oocytes were injected with either recombinant CaBP1 (8.5 μM final) or vehicle 30 min

prior to imaging. In control oocytes, injection of 40 nM F-

InsP3 resulted in an accumulating increase in cytosolic Ca2+

levels punctuated by Ca2+ puffs (Figure 3B), which was not observed in the majority of oocytes injected with CaBP1

(Figure 3C). At 100 nM F-InsP3, however, Ca2+ release was observed in CaBP1-injected oocytes, although puffs prior to

the Ca2+ tide were not apparent (data not shown). Furthermore, no calcium release was observed in oocytes

imaged simultaneously with injection of recombinant CaBP1 alone (Supplementary Figure 2). These data indicated that

CaBP1 did not induce Ca2+ release, did not irreversibly inhibit InsP3Rs, but significantly reduced the sensitivity of

IICR.

In previous studies we have shown the affinity of the

Ca2+-independent CaM binding site on InsP3R1 to be 2 μM

(Sienaert et al, 2002). In this study, based on the amount of

excess peptide required to prevent migration of CaBP into
gels (depicted in Figure 4C) compared to CaM under the same

conditions, we conclude that CaBP has a three-fold greater

affinity for the InsP3R than CaM. Thus, to investigate the

effects of CaBP on Ca2+ release, we used concentrations of

the protein up to one order of magnitude greater than its Kd.
To quantitate specifically the unidirectional flux of Ca2+, we

prolonged. Therefore, even in the face of maximal InsP3

increases, CaBP effected a delay in the development of the

Ca2+ transient, consistent with a decrease in the sensitivity of

InsP3Rs (Figure 2C). Similar results were obtained when

ScaBP1 was expressed in HeLa, SH-SYSY and HEK293 cells,

indicating that CaBP1 could act on IICR from a variety of cell

types expressing differing InsP3R isform complements (data

not shown). No effect on IICR was observed following

expression of EYFP alone (data not shown). Since both

LCaBP1 and ScaBP1 were inhibitory to Ca2+ release, an

NH2-terminally truncated cDNA construct spanning the con-

served regions of these two isoforms (cCaBP) was expressed

(Figure 1A). cCaBP resulted in a significant decrease in the

percentage of responding cells at 0.5 μM ATP from 63.9 ± 2.9% in control cells to 26.4 ± 3.7% in cCaBP-expressing

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effects of CaBP on Ca2+ release, we used concentrations of

the protein up to one order of magnitude greater than its Kd.
To quantitate specifically the unidirectional flux of Ca2+, we
The results represent the mean ± s.e.m. of three independent experiments, each performed in duplicate.

measured InsP3-induced \(^{45}\)Ca\(^{2+}\) flux from permeabilised COS cells in the presence and absence of 10 \(\mu\)M His-tagged CaBP1. We found that CaBP1 significantly increased the IC\(_{50}\) of ICR from 0.41 ± 0.13 to 1.83 ± 0.21 \(\mu\)M \((P < 0.005)\) (Figure 3D).

Interaction between CaBP1 and the NH2-terminal region of InsP3R1

To map the location of the CaBP-binding region within the NH2-terminus of InsP3R1, pull-down experiments using GST fusions of different NH2-terminal domains of the InsP3R1 were performed (Figure 4A). GST-(1–604) and GST-(1–225) but not GST-(226–604) or the GST control retained the purified ScBP1. Similar results were obtained using the LCaBP-1 variant or proteins expressed without the HIS tag (data not shown). To identify the site(s) where CaBP1 interacts with the first 225 aa of the InsP3R, we performed band-shift experiments using a series of synthetic peptides spanning this region (Sienaert et al., 2002) (Figure 4Bi). A 15% nondenaturing gel illustrating the migration of ScBP1 into the gel following incubation with a 10-fold molar excess of each of the five different peptides in either 200 \(\mu\)M free Ca\(^{2+}\) or 1 mM EGTA is shown (Figure 4Bi). The InsP3R peptides alone did not enter the gel because they were positively charged. Figure 4Bi shows that CaBP1 binds to peptide B independently of Ca\(^{2+}\), resulting in the formation of a complex that does not enter the gel. In a similar assay, CaM and apoCaM also interacted with peptide B (Sienaert et al., 2002), suggesting a common binding site for CaM and CaBP1. CaM and apoCaM, however, also interacted with peptide E (Sienaert et al., 2002). Since the peptide B/CalM interaction was Ca\(^{2+}\)-dependent (Sienaert et al., 2002), the Ca\(^{2+}\)-dependence of the interaction of peptide B with ScBP1 was investigated further.

Band-shift analysis of this interaction in the presence of an increasing ScBP1:peptide molar ratio revealed that the interaction was equally effective in the presence of 200 \(\mu\)M Ca\(^{2+}\) or 1 mM EGTA, and was thus Ca\(^{2+}\)-independent (Figure 4C). Since our overexpression studies were carried out in COS-7 cells, we determined whether CaBP1 interacted with InsP3R3. By co-immunoprecipitation (co-IP), we demonstrated that both CaBP1 isoforms interact with InsP3R3 (Figure 4D). No co-IP of InsP3R3 was observed from lysates of control cells or from cells expressing YFP alone. Furthermore, the interaction between CaBP1 and InsP3Rs determined by co-IP was Ca\(^{2+}\)-independent (Figure 4E). In parallel studies, co-IP of YFP-CalM with InsP3R3 from transfected cells was more difficult to obtain (data not shown). The efficient co-IP of CaBP with InsP3R1 from the brain demonstrates the physiological relevance of the CaBP–InsP3R interaction (Figure 4F).

CaBP1 inhibits InsP3 binding to InsP3Rs

We next investigated the effects of CaBP1 on InsP3 association with the ligand-binding domain of InsP3R1 (InsP3R1 aa 1–581, Lbs-1) (Sienaert et al., 2002). The presence of 5 \(\mu\)M Ca\(^{2+}\) inhibited \(^{3}H\)-InsP3 binding to Lbs-1 by 22 ± 1.9% as previously described (\(n = 3, P < 0.05\)) (Sienaert et al., 2002). ScBP1 (10 \(\mu\)M) inhibited InsP3 binding to Lbs-1 by 36 ± 5% in the absence of Ca\(^{2+}\). Inhibition by ScBP1 was fully additive to inhibition by Ca\(^{2+}\), and amounted to 58 ± 4% in the presence of Ca\(^{2+}\) (\(n = 3, P < 0.05\)). The inhibition of InsP3 binding to Lbs-1 observed for ScBP1 was comparable to that by CaM (\(K_i\) for CaBP 1 \(\mu\)M compared with \(K_i\) of CaM 2 \(\mu\)M, data not shown). To exclude the possibility that the observed Ca\(^{2+}\)-independent ScBP1 effect was due to a constitutively occupied Ca\(^{2+}\)-binding site with high affinity in the ScBP1 protein, we used the EF hand mutant ScBP1\(_{134}\). This non-Ca\(^{2+}\)-binding mutant (10 \(\mu\)M) inhibited InsP3 association with Lbs-1 by 33 ± 4% in the absence of Ca\(^{2+}\) and 49 ± 5% in the presence of Ca\(^{2+}\). Similar results were obtained for full-length InsP3R1 (data not shown). ScBP1 had no effect on InsP3 binding to Lbs-1 \(\Delta 1–225\) (data not shown). Taken together, these results confirm that ScBP1, like CaM, inhibits InsP3 binding to the InsP3R in a Ca\(^{2+}\)-independent manner.

CaBP1 is regulated by phosphorylation

Caldendrin, the NH2-terminal extended isoform of CaBP1, is phosphorylated, although the site of phosphorylation and its...
function are not known (Seidenbecher et al., 1998). Using the PROSITE Database of protein families and domains (Sigrist et al., 2002), we identified a unique consensus site for phosphorylation by casein kinase 2 in ScBP1 (S120), which is located at the COOH-terminus of its α-helical region. The functional consequence of phosphorylation of the structurally equivalent site in CaM (S101) is a decrease in its affinity for substrates (Quadroni et al., 1998). To test whether this was the case for ScBP1, the S120 residue was mutated to an alanine (S120A). By immunoprecipitation (IP) of ScBP1-YFP from cells incubated with 32P-orthophosphate, we found that wild-type ScBP1-YFP was phosphorylated, whereas phosphorylation of ScBP1-S120A-YFP was barely detectable (Figure 5Aii). Equivalent levels of ScBP1-YFP and ScBP1-S120A-YFP in the input lysate were determined by Western blotting (Figure 5Aii) and by FACS analysis of ScBP1-YFP-expressing cells (data not shown). When stimulated with ATP, ScBP1-S120A-expressing cells displayed a significantly lower percentage of responding cells, lesser integrated Ca2⁺ signals and lower peak Ca2⁺ transient amplitude in comparison to control cells (Figure 5B–E). Furthermore, the degree of inhibition was significantly greater than that observed for cells expressing wild-type ScBP1-YFP (P < 0.05). No significant difference was observed in the amplitude of ionomycin-induced Ca2⁺ release between CaBP-S120A-expressing cells (n = 14) and controls (n = 43, P > 0.5), indicating that the ER calcium stores were intact (Figure 5F). As shown for the wild-type CaBP (Figure 3A), the ScBP1-S120A mutant also inhibited InsP₃ ester-induced Ca2⁺ release, indicating a direct effect on InsP₃Rs (Figure 5G and H).

**Discussion**

The neuronal CaBPs are a novel group of InsP₃R-regulating proteins. The results presented here demonstrate a clear functional interaction between CaBP1 and the InsP₃R Ca2⁺ release channel. Furthermore, we find that CaBP1 activity is regulated by phosphorylation. The consequences of InsP₃R inhibition by CaBP1 may have great significance for neuronal Ca2⁺ signalling, impacting on synaptic plasticity and neuronal growth.

In this study, we characterised a cellular function of CaBP1. Our findings contrast with a previous report using an in vitro assay, which suggested that CaBP1 stimulated Ca2⁺ release independent of InsP₃ (Yang et al., 2002). We found that both the long and short naturally occurring
isoforms of CaBP1 as well as the NH2-terminally truncated cCaBP inhibited Ca\(^{2+}\) release induced by application of an InsP\(_3\) generating agonist when expressed in COS-7 cells. Since CaBP1 also inhibited Ca\(^{2+}\) release induced by applica-
tion of cell-permeant InsP\(_3\) in transfected COS-7 cells, IICR from permeabilised COS cells and from intact Xenopus oocytes, we concluded that CaBP1 was directly targeting InsP\(_3\)Rs. The effects of CaBP1 were not due to Ca\(^{2+}\) buffering, since CaBP1\(_{134}\), in which the three functional EF hands had been disabled, had a similar effect as the wild-type protein. Furthermore, the effects of CaBP1 on Ca\(^{2+}\) signalling were unlike those observed for calbindin, another EF-hand-containing protein that functions solely as a Ca\(^{2+}\) buffer (John et al., 2001). In addition, CaBP1 expression did not affect caffeine-induced Ca\(^{2+}\) release through RyRs. As well as showing specificity for IICR, these data support our conclusions that CaBP1 is not causing an artefactual adaptive response in overexpressing cells. The lack of an effect on RyRs is not surprising, since the affinity of the interaction between CaBP1 and RyRs, unlike that between CaM and RyRs, is very low (S Hamilton, personal communication). Our band-shift and co-IP data, together with the data of Yang et al. (2002), which demonstrated that CaM was unable to displace CaBP1 from InsP\(_3\)Rs, suggest that the affinity of the CaBP1–InsP\(_3\)R interaction is greater than that between CaM and InsP\(_3\)Rs. We also found that when overexpressed in COS-7 cells, CaM did not inhibit IICR to the same degree as CaBP1 (MD Bootman and HL Roderick, unpublished observations).

Thus, in neurons that express InsP\(_3\)Rs and RyRs, CaBP1 may serve to inhibit IICR specifically whereas CaM may target RyRs. Indeed, by co-IP from brain tissue we readily observe an interaction between CaBP and InsP\(_3\)Rs, whereas an interaction between InsP\(_3\)Rs and CaM is more difficult to detect (Figure 4F; K Rietdorf, MD Bootman and HL Roderick, unpublished observations).

CaM has a dual role in regulating IICR. It binds in a Ca\(^{2+}\) dependent manner to the regulatory domain of InsP\(_3\)Rs, where it has been suggested to inactivate the receptor following Ca\(^{2+}\) release (Michikawa et al., 1999; Adkins et al., 2000). It also binds independently of Ca\(^{2+}\) to the NH2-terminus of InsP\(_3\)Rs, inhibiting InsP\(_3\) binding and resultant Ca\(^{2+}\) release (Patel et al., 1997; Cardy and Taylor, 1998; Sipma et al., 1999; Adkins et al., 2000; Sienaert et al., 2002). Interestingly, in this study we observe that CaBP1 also binds to the NH2-terminus of the InsP\(_3\)R resulting in inhibition of InsP\(_3\) binding. Unlike CaM however, which binds to two noncontiguous sequences in the first 159 aa of InsP\(_3\)R1 (Sienaert et al., 2002), CaBP1 bound to the first of these sequences alone.

Like other members of the neuronal Ca\(^{2+}\)-binding protein family of proteins, CaBP1 is myristoylated at its NH2-terminus (Haeseleer et al., 2000; Burgoyne and Weiss, 2001). In the case of hippocalcin and recoverin, an increase in cytosolic Ca\(^{2+}\) results in exposure of their myristoyl moieties, which results in their translocation to intracellular membranes (Burgoyne and Weiss, 2001). We find that CaBP1 is primarily localised to the plasma membrane and Golgi, but is also present in the cytosol (Figure 1B). This distribution did not change following an increase in cytosolic Ca\(^{2+}\) (data not shown). Furthermore, myristoylation is not required for CaBP1 activity since IICR is inhibited by recombinant CaBP and the NH2-terminally truncated CaBP1 (cCaBP). The similar levels of inhibition of IICR observed in cells expressing a myristoylation mutant (SCaBP-G2A-YFP) (Supplementary Figure 3), despite expression levels approximately two-fold higher than the wild-type protein (determined by FACS), suggest that myristoylation and thus membrane localisation.
increase the potency of CaBP in inhibiting IICR. Although concentrated at the plasma membrane and Golgi apparatus, CaBP clearly affects InsP3Rs. Since the Golgi apparatus is a functional InsP3-sensitive Ca\(^{2+}\) store (Pintor et al., 1998), CaBP is strategically located to inhibit release from this organelle. In addition, it is possible that in the dendrites where CaBP1 is endogenously expressed, myristoylation serves to target CaBP1 to the cellular membranes where its function in regulating IICR is performed. In neurons, regions of the ER lie in close proximity to the plasma membrane, which may thus facilitate an interaction between InsP3Rs and CaBP (Blaustein and Golovina, 2001). Furthermore, CaBPs may modulate other processes at the plasma membrane, for example Ca\(^{2+}\) influx into neurons through Ca\(_2.1\) channels (Lee et al., 2002).

The existence of a constitutive inhibitor of InsP3Rs in neurons suggests that IICR is strictly governed. As suggested for CaM, this effect may contribute to the low sensitivity of InsP3Rs reported in Purkinje neurons, which require InsP3 concentrations several orders of magnitude greater than that needed for channel opening in peripheral tissues (Khodakhah and Ogden, 1993; Cardy and Taylor, 1998; Fujiwara et al., 2001). A mechanism to allow Ca\(^{2+}\) release under conditions of repetitive stimulation such as during memory formation would, however, be advantageous to the cell. We identified a casein kinase 2 consensus site in ScaBP1 (S120) that is structurally conserved with a previously identified site in CaM (S101). Phosphorylation of this serine in CaM has been shown to decrease its affinity for substrates (Quadroni et al., 1998). In this study, we find that S120 is the predominant site for phosphorylation in CaBP1. Furthermore, the potency of CaBP1 in inhibiting IICR was increased by mutation of S120 to alanine. Thus, the phosphorylation status of CaBP1 may contribute to the dynamic regulation of InsP3R sensitivity in cells where it is expressed.

The data presented here contrast with that previously published (Yang et al., 2002). In that study, a truncated form of CaBP1 (cCaBP) spanning the conserved regions of long and short CaBP1 and caldendrin was used (Figure 1A). Since the NH\(_2\)-terminus of other neuronal Ca\(^{2+}\)-binding proteins is known to be important in their function, we speculated that this might also be the case for CaBP1. cCaBP, however, had a similar effect on Ca\(^{2+}\) signalling as the full-length wild-type protein. We also considered that a possible drawback of the heterologous expression approach used in this study is that the cell may adapt to CaBP1 overexpression, possibly by decreasing the abundance of InsPsRs. This appeared not to be the case since IICR in Xenopus oocytes and COS cell microsomes was inhibited by recombinant CaBP. Furthermore, when IICR was investigated at earlier time points following transfection, similar, yet less dramatic, effects on Ca\(^{2+}\) release were observed (data not shown). In addition, as shown in Figure 1, we were also unable to detect any change in InsP3R levels.

In summary, in this study we have characterised a novel mechanism of action of a newly described InsP3, R-associated neuronal Ca\(^{2+}\)-binding protein. CaBP1 forms part of a wide array of proteins associated with InsP3Rs, which enable them to integrate multiple signalling inputs modulating Ca\(^{2+}\) release activity and subsequent intracellular Ca\(^{2+}\) homeostasis (Roderick and Bootman, 2003). In addition, CaBP1 may prove to be an important determinant of neuronal function regulating the activity of multiple partners yet to be identified. Future studies involving gene knockout and transgenic approaches will be required to resolve these issues.

**Materials and methods**

**Generation of YFP expression vectors**

cDNAs for both long and short CaBP1 were amplified by PCR from p58-CaBP1-GFP and pLB-CaBP1-GFP, respectively (Haseelwer et al., 2000), using primers containing an NheI site at the 5' end and a HindIII site at the 3' end. The resultant PCR products were digested with NheI and HindIII, gel purified and ligated into similarly digested pEYFP-N1 (Clontech). CaBP134 (EF hand mutant) was amplified by PCR using pGEX-CaBP1-cl-mSEH as template and primers to introduce EcoRI restriction sites at both the 5' and 3' ends of the sequence. The PCR product was digested with EcoRI, gel purified and ligated into similarly digested pEYFP-N1-SCaBP1. The G2A (myristoylation site) mutant and the casein kinase 2 mutant CaBP1-S120A were generated using the Quick Change mutagenesis protocol (Stratagene). The presence of both mutations was confirmed by sequencing. To generate YFP-CaM and YFP-CaM1234, cDNAs were amplified by PCR using pAE4-hCaM and pET21B-CaM1234, respectively, as templates and primers to introduce BamHI sites at either end of the sequence. The PCR products were digested with BamHI and ligated into similarly digested pEYFP-C1 vector (Clontech). The calbindin cDNA was generously provided by Dr B Schwaller (University of Freiburg).

**Expression of CaBP-YFP fusion proteins**

COS-7 cells were plated on 22 mm coverslips in 35 mm dishes at 50–60% confluence 24 h prior to transfection. Cells were transfected using the GeneJuicer™ transfection reagent (Novagen) using 1 μg of DNA per well. Experiments were performed at 48 h post-transfection unless otherwise stated.

**Fura-2 imaging of transfected cells**

Videoimaging of Fura-2-loaded cells was performed as previously described using a Sutter (Lambda Technologies, Brattleboro) filter wheel-based imaging system (Peppiatt et al., 2003), except that the cells were loaded with 1 μM Fura-2 AM (Molecular Probes). In addition, images of YFP-positive and -negative cells were captured using excitation at 488 nm and emission > 520 nm and saved as reference.

**Imaging of Xenopus oocytes**

Oocytes were extracted from albino Xenopus laevis following euthanasia with 0.4% tricaine methane sulphonate (MS222). Oocytes were isolated and maintained as previously described (Camacho and Lechleiter, 2000). Oocytes were injected with Fluo-4 (Molecular Probes) to a final concentration of 40 μM 30 min prior to imaging. Imaging was performed using a Noran Oz confocal microscope attached to a Nikon TE200 microscope equipped with a 40 x 1.4 n.a. oil immersion S-Fluor objective. The Ca\(^{2+}\) dye was excited by laser illumination at 488 nm. Images of S12 x S12 pixels at a 0.8 zoom were collected at 15 frames per second with a jump average of 2 and a slit width of 25 μm. Ca\(^{2+}\) release was initiated by injection following transfection reagent (Novagen) using 1 μg of DNA per well. Experiments were performed at 48 h post-transfection unless otherwise stated.

**45Ca\(^{2+}\) fluxes**

45Ca\(^{2+}\) fluxes were performed on saponin-permeabilised COS-1 cells as previously described (Missiaen et al., 2001). Here, 2 μM thapsigargin was added during the efflux to block the ER Ca\(^{2+}\) pumps. The efflux medium was replaced every 2 min during 18 min, and the efflux was performed at 25°C. The additions of Ca\(^{2+}\) and InsPsRs were indicated in the legends to the figures. At the end of the experiment, the 45Ca\(^{2+}\) remaining in the stores was released by incubation with 1 ml of a 2% sodium dodecyl sulphate solution for 30 min. Ca\(^{2+}\) release is plotted as the fractional loss, that is, the
amount of Ca\textsuperscript{2+} released in 2 min divided by the total store Ca\textsuperscript{2+} content at that time. The latter value was calculated by summing in retrograde order the amount of tracer remaining in the cells at the end of the efflux and the amount of tracer collected during the successive time intervals.

**Preparation of His-tagged CaBP1**

Recombinant His-tagged long and short CaBP1 isoforms were prepared as described previously (Nadif Kasri et al., 2003).

**Expression of GST fusion proteins encoding the NH\textsubscript{2}-terminal part of InsP\textsubscript{3}R1**

cDNA fragments encoding different regions of the NH\textsubscript{2}-terminal part of InsP\textsubscript{3}R1 (aa 1–159, 1–225, 1–604 and 224–604) were subcloned by PCR amplification using the mouse InsP\textsubscript{3}R1 (p400C1 plasmid kindly provided by Dr K Mikoshiba, University of Tokyo) as a template into the pGEX6p2 vector. The PCR products were digested with BsmHI and EcoRI, cloned in the pGEX6p2 vector and expressed in BL21(DE3) host cells. Expression and purification of GST fusion proteins was carried out as described previously (Sienaat et al., 1997).

**Interaction between ScBP1 and the NH\textsubscript{2}-terminal part of InsP\textsubscript{3}R1**

For the pull-down assay, 100 µg of purified and dialysed GST fusion protein was rebound to glutathione-sepharose 4B beads for 2 h at 4°C. After washing (TBS/lysis buffer, Pierce, Belgium), 100 µg His-tagged ScBP1 was added to the immobilised GST fusion proteins in the presence of 200 µM free Ca\textsuperscript{2+}. Following an incubation of 2 h at 4°C, the protein complexes were washed extensively with binding buffer containing 200 µM of free Ca\textsuperscript{2+}, and the retained protein was eluted by 100 mM glutathione in TBS/lysis buffer. Analysis of the eluted proteins was performed on NuPAGE\textsuperscript{TM} gels, 4–12% linear gradient gels. Gels were fixed, dried and exposed to the film for 24 h.

**Band-shift assays by nondenaturing gel electrophoresis**

The band-shift assays were performed as described previously (Sienaat et al., 2002).

**InsP\textsubscript{3} binding assay**

3\textsuperscript{H}InsP\textsubscript{3}, binding to the NH\textsubscript{2}-terminal (aa 1–581) part of InsP\textsubscript{3}R1 (Lhs-1) was performed as described previously (Sipma et al., 1999). In all, 4 µg of purified Lhs-1 protein was used.

**Western blotting**

For CaBP expression analysis, cell lysates were prepared as described previously (Roderick et al., 2000). Extracts were prepared from either subconfluent cells isolated by trypsinisation or from YFP-positive cells isolated by FACS. A measure of 10–20 µg of the cell extract was analysed using the NuPAGE\textsuperscript{TM} gel system on 4–12% linear gradient gels, transferred to nitrocellulose and probed for CaBP-YFP using either polyclonal anti-GFP antibody (dilution 1:2000; Roderick et al., 1998) or a polyclonal anti-CaBP1 antibody (dilution 1:5000, UW72; Haeseleer et al., 2000). Enhanced chemiluminescence (ECL, Pierce) was used to detect immunoreactive bands after incubation of secondary antibodies conjugated to horseradish peroxidase (HRP) (Jackson ImmunoResearch, dilution 1:10 000). For InsP\textsubscript{3}R analysis, COS-7 cell lysates were boiled for 2 min in Laemmli sample buffer, analysed on NuPAGE\textsuperscript{TM} Tris-acetate 3–8% gradient gels, transferred to Immobilon-P and probed with an isoform-specific antibody against InsP\textsubscript{3}R3 (31L220, Transduction Laboratories, dilution 1:5000). Quantification of the immunoreactive bands was performed after incubation with secondary antibodies coupled to alkaline phosphatase, detection using Vistra\textsuperscript{TM} ECF (Amersham Pharmacia Biotech) (Vanliening et al., 1997).

**3\textsuperscript{P} labelling of CaBP-YFP**

At 24 h postseeding in 60 mm Petri dishes, COS-7 cells were transfected with the appropriate CaBP1-YFP vectors. At 40 h post-transfection, cells were washed × 1 in phosphate-free MEM (ICN, cat. #1642349) containing 10% dialysed calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (labelling media). A measure of 1.5 ml of labelling media containing 0.375 µCi/ml 3\textsuperscript{P}-phosphorous (orthophosphate in acid-free aqueous solution, Amersham, cat. #P813) was added to the cells and incubated for 6 h at 37°C. Following incubation in ice-cold TBS, cells were washed 2 min in Laemmli sample buffer, analysed on NuPAGE\textsuperscript{TM} 4–12% linear gradient gels. Gels were fixed, dried and exposed to the film for 24 h.

**Co-IP of InsP\textsubscript{3}Rs and CaBP**

Lysates were prepared as described for Western blotting from 10 cm dishes of COS-7 cells expressing appropriate CaBP1-YFP constructs. Following clarification, 30 µl of each lysate was retained for Western blot analysis and the remainder diluted four-fold in lysis buffer without detergent. IP using 5 µl of anti-GFP antibody was performed and immune complexes were captured by incubation with 30 µl protein A/G sepharose. Immunoprecipitates were boiled in 30 µl sample buffer and analysed by SDS–PAGE and Western blot using a monoclonal antibody directed against the InsP\textsubscript{3}R3. Immunoreactive bands were visualised by ECL following incubation with an HRP-conjugated secondary antibody. For IP from rat brain, whole rat brain was disrupted with a Dounce homogeniser in cell lysis buffer described above. Following 1 h incubation on ice, the insoluble matter was isolated by centrifugation at 5000 g for 10 min. A measure of 2 mg of cell lysate was used for each IP. A polyclonal antibody against InsP\textsubscript{3}R1 was used to immunoprecipitate InsP\textsubscript{3}Rs (Farys et al., 1995).

**Data analysis**

For the percentage of responding cells, an average of the responding cells per coverslip was taken. A response was characterised as a deflection greater than 25 nM from baseline. The integrated Ca\textsuperscript{2+} response is the area under the Ca\textsuperscript{2+} transient minus baseline. Statistical analysis was by Student’s t-test or χ\textsuperscript{2} test. Data are presented as mean±s.e.m. Significance was accepted at P<0.05 and is indicated by an asterisk.

**Supplementary data**

Supplementary data are available at The EMBO Journal Online.

**Note added in proof**

During review of this manuscript, a paper appeared in the press that described an inhibitory effect of CaBP on IICR in PC12 and HeLa cells (Haynes et al., 2003). Their findings complement our data presented showing an inhibition of Ca\textsuperscript{2+} release using direct stimulation of InsP\textsubscript{3}Rs.

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