

Identification and characterization of two distinct calmodulin-binding sites in the Trpl ion-channel protein of *Drosophila melanogaster*

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Two putative light-sensitive ion channels have been isolated from *Drosophila*, encoded by the transient-receptor-potential (*trp*) and transient-receptor-potential-like (*trpl*) genes. The cDNA encoding the Trpl protein was initially isolated on the basis that the expressed protein binds calmodulin. Using both fusion proteins and a synthetic peptide, we now show that two calmodulin-binding sites are present in the C-terminal domain of the Trpl protein, CBS-1 and CBS-2. CBS-1 binds calmodulin in a Ca²⁺-dependent fashion, requiring Ca²⁺ concentrations above 0.3–0.5 μ M for calmodulin binding. In contrast, CBS-2 binds the

Ca²⁺-free form of calmodulin, with dissociation occurring at Ca²⁺ concentrations between 5 and 25 μ M. Phosphorylation of a serine residue within a peptide encompassing CBS-1 by cyclic AMP-dependent protein kinase (PKA) abolishes calmodulin binding, and phosphorylation of the adjacent serine by protein kinase C appears to modulate this phosphorylation by PKA. Interpretation of these findings provides a novel model for ion-channel gating and modulation in response to changing levels of intracellular Ca²⁺.

INTRODUCTION

The *trpl* gene of *Drosophila* was isolated in a screen for calmodulin-binding proteins [1]. Analysis of the protein encoded by the *trpl* gene revealed similarity to the *Drosophila* phototransduction gene *trp* and to ion-channel proteins. The Trp and Trpl proteins show similarity to vertebrate voltage-gated Na⁺ and Ca²⁺ channels. Both proteins have six transmembrane regions; however, the S4 transmembrane regions in the Trp and Trpl proteins lack the positively charged residues presumed responsible for voltage gating [2], thus the Trp and Trpl channels are likely to be second-messenger-gated rather than voltage-gated.

The similarity of the Trp and Trpl proteins to voltage-gated ion channels, together with their retina-specific expression, led to the proposal that they encode light-sensitive ion channels involved in phototransduction [1,3]. The phototransduction pathway in *Drosophila* (for reviews see refs. [4] and [5]) is initiated by light-activated rhodopsin, which activates phospholipase C (PLC) via a G-protein. PLC catalyses the conversion of a membrane phospholipid into the second messengers inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol. It is known that PLC is required for phototransduction, as mutations in the gene encoding PLC, *norpA*, completely abolish the light response [6,7]. IP₃ is thought to bind to receptors in the endoplasmic-reticulum Ca²⁺ stores, leading to Ca²⁺ release and a concomitant influx of Ca²⁺ and Na⁺ from the extracellular space through cation channels. This results in a biphasic increase in intracellular Ca²⁺ similar to that seen in many types of mammalian non-excitable cells [8], suggesting that similar proteins may be involved. In both *Drosophila* photoreceptors and mammalian non-excitable cells, the mechanisms of IP₃ production are well defined; however, the channels and the mechanism of cation influx have not been identified. It has been suggested that the *trp* and *trpl* genes encode such channels.

This hypothesis is supported by recent studies in which both the *trp* and *trpl* genes have been expressed in insect Sf9 cells

[9–12] and shown to form functional ion channels with features specific to each type of channel. The expressed Trpl channels are: non-selective for Na⁺, Ca²⁺ and Ba³⁺; insensitive to Gd³⁺; activated by a receptor-dependent mechanism; not activated by depletion of the internal Ca²⁺ stores by thapsigargin; and constitutively active under basal non-stimulated conditions. The *trpl* gene has also been expressed in *Xenopus* oocytes (G. Barritt, personal communication), and is constitutively active in this cell type. In contrast, Trp channels are: more selective for Ca²⁺ than Na⁺; blocked by low concentrations of Gd³⁺; and activated by thapsigargin. The fact that Trp channels are activated by thapsigargin makes the Trp protein the better candidate for being a homologue of the mammalian channels involved in Ca²⁺-activated Ca²⁺ entry.

Several lines of evidence exist for the presence of two light-sensitive ion channels in *Drosophila*. Mutations in the *trp* gene result in a transient, rather than sustained, depolarization of photoreceptor cells in response to high-intensity light. Low-level light, however, produces a nearly normal response [6,13,14]. The residual response seen in *trp* mutant photoreceptors could be explained by the presence of another channel. Also, whole-cell voltage-clamp experiments on wild-type photoreceptors found there was not a unique reversal potential, but rather biphasic currents were observed, indicating the presence of more than one channel [15]. In contrast, when *trp* mutant photoreceptors were examined, they were found to have a unique reversal potential with a greatly reduced dependence on extracellular Ca²⁺. The absence of a Ca²⁺-selective channel in *trp* mutants is supported by the observation that the Ca²⁺ channel blocker La³⁺ mimics the *trp* mutant phenotype [3,16], and is consistent with the expression studies.

Given that the *trpl* gene possibly encodes a light-sensitive ion channel, we are interested in how this channel might be gated and modulated. As the *trpl* gene was isolated on the basis of it encoding a calmodulin-binding protein, an obvious candidate for gating the Trpl channel is calmodulin. An indication that calmodulin is involved in phototransduction is provided by the

ninaC mutation. The *ninaC* gene encodes an unconventional myosin/kinase expressed only in photoreceptor cells and thought to be involved in the subcellular localization of calmodulin. In *ninaC* mutants the amount of calmodulin in the photoreceptors is reduced twofold, and is primarily located extracellularly [17]. Null mutations at this locus result in an abnormal electroretinogram, which shows an increased initial response, a reduced off-transient, and slow termination of the response [17]. The prolonged depolarizing afterpotential is also reduced in these flies [6], and it appears that the adaptive response is severely reduced. These phenotypes clearly indicate an effect of the *ninaC* mutation on phototransduction and indicate that calmodulin may be involved both in termination of the light response and in the adaptive response.

Calmodulin is a small acidic Ca^{2+} -binding protein that is expressed ubiquitously in the nervous system. It can bind up to four Ca^{2+} ions, and undergoes sequential conformational changes on binding of each ion [18,19]. Unlike many Ca^{2+} -binding proteins, calmodulin regulates an array of target proteins and as such is involved in regulation of many cellular processes [20]. Calmodulin has been reported to modulate a number of ion channels, for example the vertebrate olfactory and rod cyclic GMP-gated cation channels' affinity for cyclic GMP is reduced by calmodulin [21,22], whereas the *Paramecium* K^+ and Na^+ channels are activated by calmodulin [23].

The investigation of calmodulin-regulated proteins has been difficult owing to the lack of a specific primary amino acid sequence motif in the binding site for calmodulin. For a number of sites, it appears that calmodulin recognizes and binds to an amphiphilic α -helix in the target protein [24]. Such helices have one face positively charged and the other face highly hydrophobic. Although not all calmodulin-binding sites form such helices, searching for such motifs is a useful initial approach to identifying calmodulin-binding sites. This paper describes the identification of two calmodulin-binding sites in the C-terminal domain of the Trp1 protein, CBS-1 and CBS-2, and the investigation of the effect of the Ca^{2+} concentration on binding of calmodulin to these sites. The effect of phosphorylation of CBS-1 on calmodulin binding is also described, as are the implications of this study for possible gating and modulation of the Trp1 cation channel.

EXPERIMENTAL

Fusion protein production and purification

Fusion proteins composed of maltose-binding protein and parts of the Trp1 protein were constructed using the pMal-c expression vector system (New England Biolabs). Small-scale fusion protein induction was performed according to the manufacturer's directions. For isolation on affinity columns, cells from large-scale inductions were harvested by centrifugation at 4000 g for 10 min, then resuspended in a lysis buffer consisting of 10 mM sodium phosphate, pH 7.2, 30 mM NaCl, 0.25% Tween 20, 10 mM 2-mercaptoethanol, 10 mM EDTA, pH 8.0, 10 mM EGTA, pH 7.0 and 10 mM PMSF. The cells were lysed on ice by sonication for a total of 2 min using the MSE 150 W Ultrasonic Disintegrator Mk2, then centrifuged at 9000 g for 10 min. The fusion protein was then purified from the supernatant with amylose resin, and eluted from the resin with 10 mM maltose.

Calmodulin overlays

SDS/PAGE was performed with 10% acrylamide gels using a Bio-Rad minigel apparatus. Electroblothing was performed on the LKB Multiphor II Novablot apparatus, the buffer consisting

of 48 mM Tris/HCl, pH 8.5, 39 mM glycine, 0.037% SDS and 20% methanol. Nitrocellulose filters were blocked for at least 1 h with 1% BSA in 50 mM Tris/HCl, pH 7.5, containing 150 mM NaCl, 0.5 mM CaCl_2 and 50 mM MgCl_2 (TBS/CaMg). Filters were then incubated for 2 h with 100 ng/ml biotinylated calmodulin (Gibco/BRL) in the same buffer. This was followed by two 15 min washes in TBS/CaMg then incubation with either streptavidin-alkaline phosphatase (Gibco/BRL) or streptavidin-horseradish peroxidase (Amersham) at a dilution of 1:10000 in the same buffer. Filters were then washed (3 × 15 min) in TBS/CaMg and secondary antibody detected. For alkaline phosphatase, colour was developed by incubation for 15 min to 1 h (light-protected) with 0.33 mg/ml Nitro Blue Tetrazolium and 0.175 mg/ml 5-bromo-4-chloro-indolyl phosphate. Horseradish peroxidase activity was detected using Amersham's enhanced chemiluminescence (ECL) system according to the manufacturer's directions.

Binding of peptide/fusion protein to calmodulin-Sephacrose

CBS-1 peptide (VKWVIRIFRKSSKTI-biotinylated; Chiron Mimitopes; 5 μg) or B fusion protein (1 μg) (see Figure 1) was bound to 100 μg of calmodulin-Sephacrose (Pharmacia) for 1 h at 4 °C on a rotatory platform in TBS (50 mM Tris/HCl, pH 7.5, 150 mM NaCl) at the Ca^{2+} concentration described. Unbound peptide/protein was then removed by washing the calmodulin-Sephacrose (8–10 × 10 min) in 1 ml of the same buffer, washing being removed by centrifugation at 1500 g (3000 rev./min) for 30 s. Washes in TBS at each Ca^{2+} concentration shown in the Figures were then performed (2 × 10 min) in 1 ml of buffer. Washings were then concentrated on to nitrocellulose using a slot-blot apparatus (Schleicher and Schuell), and the presence of peptide or fusion protein was detected as follows. For unphosphorylated peptide, the peptide was linked to streptavidin-horseradish peroxidase, the presence of which was detected using the ECL system. For phosphorylated peptide, [γ - ^{33}P]ATP was detected by autoradiography. For fusion protein B, detection was carried out by probing with anti-Trp1 serum at 1:500 dilution overnight at 4 °C in TBS/5% skimmed milk powder. The filter was then washed (2 × 10 min) in TBS/0.05% Nonidet P40 and incubated with anti-rabbit IgG-horseradish peroxidase (Amersham) at 1:10000 dilution in TBS/5% skimmed milk powder. The filter was washed (3 × 10 min) in TBS/0.5% Nonidet P40 and detected with the ECL system. The density of the bands on all blots was then determined using the Molecular Dynamics Image Quant Fast Scan system.

Peptide phosphorylation

Phosphorylation reactions were carried out for 2 h at 30 °C in a 100 μl reaction volume. The protein kinase C (PKC) reaction mixture consisted of 20 mM Tris/HCl, pH 7.5, 10 mM MgCl_2 , 0.5 mM CaCl_2 , 100 $\mu\text{g/ml}$ phosphatidylserine, 20 $\mu\text{g/ml}$ 1,3-dioclein, 10 μg of CBS-1 peptide, 0.05 m-unit of PKC (Boehringer-Mannheim) and 100 μM [γ - ^{33}P]ATP (74 TBq/mmol; Bresatec). The cyclic AMP-dependent protein kinase (PKA) reaction mixture consisted of 20 mM Tris/HCl, pH 6.5, 4 mM MgCl_2 , 4 μM NaF, 1 μM EGTA, 10 μg of CBS-1 peptide, 0.05 unit of PKA (catalytic subunit; Sigma) and 100 μM [γ - ^{33}P]ATP (74 TBq/mmol; Bresatec). Phosphorylation was quantified using 0.1 vol. of the reaction mixture. Peptide was separated from unincorporated [γ - ^{33}P]ATP using size-exclusion chromatography. The amount of [γ - ^{33}P]ATP present in the peptide fractions was calculated and the degree of phosphorylation calculated as mol of $^{33}\text{PO}_4$ /mol of peptide. The double-phosphorylation experiment used PKC to phosphorylate 10 μg

of peptide in the PKC reaction mixture with unlabelled ATP; the enzyme was then inactivated by heating at 65 °C for 10 min. PKA, [³²P]ATP and 1 mM EGTA were then added and PKA phosphorylation was carried out.

RESULTS

Identification of the calmodulin-binding sites

As the result of searching the amino acid sequence for amphiphilic α -helices, the *Trp1* protein was originally postulated to contain two calmodulin-binding sites in the C-terminal domain [1]. Given that the *Trp1* protein has six transmembrane domains, the C-terminal domain should be on the intracellular face of the membrane. As residues 710–728 could form an ideal amphiphilic α -helix, this region was proposed to be the first calmodulin-binding site (CBS-1). A second putative site, CBS-2 (residues 809–825), was proposed to be present on the basis that one of the group of *trpl* cDNAs originally isolated, called 3.9, begins C-terminal to CBS-1 (Figure 1), and the fusion protein it produces has the ability to bind calmodulin on Western blots.

The initial method used in this study to identify the calmodulin-binding sites was the production of fusion proteins containing the putative sites using the pMal expression vector. These fusion proteins were purified on an amylose-affinity resin, electrophoresed, transferred to nitrocellulose and overlaid with biotinylated calmodulin. Fusion proteins containing each putative site separately were produced. The protein containing the putative CBS-1 contains residues 709–787 [Figure 1(i) insert A] and the protein containing the putative CBS-2 contains residues 787–1124 [Figure 1(i) insert B]. Both fusion proteins A and B bound calmodulin (Figure 2), but their modes of binding differed in that fusion protein B retained the ability to bind calmodulin in the absence of Ca²⁺ (5 mM EGTA) (Figure 2B), whereas fusion protein A required Ca²⁺ to bind calmodulin (Figure 2A).

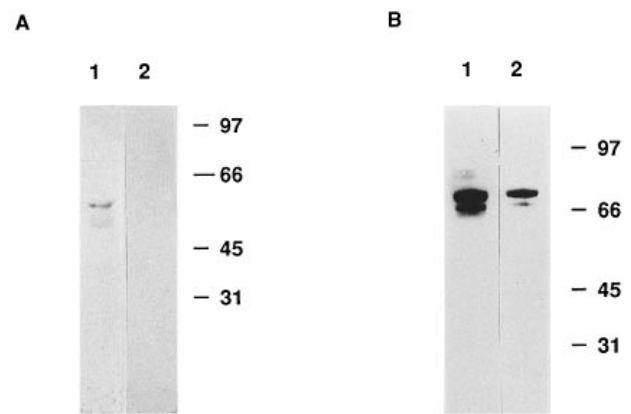


Figure 2 Binding of biotinylated calmodulin to fusion proteins A (A) and B (B)

Calmodulin overlays of 5 μ g of (A) fusion protein A and (B) fusion protein B are shown. The filters had been incubated with biotinylated calmodulin. Detection in (A) was with streptavidin–alkaline phosphatase and that in (B) was with streptavidin–horseradish peroxidase. Lane 1, 0.5 mM Ca²⁺; lane 2, 5 mM EGTA. Molecular masses (kDa) are shown on the right.

Some difficulty was encountered in the calmodulin overlays for fusion protein A, and accordingly a synthetic peptide (VKWVIRIFRKSSKTI) representing this site was synthesized (see the Experimental section). It has previously been shown that binding of basic peptides to calmodulin will retard its progress on native acrylamide gels [25]. The CBS-1 peptide was therefore incubated with Ca²⁺/calmodulin and run on a non-denaturing polyacrylamide gel (Figure 3). In the absence of the peptide a single band was observed representing pure Ca²⁺/calmodulin. At

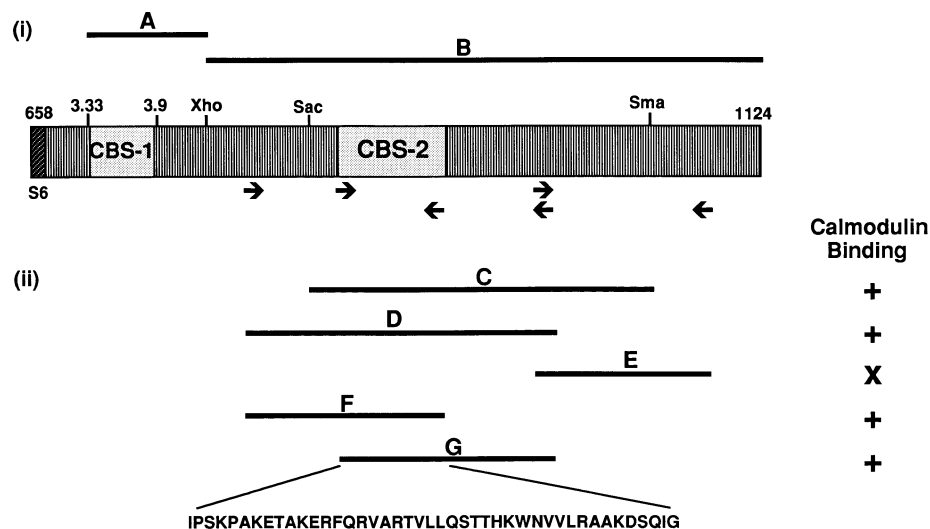


Figure 1 Localization of calmodulin-binding sites

A representation of the C-terminal region of the *Trp1* protein, amino acids 658–1124, is shown. Symbols are as follows: S6, sixth transmembrane region; 3.33 and 3.9, starts of cDNAs; Xho, Sac, Sma, positions of restriction sites. The arrows indicate PCR primers, and the locations of CBS-1 and CBS-2 are indicated. All inserts were subcloned into the pMal expression vector and fusion proteins produced (see the Experimental section). (i) Inserts A and B are restriction fragments, used to produce fusion proteins A and B. (ii) Insert C is a restriction fragment, and inserts D–G are PCR products. Fusion protein C binds calmodulin independently of Ca²⁺, excluding the original putative CBS-2 (residues 809–825). The location of CBS-2 within the insert C region was narrowed down using PCR-generated inserts. Fusion protein D binds calmodulin, whereas fusion protein E does not. Fusion proteins F and G both bind calmodulin, therefore CBS-2 is located in their overlapping region, residues 853–895. All fusion proteins that bound calmodulin (C, D, F and G) did so independently of Ca²⁺.

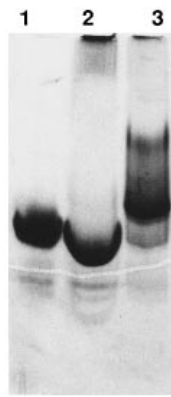


Figure 3 Retardation by CBS-1 peptide of calmodulin on a native gel

A 15% native polyacrylamide gel was used. Lane 1, calmodulin alone; lane 2, calmodulin incubated with CBS-1 peptide in the presence of 1 mM EGTA; lane 3, calmodulin incubated with CBS-1 peptide in the presence of 50 μM Ca^{2+} . Incubations were carried out using 1 μg of CBS-1 peptide and 8 μg of calmodulin (1:1 molar ratio) at 4 $^{\circ}\text{C}$ for 1 h.

a ratio of peptide to calmodulin of 1:1, and in the presence of Ca^{2+} , a single band was observed with a lower mobility, representing the peptide- Ca^{2+} /calmodulin complex. When this experiment was carried out in the presence of EGTA, retardation did not occur, demonstrating that binding of the peptide to calmodulin is Ca^{2+} -dependent. The retardation has been observed over a range of Ca^{2+} concentrations above 1 μM (results not shown). This experiment thus demonstrates that the putative CBS-1, residues 710–728, is a calmodulin-binding site, and that binding of calmodulin to this site is Ca^{2+} -dependent.

The putative CBS-2 showed less resemblance to a calmodulin-binding site than CBS-1 [1]. The binding of fusion protein B to calmodulin proved that there was a second site encoded by the cDNA sequence 3' to the *Xho*I restriction site corresponding to residue 787 (Figure 1), but did not indicate its precise location. In order to locate this site, further fusion proteins were produced, and calmodulin binding was tested [Figure 1(ii)]. These experiments showed that the suggested CBS-2 was not in fact a calmodulin-binding site. A further search of the region did not identify any sequences that seemed likely to form an amphiphilic α -helix, thus a systematic search of the region for calmodulin-binding activity was undertaken [Figure 1(ii)]. The site was eventually localized to a 43-amino acid sequence from residue 853 to residue 895. This amino acid sequence, when represented on a helical wheel, shows little resemblance to an amphiphilic α -helix. This may reflect an unusual property of this site, as all fusion proteins containing only this site bind calmodulin independently of Ca^{2+} .

Effect of Ca^{2+} concentration on calmodulin binding

Additional experiments were carried out to determine the Ca^{2+} dissociation constants of calmodulin binding for both sites (see the Experimental section). The results of these experiments are shown in Figures 4, 5 and 6.

As the binding of calmodulin to CBS-1 was Ca^{2+} -dependent, the peptide was bound to calmodulin–Sepharose in 100 μM Ca^{2+} , and then washed in decreasing Ca^{2+} . The peptide bound to calmodulin–Sepharose in 100 μM Ca^{2+} , and dissociated from the Sepharose at 0.3–0.5 μM Ca^{2+} (Figure 4). The normal free Ca^{2+} concentration in a resting cell is of the order of 0.1 μM [26]. This

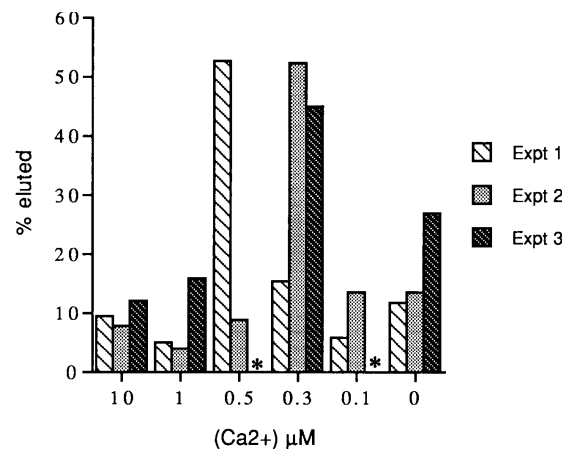


Figure 4 Effect of Ca^{2+} concentration on calmodulin binding to CBS-1

CBS-1 peptide (5 μg) linked to streptavidin–horseradish peroxidase was bound to calmodulin–Sepharose in 100 μM Ca^{2+} , then washed in 10, 1, 0.5, 0.1 μM Ca^{2+} and 5 mM EGTA. Results are expressed as the percentage of bound peptide eluted at each concentration. Concentrations not tested are indicated by an asterisk.

result therefore suggests that CBS-1 binds calmodulin only at elevated intracellular Ca^{2+} levels.

As the calmodulin overlays had shown binding of calmodulin to CBS-2 to be Ca^{2+} -independent (Figure 2B), the B fusion protein was bound to calmodulin–Sepharose in the presence of EGTA, and then washed in increasing Ca^{2+} concentrations to find at what concentration, if any, binding was abolished. As expected, the B fusion protein bound to calmodulin–Sepharose in the presence of EGTA, and was found to dissociate at approximately 5–10 μM Ca^{2+} (Figure 5A). However, the calmodulin overlays had also shown that the B fusion protein bound calmodulin at high Ca^{2+} levels (Figure 2B), therefore the B fusion protein was also bound to calmodulin–Sepharose in 100 μM Ca^{2+} , and washed in decreasing concentrations of Ca^{2+} . The B fusion protein bound to the Sepharose as expected, and was found to dissociate at 25–50 μM Ca^{2+} (Figure 5B). These two experiments therefore demonstrated that CBS-2 binds calmodulin when Ca^{2+} is absent, and at all Ca^{2+} concentrations except in the range 5–25 μM .

Effect of phosphorylation of CBS-1 on calmodulin binding

CBS-1 contains two serine residues, 721 and 722, which are present in consensus sequences for phosphorylation. This is a common feature of calmodulin-binding sites [27]. The protein kinase thought most likely to be involved in this phosphorylation *in vivo* is PKC, as it is activated by diacylglycerol and Ca^{2+} , products of the phototransduction pathway. The consensus sequence for phosphorylation by PKC is quite loose, namely (S/T)X(R/K) [28]. However, Ser-721 is present in such a sequence and is thus a candidate for PKC phosphorylation. The second serine, residue 722, is present in a consensus sequence for phosphorylation by PKA, namely (R/K)(R/K)XS [29]. We therefore investigated, first, whether these two protein kinases were able to phosphorylate the CBS-1 peptide, and, secondly, the effect of this phosphorylation on calmodulin binding.

The peptide acted as a substrate for both PKC and PKA, and

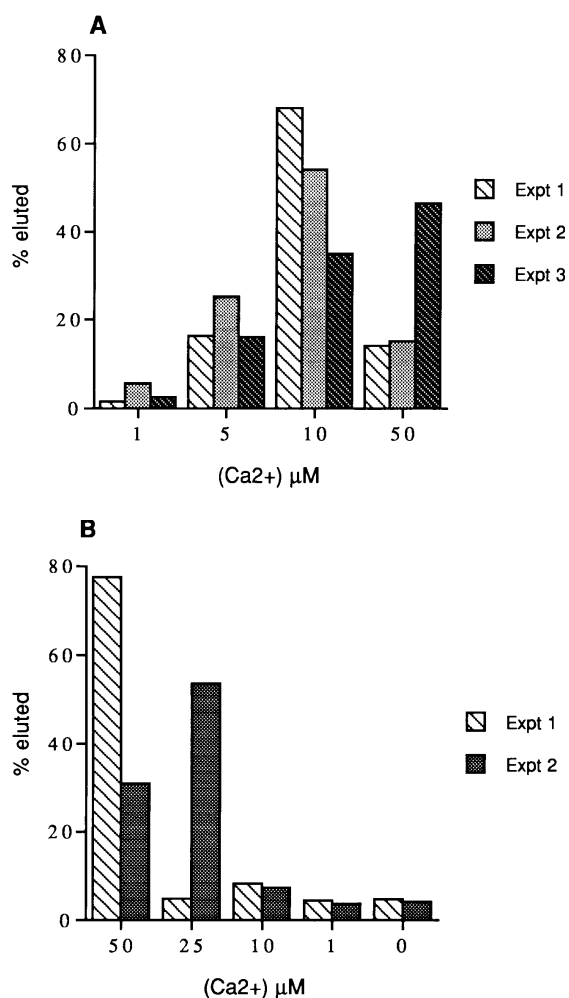


Figure 5 Effect of Ca^{2+} concentration on calmodulin binding to CBS-2

(A) Fusion protein B (1 μg) was bound to calmodulin–Sepharose in 5 mM EGTA, then washed in 1, 5, 10 and 50 μM Ca^{2+} . (B) Fusion protein B (1 μg) was bound to calmodulin–Sepharose in 100 μM Ca^{2+} , then washed in 50, 25, 10, 1 μM Ca^{2+} and 5 mM EGTA. Results are expressed as the percentage of bound protein eluted at each concentration.

in each case phosphorylation occurred to a maximum of 0.8 mol of phosphate/mol of peptide. When the PKC-phosphorylated peptide was used in calmodulin-binding studies, it retained its ability to bind to calmodulin–Sepharose, and was eluted at the same Ca^{2+} concentration as the unphosphorylated peptide (Figure 6A). In contrast, phosphorylation of the peptide by PKA abolished its affinity for calmodulin–Sepharose (Figure 6B). This suggests that phosphorylation of one of the two serine residues prevents calmodulin binding.

Given the close proximity of the two serine residues within CBS-1, it is possible that phosphorylation of one serine residue interferes with subsequent phosphorylation of the second. The observation that phosphorylation by PKC has no effect on the binding of calmodulin to the CBS-1 peptide led us to investigate whether PKC prephosphorylation had any effect on subsequent PKA phosphorylation (see the Experimental section). The pre-phosphorylation by PKC was found to cause a 70% reduction in the level of PKA phosphorylation of the peptide as compared with control values (results not shown). This suggests that PKC

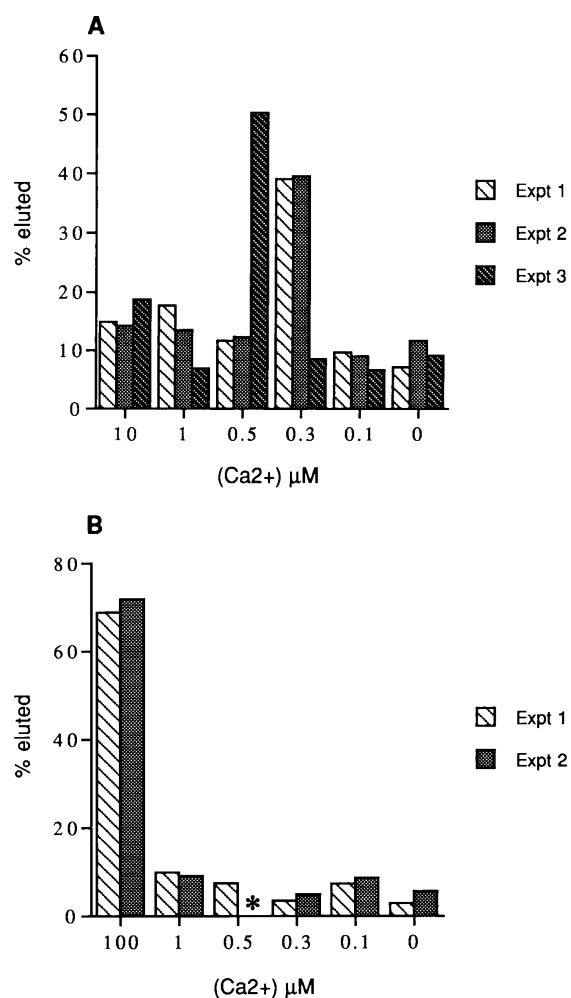


Figure 6 Effect of phosphorylation of CBS-1 on calmodulin binding

(A) CBS-1 peptide (5 μg) phosphorylated by PKC was bound to calmodulin–Sepharose in 100 μM Ca^{2+} , then washed in 10, 1, 0.5, 0.3, 0.1 μM Ca^{2+} and 5 mM EGTA. Phosphorylation in each experiment occurred to a maximum of 0.8 mol of phosphate/mol of peptide. Results are expressed as the percentage of bound peptide eluted at each concentration. (B) CBS-1 peptide (5 μg) phosphorylated by PKA was bound to calmodulin–Sepharose in 100 μM Ca^{2+} , then washed in 1, 0.5, 0.3, 0.1 μM Ca^{2+} and 5 mM EGTA. The 100 μM column represents the unbound fraction in which most of the peptide remained. Phosphorylation in each experiment occurred to a maximum of 0.8 mol of phosphate/mol of peptide. Results are expressed as the percentage of total peptide eluted at each concentration. Concentrations not tested are indicated by an asterisk.

phosphorylation of one of the serine residues effectively inhibits PKA phosphorylation of the other.

DISCUSSION

This study has identified two calmodulin-binding sites in the C-terminal domain of the Trp1 protein, termed CBS-1 and CBS-2. Other calmodulin-binding proteins have been shown to contain two or multiple calmodulin-binding sites, for example muscle phosphofructokinase, phosphorylase kinase and unconventional myosins [29–31]. However, the Trp1 protein appears to be unusual in that it has two calmodulin-binding sites which bind calmodulin in distinctly different ways. In phosphofructokinase and phosphorylase kinase, calmodulin binding at both sites occurs in a Ca^{2+} -dependent manner, whereas in most myosins

calmodulin binding at all sites occurs in a Ca^{2+} -independent manner. However, one particular myosin, myr4, a myosin I from rat, contains two sites which bind calmodulin independently of Ca^{2+} , and one site to which binding is Ca^{2+} -dependent [31].

Binding of calmodulin to CBS-1 of the Trp1 protein is Ca^{2+} -dependent, occurring at Ca^{2+} concentrations higher than 0.3–0.5 μM where calmodulin should be fully saturated with Ca^{2+} . In contrast, the Ca^{2+} -free form of calmodulin can bind to CBS-2, but binding does not occur between 5 and 25 μM Ca^{2+} , and possibly reoccurs at Ca^{2+} concentrations higher than 25 μM . Within the 43-amino acid sequence of CBS-2 the precise amino acid sequences to which calmodulin binds in the presence and absence of calmodulin have not been determined.

Binding of calmodulin to proteins independently of Ca^{2+} has been observed in the γ -subunit of phosphorylase kinase [30], in adenylate cyclase [32] and in unconventional myosins in which binding occurs via an IQ motif [31]. CBS-2 of the Trp1 protein does not contain such an IQ motif, nor do the calmodulin-binding sites of phosphorylase kinase and adenylate cyclase. Interestingly these calmodulin-binding sites also show little resemblance to amphiphilic α -helices. CBS-2 is therefore a member of a distinct class of calmodulin-binding sites to which calmodulin binding occurs via a mechanism different from that previously described [24,33], and in which the Ca^{2+} -induced allosteric transition of calmodulin is not required for binding. A different elongated structural form of calmodulin associated with binding to phosphorylase kinase and cyclic AMP phosphodiesterase has been observed in X-ray crystallographic and small-angle X-ray scattering experiments in addition to the compact structure observed in calmodulin bound to proteins such as myosin light chain kinase [34,35].

Dissociation of calmodulin from CBS-2 is at Ca^{2+} levels well in excess of those needed to saturate calmodulin, suggesting that Ca^{2+} must act at some level on the Trp1 protein other than on calmodulin alone. This is also indicated by the reassociation of calmodulin with CBS-2 at Ca^{2+} levels above 25 μM . Although the physiological significance of the increased affinity of CBS-2 for calmodulin at such high Ca^{2+} concentrations is debatable, microdomains of Ca^{2+} at a concentration higher than 100 μM have been observed in some cells, for example the presynaptic terminal of the squid giant synapse [36].

The binding of calmodulin to CBS-2 in the absence of Ca^{2+} may explain the constitutive activity observed when Trp1 channels were expressed in Sf9 cells and *Xenopus* oocytes ([9]; G. Barritt, personal communication). The constitutive activity in Sf9 cells was not observed until 3 days after infection [9], and several possible reasons for this were suggested, one being that there was a titration of some endogenous factor in the Sf9 cell which is necessary to inhibit activity of the newly formed channels. Given that calmodulin binds to CBS-2 of the Trp1 protein in the absence of Ca^{2+} , calmodulin is a possible candidate for this factor. A recent experiment in which calmodulin was microinjected into oocytes expressing Trp1 channels considerably reduced the constitutive activity (G. Barritt, personal communication). Thus, in a resting cell, calmodulin may bind to CBS-2 and maintain the Trp1 channel in a closed state. This may mean that calmodulin forms an integral subunit of the Trp1 channel, as seen in phosphorylase kinase [30].

If binding of calmodulin at CBS-2 inhibits channel activity, the question then arises as to the function of calmodulin binding to CBS-1 in gating of the channel. The above discussion implies that, in order to open the channel, CBS-2 must be vacated, but it requires a Ca^{2+} concentration above 5 μM to dissociate calmodulin from this site. The finding that this concentration is much higher than that needed to saturate calmodulin fully might

be explained by the properties of CBS-1. Calmodulin will bind to CBS-1 at Ca^{2+} concentrations above 0.5 μM . It is therefore possible that the close proximity of CBS-1 to CBS-2 causes the Ca^{2+} -saturated form of calmodulin to vacate CBS-2 in favour of CBS-1. This could be termed a 'calmodulin shuttle'. This would cause the channel to open at Ca^{2+} concentrations higher than 0.5 μM , corresponding to elevated intracellular Ca^{2+} levels, where calmodulin would be fully saturated with Ca^{2+} . Vacation of CBS-2 by calmodulin in favour of CBS-1 may thus be the trigger for the channel to open. This would mean that the Trp1 channel is open at the range of Ca^{2+} concentrations between 0.5 and 5 μM . Such Ca^{2+} levels may be achieved by release of Ca^{2+} from intracellular stores or by influx through other Ca^{2+} channels.

If calmodulin is shifted from CBS-2 to CBS-1 of the Trp1 protein in order to activate the channel, any other mechanism that dissociates calmodulin from CBS-2 would also activate the channel. This would allow for the possible involvement of other second messengers in channel activation. For example, cyclic GMP has been implicated as a second messenger in invertebrate phototransduction, as cyclic GMP-gated channels have been found in highly localized regions in *Limulus* photoreceptors [37]. However, any other second messengers that are involved must act downstream of PLC, as *norPA* mutants completely lack light-activated currents [7].

Of potential interest is the finding that phosphorylation of CBS-1 by PKA prevents binding of calmodulin to this site. This is not without precedent: when a serine residue in the calmodulin-binding site of myosin light chain kinase is phosphorylated by PKA, the affinity of the site for calmodulin is reduced [38], and when neuromodulin is phosphorylated at Ser-41 in its calmodulin-binding site by PKC, calmodulin-binding is abolished [39]. The protein kinase that phosphorylates CBS-1 *in vivo* may not be PKA, but another kinase with similar substrate specificity. An eye-specific PKC of unknown substrate specificity is present in *Drosophila* [40] and there are likely to be other novel protein kinases in photoreceptor cells.

The effect of phosphorylation of CBS-1 by PKA suggests a mechanism for modulation of channel activity. Phosphorylation at this serine caused CBS-1 to lose its affinity for Ca^{2+} /calmodulin. Thus in the above model it would prevent calmodulin from moving to CBS-1, leaving it bound at CBS-2 and keeping the channel closed. This situation would be maintained unless Ca^{2+} levels in excess of 5 μM were reached, at which point calmodulin would dissociate from CBS-2. Thus phosphorylation, and subsequent dephosphorylation, of CBS-1 may modulate Trp1 channel activity. This phosphorylation and dephosphorylation may occur in response to changing levels of intracellular Ca^{2+} , as several kinases and phosphatases are known to be regulated by Ca^{2+} levels [41,42].

The effect of phosphorylation by PKC at CBS-1 is less clear. No effect on calmodulin binding was observed, but when both kinases were used to phosphorylate CBS-1 it was found that phosphorylation by PKC resulted in a significant reduction in phosphorylation by PKA. Phosphorylation of CBS-1 by PKC *in vivo* may therefore regulate the phosphorylation of CBS-1 by PKA, thus affecting its modulation of channel activity.

In summary, this paper has described the identification and characterization of two very different calmodulin-binding sites in the *Drosophila* Trp1 protein. CBS-2 may be involved in gating the channel in response to changing intracellular Ca^{2+} /calmodulin levels, whereas CBS-1 may modulate this gating by providing an alternative binding site for calmodulin under particular conditions of Ca^{2+} concentration and phosphorylation. The Trp1 channel may thus provide us with a unique example of a channel that is both gated and modulated by calmodulin.

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