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INVITED REVIEW

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Multiple roles of calmodulin and other Ca^{2+}-binding proteins in the functional regulation of TRP channels

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Abstract Transient receptor potential channels (TRP) have emerged as cellular sensors of various internal and external cues. Generally, the activation of TRP canonical (TRPC) channels is triggered by the stimulation of phospholipase C; however, multiple factors are involved in the regulation of these channels. Among them, Ca^{2+}-mediated feedback channel modulations are often mediated by calmodulin (CaM) and other Ca^{2+}-binding proteins. In vitro binding studies have revealed multiple CaM-binding sites on TRPC proteins. Among them, a common CaM/inositol 1,4,5-trisphosphate receptor-binding site is found at the carboxyl terminus of every TRPC isoform. Additional non-conserved CaM-binding sites are present at the amino and carboxyl termini of several TRPC proteins. Likewise, multiple CaM-binding sites were found in other TRP proteins. These, together with the presence in close vicinity of the interaction sites for the related neuronal Ca^{2+}-binding proteins, such as CaBP1, suggest a multitude of diverse intracellular Ca^{2+}-dependent regulations of TRP channels. Functional studies have begun to reveal the unique roles of CaM and CaBP1 binding to several TRP channels. This review will focus on the CaM- and CaBP1-mediated regulations of TRPC channels. Related studies on TRPM and TRPV channels will also be highlighted.

Keywords G protein · TRPC · TRPV · TRPM calmodulin · CaBP1 · Growth cone · Capacitative Ca^{2+} entry · Cation channel

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Introduction

The original drive behind the cloning of mammalian transient receptor potential canonical (TRPC) homologues was the possibility that these proteins form channels that mediate capacitative or store-operated Ca^{2+} entry (SOCE) [6, 106]. Seven non-allelic *trpc* genes are present in mammals. Among them, *trpc*2 is a pseudogene in human. Although a fair amount of controversy was generated over the issue whether or not TRPCs form store-operated channels, the idea that these proteins participate in SOCE remains viable (see recent reviews in references [44, 45, 49, 58, 71, 87]). Relevant studies can be divided into two categories. One is the manipulation of endogenous expression of TRPC levels. Numerous studies have shown that disruption of TRPC protein expression, either through RNA suppression with antisense oligonucleotides, antisense RNA, or RNAi or via gene knockout, resulted in a reduction or complete elimination of SOCE [8, 14, 40, 43, 46, 55, 63, 75, 81, 84, 85, 89, 93, 94, 106]. In pulmonary artery smooth muscle cells, up-regulation of TRPC1 and TRPC6 expression was accompanied by an increase in SOCE [15, 98]. In cardiac myocytes, knockdown of the type 2 sarco-/endoplasmic reticulum Ca^{2+}/ATPase expression resulted in a paralleled compensatory increase in the expression of TRPC4/5 and activity of SOCE [67]. By contrast, the heterologously expressed TRPC channels often do not behave in a store-operated manner [7, 53, 54, 64, 73, 74, 105]. The biophysical properties of ectopically expressed TRPC channels are also quite different from that of native channels. This has raised the possibility that native store-operated channels are heteromers composed of TRPC and other unidentified subunits. However, attempts to reconstitute SOCE by mixing and matching different TRPC subunits were unsuccessful [73, 74].

On the other hand, native TRPC-like channel activities were found in human submandibular gland cells [43], growth cones of *Xenopus* spinal neurons [88] and

rat hippocampal neurons [17], and several types of smooth muscles [1, 23–25, 29, 38, 103]. In agreement with the ectopically expressed TRPCs, these channels are cation non-selective and are activated following the stimulation of phospholipase C pathway, through either G proteins or receptor tyrosine kinases. As a consequence, the activation of TRPC channels evokes not only Ca^{2+} influx but also membrane depolarization [12, 30, 32, 88, 92], which in excitable cells, may be important for triggering action potentials and activation of voltage-gated Ca^{2+} channels. Consistent with this, functional significance has been found for TRPC channels in smooth muscle and vascular tones in blood vessels, neurotransmitter release, synaptic transmission, growth cone morphology, and growth cone guidance [17, 32, 39, 47, 88, 92].

It remains a mystery which step or component of the phospholipase C pathway is critical for TRPC activation. Diacylglycerol was shown to activate TRPC3/6/7, and perhaps, TRPC1 (only under Ca^{2+}-free conditions; [21, 41, 53]), but it is unlikely to be the sole player for the receptor-induced channel activation as it still synergizes with receptor agonist and inositol 1,4,5-trisphosphate (IP_3) [1, 12]. Ca^{2+} plays a peculiar role on TRPC channels. These regulations include both inhibition and facilitation from the extracellular as well as the intracellular sides of the plasma membrane [30, 54, 64, 68, 69, 101]. Activation by multiple factors is common among TRP channels. For example, TRPV1 is activated by heat, protons, vanilloid compounds, and several endogenous substances [76, 83]. The activation is also regulated by protein phosphorylations [4, 5, 52], phosphatidylinositol 4,5-bisphosphate hydrolysis [11], and products of arachidonic acid metabolism [70]. These activation mechanisms also synergize with each other's function, making the channel a coincidence detector. Thus, it is anticipated that TRPC channels are activated through multiple mechanisms. The purpose of this review is to provide a comprehensive overview about the current progress on the regulation of TRPC channels by calmodulin (CaM) and one of its neuronal specific homologues, Ca^{2+}-binding protein 1 (CaBP1). Studies on CaM binding and regulation of TRPV and TRPM channels are also included for comparisons.

Presence of multiple CaM-binding sites on TRPC proteins

CaM is involved in the Ca^{2+}-dependent regulation of many proteins, including ion channels [13, 36, 59, 86, 95, 100]. One can date the connection between CaM and TRPC all the way back to 1992 for the expression cloning of the second TRPC (TRP-Like) from fruit flies, for which CaM was used as the probe [56]. It was later demonstrated that CaM also interacted with the *Drosophila* TRP [10], the prototypical channel after which all TRP proteins are named. However, besides from knowing where CaM may bind to TRP [amino acids (aa) 683–695] and TRP-Like (aa 710–725, aa 854–875), functional significance of CaM binding on these channels has not been revealed [80, 90]. In addition, it was not known whether CaM binding is conserved among TRPC proteins.

In order to gain further insights on CaM-mediated channel regulation, we have searched for CaM-binding sites on mammalian TRPC proteins. We have taken an experimental approach using in vitro binding assays to identify regions on TRPC proteins that are capable of interacting with CaM. This allowed us to find more potential CaM-binding sites than using in silico motif search, which was proven to be of limited value in our hands. Because most CaM-binding sites are restricted to a short, continuous α-helical sequence [60], it is possible to isolate them through screening a series of overlapping fragments generated from the protein of interest. Initially, we screened for CaM-binding sites on murine TRPC4α, a longer and less-active version of the two functional isoforms found for TRPC4 [65, 78]. The full-length TRPC4α was divided into the N terminus, the transmembrane region, and the C terminus. The DNA fragments were either prepared with the use of existing restriction sites on the cDNA or generated by polymerase chain reactions in between any two desired locations. After being placed in a prokaryotic expression vector with a T7, T3, or SP6 promoter, the encoded protein fragments were synthesized in vitro using the transcription and translation-coupled rabbit reticulocyte lysate system. A small amount of [^{35}S]Met and [^{35}S]Cys was included in the reaction to label the newly synthesized proteins. The advantage of this method is that hydrophobicity and other structural issues usually do not interfere with the efficiency of the in vitro synthesis, allowing transmembrane and hydrophobic regions to be synthesized equally well as the soluble fragments. The lysate was used directly in pull-down assays without any purification. The ribosomal and other unlabeled proteins present in the lysate served as good blocking agents for nonspecific interactions. To test the interaction with CaM, we incubated the lysate with CaM-agarose beads in the absence and presence of varying concentrations of free Ca^{2+}. After incubation, the bound proteins were separated from the unbound ones by a short, low-speed centrifugation. The beads were washed with the same buffer and bound proteins were visualized by X-ray autoradiography after SDS-PAGE. Because the proteins were radioactively labeled, quantification was possible with the use of a phosphorimager. Once a protein region was found to bind to CaM-agarose, it was divided further into smaller fragments, which were tested using the same binding assay. This was repeated until further deletion of one or two amino acids from either end of a small fragment always significantly reduced the binding. In order to increase the labeling and to be able to resolve the small fragments shorter than 50 aa on the SDS-PAGE, we tagged them with either blue fluorescence protein or the first 322 aa of the bacterial maltose-binding protein. This strategy was proven to be very

useful for uniformly labeling different short segments. Examples of these experiments were published [50, 77, 101].

Using the above approach, we have identified three CaM-binding sites on TRPC4α, one at the N terminus and two at the C terminus (Fig. 1). The N-terminal CaM-binding (NCB) site is located immediately before the first transmembrane segment. The NCB sequence is relatively conserved among TRPC isoforms. However, except for TRPC1, C4, and C5, which contain five, four, and five basic residues, respectively, other TRPCs only contain two positively charged residues within this motif. Whether or not and under what conditions they bind to CaM remain to be tested. Most of our functional studies have focused on the C-terminal CaM-binding (CCB) sites. Interestingly, the first CCB site is also bound to an N-terminal region of IP_3 receptors (IP_3Rs) and was therefore named the CaM- and IP_3R-binding (CIRB) site [77, 101]. The CIRB site is conserved among TRPC proteins including the three *Drosophila* proteins. However, this motif is separated from the main conserved core region (Fig. 1, shaded areas) of TRPCs by a nonconserved area of variable lengths. In TRPC1, C4, C5, and the *Drosophila* TRPs, the CIRB site begins at the start of an exon (Fig. 2). In TRPC2, C3, C6, and C7, a new exon starts not too far from the beginning of the CIRB sites. It is also notable that in TRPC2-7, the CIRB site is encoded by one or two very short exons. These features suggest that there is a high selection pressure to maintain the CIRB site through evolution.

Downstream from the CIRB site, there are two CCB sites in TRPC4α and one each in TRPC1, C2, and C5 (Fig. 1). In TRPC4α, the earlier CCB site (787–812) was identified by binding studies [77], whereas the later site (829–853) was determined through motif search [79]. The location of the later site also coincides with that of the second CCB (CCBII) site of TRPC5. However, the homology between the two sequences is quite low (16% identical and 40% similar, see Fig. 3 for sequences). Interestingly, both CCB sites are absence in TRPC4β, suggesting a more complex control by Ca^{2+}/CaM on TRPC4α than TRPC4β. The CCBII sequence of TRPC1 and that of TRPC2 are unique to themselves. In addition, an N-terminal region of TRPC2 (109–161), which is located outside the conserved areas of TRPCs, has also been shown to bind to CaM [97]. However, the minimal sequence for this NCB site has not been determined.

The sequences for all CaM-binding sites of TRPCs identified to date are listed in Fig. 3. For comparison, those of TRPM4 and TRPV1, V4, and V6 are also listed. Each sequence conforms to at least one of the three known consensus CaM-recognition motifs [60]. More interestingly, the CIRB sites of TRPC3-7 contain all three CaM motifs. A Tyr replaces the Ile in the IQ (IQXXXRGXXXR) motif, and it also serves as the first hydrophobic residue in the 1-8-14 motif of type A (1-5-8-14). The IQ (or YQ) motif is also found in *Drosophila* TRPγ, but the 1-8-14 motif is slightly disrupted by a Thr at the 14th position. In TRPC2 and *Drosophila* TRP and TRP-Like, Gln is missing from the IQ motif; in TRPC1, the first basic residue is replaced by a Cys. The 1-8-14 motif of type A in TRPC1 is also disrupted by the last Thr. In TRPC2 and *Drosophila* TRP, this motif is shifted 1 aa forward as compared to other CIRB sites, and it shares the two hydrophobic residues (positions 5 and 14) with the 1-5-10 motif. Nonetheless, a perfect 1-5-10 motif is found in all CIRB sites. The residue at the fifth position is often shared with the eighth residue of the 1-8-14 motif. Thus, most of the conserved residues in the consensus CIRB sequence [YQx(V/I)(MI)(KR)xL(V/I)xRY(VIL)x, Fig. 2] appear to be important for binding to CaM one way or the other through the three recognition mechanisms. Two of these residues, V/I and

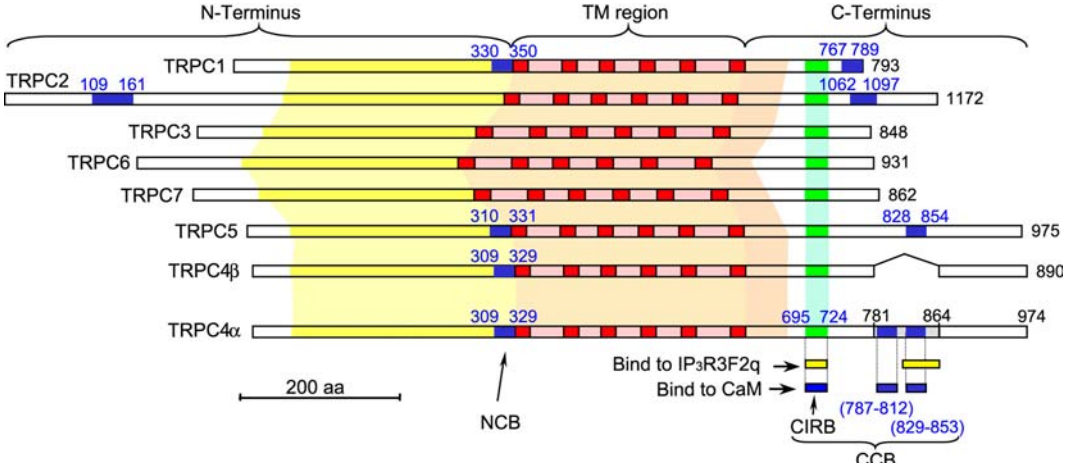

Fig. 1 Inositol 1,4,5-trisphosphate receptor (*IP_3R*)- and calmodulin (*CaM*)-binding sites identified on transient receptor potential canonical (*TRPC*) proteins. The diagram shows locations of CaM- and IP_3R-binding (*CIRB*) sites (*green*) and other CaM-binding sites (*blue*) found on TRPC proteins. *Red boxes* indicate transmembrane (*TM*) segments. Conserved areas are *shaded*. Except for amino acids (*aa*) 829–853 of TRPC4α [79], all binding sites were identified using in vitro binding assays. TRPC4β is a shorter and more active form of TRPC4 than TRPC4α [65]. It lacks 84 aa at the C terminus, which also includes two CCB sites

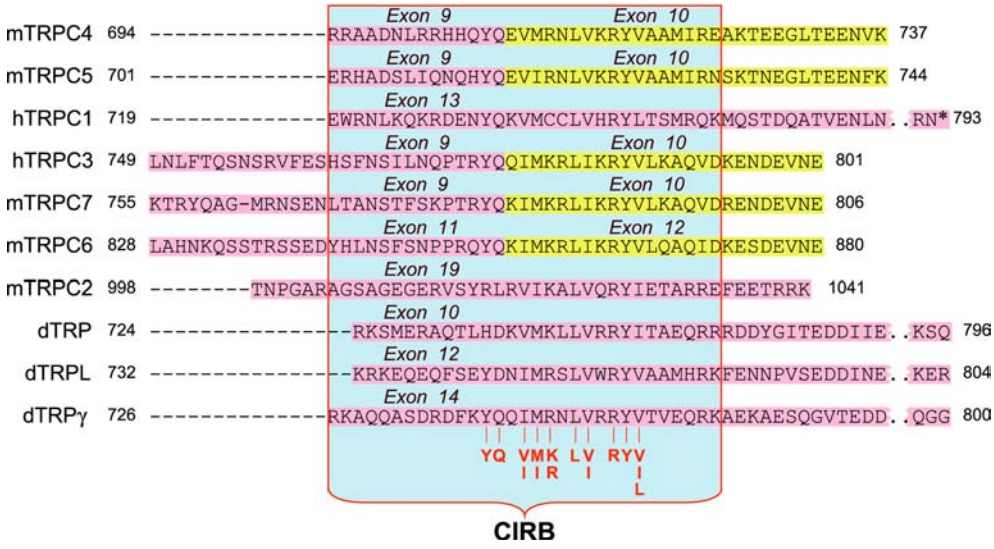

Fig. 2 Exons encoding the CIRB sites of TRPC proteins. Intron/exon boundaries were determined by comparing the cDNA with the genomic sequence for each TRPC. Deduced sequences from only those contribute to the CIRB sites are shown and *highlighted in different colors* when two exons are involved. Sequences for TRPC1 and *Drosophila* TRPs are *shortened* to save space. Conserved residues in the CIRB sequences are indicated at the *bottom*. *h*, *m*, and *d* denote, human, murine and *Drosophila*, respectively

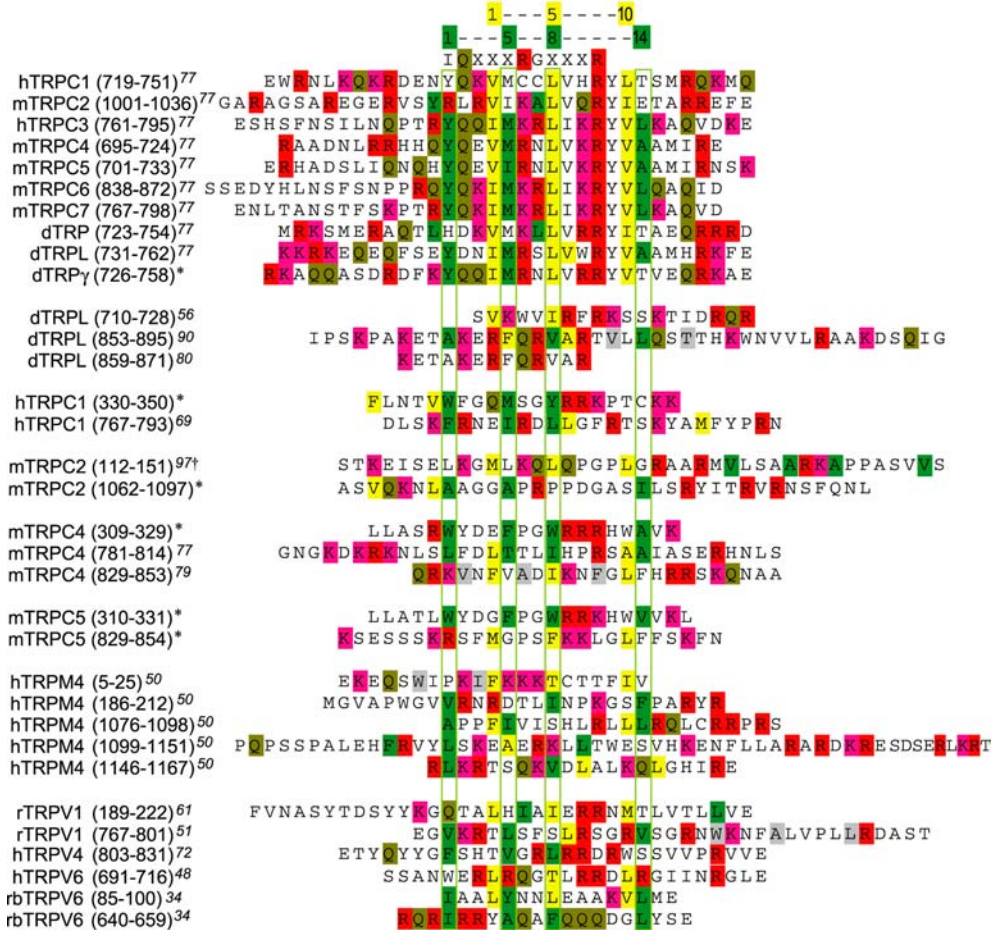

Fig. 3 CaM-binding sequences found in TRP proteins. Arg, Lys, and Gln residues are *highlighted in red*, *pink*, and *olive*, respectively. Sequences are aligned primarily based on the identified 1-8-14 (*green*) and secondarily on the 1-5-10 (*yellow*) CaM-recognition motifs [60]. The location of the IQ (*YQ*) motif found in some CIRB sites of TRPCs is indicated at the *top*. Alternative motifs that conform to the 1-8-14 or 1-5-10 patterns are indicated in *gray*. *rb* Rabbit. References are indicated *after the parentheses*, which show the positions of these sites on full-length proteins. *Asterisks* Unpublished, *daggers* predicted based on locations of 1-8-14 and 1-5-10 motifs within the region shown to bind to CaM [97]

Y (underlined above), are not included in the CaM-recognition motifs and, thus, probably are important for binding to IP_3Rs. The other CaM-binding sites identified for TRPs conform either the 1-8-14 or the 1-5-10 motif and some times both. No IQ motif is present in these sequences. In several cases, the 1-8-14 or 1-5-10 motifs contain Gln, Gly, Pro, Ser, and Thr at the hydrophobic positions, indicating that these sites may be atypical. Particularly, the three CCB sites of TRPM4 do not match perfectly with any known CaM motif. These sites are connected to each other in a continuous sequence, suggestive a binding pattern analogous to the

type 2 small conductance Ca^{2+}-activated K^+ channel (SK2), in which two CaM molecules are bound to two channel subunits, each providing three α helices arranged in an anti-parallel manner [66]. This is supported by the finding that deletion of any region within the three continuous CaM-binding sites severely reduced the Ca^{2+} sensitivity of the TRPM4 channel [50].

Functional divergence of different CaM-binding sites

The inhibitory function of the CIRB site

In binding studies, we have demonstrated that CaM and IP_3Rs competed with each other for binding to the CIRB site of any TRPC [77, 101]. This competition is Ca^{2+} dependent, as CaM only binds to the CIRB site in the presence of Ca^{2+}. Therefore, at low Ca^{2+} concentrations, the CIRB site is most likely bound to IP_3Rs, allowing the TRPC channel to be activated through the conformational coupling mechanism [2, 26]. Ca^{2+} influx through the channels will increase the local Ca^{2+} concentration and thus enhance the affinity of CaM to the CIRB site. Whether or not CaM competes off IP_3Rs from TRPC channels in the continued presence of IP_3 is unclear, because binding studies were performed with short IP_3R peptides that do not contain the IP_3-binding motif. However, at high enough concentrations, a peptide that represents the TRPC-binding site of type 3 IP_3R (IP_3R3) inhibited CaM binding to the CIRB sites in the presence of high Ca^{2+}, suggesting that activated IP_3Rs can stay bound to TRPCs even when CaM is most active [77, 101].

Electrophysiological studies have demonstrated that Ca^{2+} and CaM inhibited the function of TRPC3 expressed in HEK293 cells. In inside-out patches, application of the IP_3R3 peptide to the cytoplasmic side caused TRPC3 activation, which was inhibited by Ca^{2+}/CaM. Furthermore, simply inactivating or removing CaM from the excised patches was able to activate TRPC3. This was shown with the use a CaM antagonist, calmidazolium, a high-affinity CaM-binding peptide, and a CaM mutant that had lost all Ca^{2+}-binding sites [101]. Similar results were obtained for TRPC4 but with a higher concentration of the IP_3R3 peptide, a finding consistent with the observation that for TRPC3/6/7, the affinities of IP_3R to the CIRB sites were higher than for TRPC1/2/4/5 [77]. Thus, CaM is bound to the CIRB sites to keep TRPC channels from being activated. Its displacement by IP_3Rs or other mechanisms leads to channel activation. This competitive modulation by IP_3Rs and Ca^{2+}/CaM has also been demonstrated for native TRPC1-like channels in CHO cells [82].

There are quantitative and qualitative differences in CaM binding with the CIRB sites among TRPCs. First, the affinities to CaM range from 10 n*M* to 290 n*M* [77], suggesting that CaM binds to certain TRPC channels more tightly than to others. Second, the apparent Ca^{2+} affinities for this interaction are also different, ranging from 1.6 μ*M* to 44 μ*M* [77]. Therefore, certain TRPC channels are inhibited by CaM at lower Ca^{2+} concentrations than others are. Third, different CaM mutants were examined for interaction with TRPC CIRB sites in vitro. These mutants lack Ca^{2+} binding at one or two of the four EF hands and were made by a Glu-to-Ala substitution in the last Ca^{2+} ligand position of the EF hand. As shown in Fig. 4, TRPC2, TRPC3, and TRPC6 tolerated very well the single mutation at EF1, EF2, or EF3, and moderately well the mutation at EF4 as well as those at EF1 and EF2 together (EF1,2). However, mutations at EF3 and EF4 together (EF3,4) greatly reduced the binding, suggesting that the CIRB sites of TRPC2, TRPC3, and TRPC6 are preferentially bound to the C-terminal lobe of CaM. In contrast, the CIRB sites of TRPC1 and C4 did not tolerate mutations in either the single or double EF hands, indicating that both N- and C-terminal CaM lobes are involved in the binding. More interestingly, the CIRB site of TRPC5 appeared to be bound to the N-terminal lobe of CaM, because it endured mutations at EF3 and EF4 but not EF1 and EF2. Because the N-terminal lobe of CaM has a much lower Ca^{2+} affinity than the C-terminal lobe [27], this result is consistent with the finding that TRPC5 CIRB site required a much higher Ca^{2+} concentration than other CIRB sites to bind to CaM [77]. These differences warrant more detailed studies for each TRPC isoform on how and under which condition CaM binding to the CIRB site is involved in regulating the channel function.

Diverse functions of nonconserved TRPC CCBII sites

For other TRPCs CaM-binding sites, functional analyses were carried out for the CCBII sties of TRPC1 and

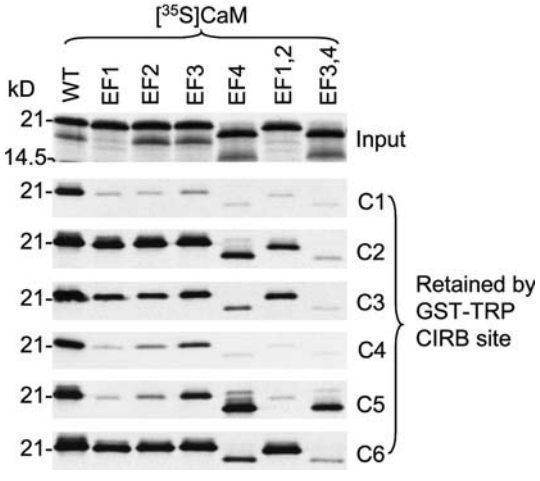

Fig. 4 Interaction of TRPC CIRB sites with the N- and C-terminal lobes of CaM. ^{35}S-labeled wild-type CaM or CaM carrying a single Glu-to-Ala mutation at position 31 (*EF1*), 67 (*EF2*), 104 (*EF3*), or 140 (*EF4*) or double mutations at 31 and 67 (*EF1,2*) or 104 and 140 (*EF3,4*) was incubated with a glutathione S transferase (*GST*) fusion protein containing the CIRB sequence of TRPC1-6. Autoradiograms show the input CaM and its mutants (*top*) and those retained by GST-CIRB sites of TRPCs. Note this is a reciprocal experiment as those described earlier in the text and shown in references [50, 77, 101]

TRPC5. As expected from the lack of sequence homology, these sites showed very different functional effects. The TRPC1 CCBII site is located at the very C-terminal end of the protein. In human submandibular gland cells, the expressed TRPC1 channels were studied by infusing IP_3 into the cells through the patch pipet. The inward TRPC1 currents (0–120 mV) displayed a Ca^{2+}-dependent slow inactivation that was removed by omitting Ca^{2+} from the extracellular solution, including a high concentration of 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid in the patch pipet, or coexpressing TRPC1 with the EF1234 CaM mutant, indicating that Ca^{2+} entry through the open channel causes a CaM-mediated feedback inhibition. It was found that deletion of the CCBII but not the CIRB site eliminated the Ca^{2+}-dependent slow inactivation [69]. This suggests that CCBII of TRPC1 is important for the feedback channel inhibition induced by Ca^{2+}/CaM.

By contrast, our recent results suggest that the CCBII site of TRPC5 has a positive effect on channel activation. We have studied the activation of TRPC5 current through thrombin and bradykinin receptors. Application of thrombin caused a slow activation of TRPC5 currents that reached peak levels in 7–9 min. Inclusion of 10 μ*M* CaM in the patch pipet greatly enhanced the rate of agonist induced channel activation, showing that CaM facilitates the opening of TRPC5 channels. Deletion of the CCBII site from TRPC5 slightly reduced the rate of channel activation, but more importantly, it eliminated the facilitation effect of CaM, indicating that the CCBII site is critical for the Ca^{2+}/CaM-mediated acceleration of receptor-evoked activation of TRPC5 [L. Vaca and M.X. Zhu, unpublished results]. In these experiments, the intracellular Ca^{2+} was buffered to 0.4 μ*M* and the extracellular Ca^{2+} was 2 m *M*. It was shown that the activation of TRPC5 is dependent on both extracellular and intracellular Ca^{2+} [28, 33, 54, 64, 99]. The extracellular effect of Ca^{2+} was determined to be mediated by three negatively charged amino acids, Glu^{543}, Glu^{595} and Glu^{598}, located at the pore loop [28]. It is likely that Ca^{2+}-induced CaM binding at the CCBII site is responsible, in part, for the intracellular Ca^{2+} dependence of TRPC5 activation. Because the CCBII mutant is still active, another Ca^{2+}-dependent site may also exist. This could be the NCB site or a non-CaM-binding site.

Regulatory functions of CaM on TRPV and TRPM channels

Ca^{2+}/CaM is involved in the desensitization of TRPV1 channels [51, 61]. During repetitive short (10 s) capsaicin stimulations with a 30-s interval, the response to the second stimulation was about 24% of the first stimulation. The inhibition was completely removed by omitting Ca^{2+} from the external solution, as well as by deleting the CCB site from TRPV1. However, in the continued presence of capsaicin (40 s), the deletion of the same site only partially rescued the Ca^{2+}-dependent desensitization, indicating that the CCB site is mainly responsible for the fast, but has a minor contribution to the slow, component of Ca^{2+}-induced TRPV1 desensitization [51]. The CCB site of TRPV1 binds to CaM in the absence of Ca^{2+}, suggesting that CaM is anchored to TRPV1 at the C- terminus. A Ca^{2+}-dependent NCB site has also been found for TRPV1. However, how it is involved in the Ca^{2+}/CaM-mediated desensitization of TRPV1 remains to be elucidated, because in the original analysis, the deletion mutants failed to form functional channels [61].

Activation of TRPV4 by hypotonic solutions, heat, or phorbol ester agonists was facilitated by a rise in intracellular Ca^{2+}, which accelerated the rate of current development and increased the level of current amplitude. Disrupting the C-terminal Ca^{2+}-dependent CaM-binding site of TRPV4 eliminated facilitation effect of intracellular Ca^{2+}, indicating that the CCB site is critical for the CaM-mediated potentiation of TRPV4 function [72]. In addition, the Ca^{2+}-dependent facilitation of TRPV4 was also followed by a Ca^{2+}-dependent inhibition, which brought the activity to levels lower than those before stimulation. This provides a negative feedback mechanism to control the level and duration of Ca^{2+} rise during prolonged stimulation of TRPV4 channels. Interestingly, the Ca^{2+}-dependent inhibition persisted in TRPV4 mutants that had lost the CCB site [72]. Either CaM is not involved in the Ca^{2+}-induced channel inactivation or additional CaM-binding sites are present to mediate this effect. Indeed, using in vitro binding assays, we have isolated three NCB sites and one additional CCB site from TRPV4 (J. Tang and M.X. Zhu, unpublished result). Functional studies will reveal whether one of them is involved in the Ca^{2+}-dependent channel inactivation.

Multiple CaM-binding sites were identified for TRPV6 (Fig. 3; [34, 48]). A CCB site found in human TRPV6 has been shown to mediate Ca^{2+}-dependent channel inactivation. CaM binding at this site is competitively regulated by the phosphorylation of a threonine residue by protein kinase C. The fast Ca^{2+}-dependent inactivation was greatly attenuated with the disruption of the CCB site but accelerated with a Thr-to-Ala mutation that prevented its phosphorylation. Notably, the regulation by protein kinase C at the CCB is not conserved in rat, because the Thr is substituted by Ala [48]. On the other hand, constitutive association of CaM with rabbit TRPV6 at the NCB and another CCB sites is involved in the potentiation of TRPV6 function. This effect may take place at resting Ca^{2+} concentrations, because the binding occurred at as low as 30 n*M* Ca^{2+}, and the C-terminal lobe of CaM is mainly responsible for the regulation. Interestingly, even though TRPV5 contains CaM-binding sites at similar locations, it did not show a CaM-mediated functional potentiation as TRPV6 [34].

Two members of the TRPM subfamily, TRPM4 and TRPM5, are activated by Ca^{2+} from the cytoplasmic side of the plasma membrane [20, 35, 42, 57]. Using in

vitro binding assays, we have isolated five CaM-binding sites, two at the N terminus and three at the C terminus, from TRPM4. Studies with deletion mutants suggest that the CCB, but not the NCB, sites are important for the Ca^{2+}-induced activation of TRPM4 [50]. However, there may still be other Ca^{2+}-sensitive sites on TRPM4, because the CCB site mutations only greatly reduced, but did not eliminate, the activation by Ca^{2+}. Similar results were obtained with the use of EF1234 CaM mutant [50]. Whether or not TRPM5 is activated through CaM binding remains to be investigated.

Therefore, it appears that Ca^{2+}/CaM exerts multiple regulatory roles on TRP channels. One can anticipate that more CaM-binding sites will be identified from different TRP proteins and the functional implications of these interactions will be very complex, ranging from channel activation, facilitation, inactivation/desensitization, to presumably subunit assembly, surface expression, recycling, and other regulations. For instance, CaM is involved in inhibiting the kinase activity of TRPM7, although the CaM-binding site(s) remain(s) to be identified [62]. CaM was implicated in the surface expression of SK2 channels [37]. Whether any of the CaM-binding sites in TRP channels is involved in this function is not known, but reduced total and cell surface expression of TRPV1 was found for NCB site deletions [61]. Deletions of the CIRB site from TRPC3 also hampered its cell surface expression [91].

Regulatory functions of other Ca^{2+}-binding proteins on TRP channels

Unlike the ubiquitously expressed CaM, many other four EF-hand motif proteins, e.g., the neuronal Ca^{2+}-binding proteins, are more specifically expressed in neurons and serve functions either similar to or distinct from CaM [9, 18]. CaBP1 has been shown to regulate voltage-gated Ca^{2+} channels and IP_3Rs in a different fashion than CaM [19, 31, 36, 96, 104]. For both channel types, the CaBP1-binding sites are closely localized with those for CaM binding [31, 36, 104]. CaBP1 has been shown to compete with CaM for binding to the L-type Ca^{2+} channels [104]. In a recent study, we showed that in *Xenopus* oocytes, coexpression of CaBP1 inhibited the activation of TRPC5 by $G_{q/11}$-couple M5 muscarinic receptors and by ionomycin [33]. The effect was not mimicked by an EF-hand mutant of CaBP1, indicating that Ca^{2+}-dependent binding of CaBP1 is required for the inhibitory action. Neither CaM nor the EF1234 CaM mutant had a similar effect as CaBP1. We have found that CaBP1 binds to both the N and C termini of TRPC5. At the C terminus, the CaBP1-binding sites are very close, but not identical, to the CCB sites. In fact, the similar strategy used to locate CaM-binding sites did not work well with CaBP1, indicating that discontinuous regions may have to fold together to coordinate the CaBP1–TRPC5 interaction. Nonetheless, the CaBP1–TRPC5 interaction was dose-dependently inhibited by the increasing concentrations of CaM. A survey with other TRPC proteins showed that CaBP1 was able to bind to all TRPCs (M. Kinoshita-Kawada, J. Tang, and M.X. Zhu, unpublished results). Therefore, competitive interactions between CaM and CaBP1 to TRPC channels in neurons may have significant functional implications.

The conformation of CaBP1 is different from that of CaM, because the second EF hand of CaBP1 is not regulated by Ca^{2+} [18], CaBP1 has an N-terminal myristoylation site, and it contains an extra turn at the helical domain that links the N- and C-lobes [18, 19]. This may explain why CaBP1 binding to the same or nearby region as CaM often resulted in different functional consequences. Recent studies suggest that TRPC5 plays an important role in controlling neurite outgrowth and growth cone morphology of hippocampal neurons [17]. However, TRPC5 proteins are distributed in the neuronal arborization as well as growth cones [17]. In the cell body and neuronal processes, TRPC5 is localized to the cytoplasmic transport packets, which transport the cation channels along with other membrane proteins to the growth cones. CaBP1 is expressed in somatodendritic regions of principal neurons throughout the brain. Immunocytochemical studies indicate that the protein is enriched at the cell body and neuronal processes but rare at the nerve terminals [36]. The presence of CaBP1 in the cell body and neuronal processes may help prevent spontaneous channel activation during the transport of TRPC5 from the somata to the growth cones.

Concluding remarks

CaM has been shown to regulate TRP channels, with diverse consequences. In most cases, the regulation is achieved through direct binding of CaM on the cytoplasmic side of the channel protein. Multiple CaM-binding sites were identified on different TRP proteins. Functional implications were resolved for some, but are largely missing for many others, CaM-binding sites. There are many differences among various CaM-binding sites in terms of affinities to CaM and to Ca^{2+}, as well as the precise conformations and the regions of CaM that are bound to these sites. The challenge that electrophysiologists are facing is to find conditions for each channel type that will reveal multiple Ca^{2+}-dependent, and sometimes Ca^{2+}-independent, processes that may require CaM. This is not always possible if recordings are carried out under the same conditions. In neurons, additional regulations may be achieved through neuronal Ca^{2+}-binding proteins that are structurally related to but often exert different effects than CaM.

The physiological significance of multiple feedback mechanisms through CaM and other Ca^{2+}-binding proteins can be fascinating, given the multifaceted roles Ca^{2+} has on many cellular processes. Several recent studies have demonstrated an essential role for TRPC channels in growth cone extension and turning [3, 17, 39, 88]. Ca^{2+} entry through TRPC channels and the con-

sequent activation of voltage-gated Ca^{2+} channels [88] provides the spatial and temporal variations of Ca^{2+} signal that either promotes or inhibits neurite outgrowth [16]. These variations are also responsible for both the attractive and repulsive effect of guidance cues on growth cone turning [22, 102]. Because of the complex action of Ca^{2+} on the growth cone behavior, the feedback regulation of TRPC channels by Ca^{2+} becomes very important. A small change in the TRPC function is likely to affect the overall Ca^{2+} signal at growth cones and filopodia, because the activation of voltage-gated Ca^{2+} channels and ryanodine receptors may follow TRPC channel activities. The presence of multiple CaM- and CaBP1-binding sites on TRPCs is indicative of a complex feedback mechanism that includes both positive and negative responses to intracellular Ca^{2+} rises, which in turn, further influence the Ca^{2+} signal and hence the growth cone behavior.

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