Identification of Common Binding Sites for Calmodulin and Inositol 1,4,5-Trisphosphate Receptors on the Carboxyl Termini of Trp Channels*

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Homologues of Drosophila Trp (transient receptor potential) form plasma membrane channels that mediate Ca\(^{2+}\) entry following the activation of phospholipase C by cell surface receptors. Among the seven Trp homologues found in mammals, Trp3 has been shown to interact with and respond to IP\(_3\) receptors (IP\(_3\)Rs) for activation. Here we show that Trp4 and other Trp proteins also interact with IP\(_3\)Rs. The IP\(_3\)-R-binding domain also interacts with calmodulin (CaM) in a Ca\(^{2+}\)-dependent manner with affinities ranging from 10 \(\text{nM}\) for Trp2 to 290 \(\text{nM}\) for Trp6. In addition, other binding sites for CaM and IP\(_3\)Rs are present in the \(\alpha\) but not the \(\beta\) isoform of Trp4. In the presence of Ca\(^{2+}\), the Trp-IP\(_3\)R interaction is inhibited by CaM. However, a synthetic peptide representing a Trp-binding domain of IP\(_3\)Rs inhibited the binding of CaM to Trp3, -6, and -7 more effectively than that to Trp1, -2, -4, and -5. In inside-out membrane patches, Trp4 is activated strongly by calmidazolium, an antagonist of CaM, and a high (50 \(\mu\text{M}\)) concentration of the Trp-binding peptide of the IP\(_3\)R. Our data support the view that both CaM and IP\(_3\)Rs play important roles in controlling the gating of Trp-based channels. However, the sensitivity and responses to CaM and IP\(_3\)Rs differ for each Trp.

Binding of many cell surface receptors by hormones, neurotransmitters, and growth factors leads to the activation of phospholipase C, which in turn produces diacylglycerol and inositol 1,4,5-trisphosphate (IP\(_3\))\(^1\). Diacylglycerol activates protein kinase C, while IP\(_3\) triggers Ca\(^{2+}\) entry from internal stores in turn activates store-operated channels (SOCs) located on the plasma membrane, allowing Ca\(^{2+}\) influx from the extracellular space. The store-operated Ca\(^{2+}\) influx, also known as capacitative Ca\(^{2+}\) entry (1, 2), plays critical roles in controlling the duration and the frequency of cytosolic Ca\(^{2+}\) changes (2–4). In contrast to the well-defined roles of IP\(_3\) and IP\(_3\)Rs in Ca\(^{2+}\) release, the molecular makeup of channels that mediate Ca\(^{2+}\) influx and their gating mechanism(s) remain to be elucidated. Drosophila transient receptor potential (Trp) protein and its mammalian homologues have been shown to form either Ca\(^{2+}\)-selective or nonselective cation channels that mediate Ca\(^{2+}\) influx in response to phospholipase C activation (5–7). To date, seven \(\text{trp}\) genes have been cloned from mammalian species (6, 8), probably reflecting the heterogeneity of Ca\(^{2+}\) influx channels or pathways found in different cells (4, 9). Expression of individual Trp proteins in heterologous systems revealed that Trp channels may be activated by a number of intermediaries involved in the phospholipase C-stimulated signaling cascade, including Ca\(^{2+}\) (10), diacylglycerol (11), and activated IP\(_3\)Rs (12–14). Although store depletion induced by an intracellular Ca\(^{2+}\)-ATPase inhibitor, thapsigargin, appears to be sufficient to open some Trp channels (e.g. Trp1 (15), Trp2 (16), and Trp4 (17)), it remains controversial whether all Trp proteins participate in forming SOCs (18, 19). Perhaps the answer lies within the structural organization of the channel, which could be composed of four different Trp subunits (5). A recent example showed that expression of two Drosophila store-insensitive Trp proteins, Trpy and Trp-like (Trpl), led to the formation of a SOC (20). Thus, the store sensitivity may be reconstituted with the proper combination of different Trp subunits. Consistent with this idea, Trp1, Trp3, and Trp4 have been shown to be part of SOCs in human submandibular gland cells, neurons, and adrenal cortex cells, respectively (15, 21, 22).

Recent studies showed that IP\(_3\)Rs are involved in the activation of Trp3. Following the initial demonstration that human Trp3 (hTrp3) in inside-out membrane patches was activated by IP\(_3\)Rs in the presence of IP\(_3\) (12), Boulay et al. (14) identified the binding domains involved in the Trp-IP\(_3\)R interaction, which were found to be located in the N terminus of type 3 IP\(_3\)R (IP\(_3\)R3) and the C terminus of Trp3. Overexpression of short peptide fragments containing these binding sites altered the activity of endogenous store-operated Ca\(^{2+}\) influx in HEK293 cells (14). While the association with IP\(_3\)Rs has also been shown for Trp1 and Trp6 by coimmunoprecipitation (14, 23, 24), it remains to be determined whether direct interaction with IP\(_3\)Rs is common for all Trp proteins. In this study, we examined murine Trp4 (mTrp4) for interaction with the first...
and the stronger Trp3-binding domain of IP$_3$R3 (F2q; Glu$^{669}$–Asp$^{698}$) (14). In addition, we examined the interaction between Trp4 and calmodulin (CaM), which has been shown to bind to the C termini of Drosophila Trp (25) and TrpL (26, 27) and has been implicated to cause inactivation of TrpL (28). We report here the presence of two CaM-binding and at least two IP$_3$-binding sites at the C terminus of Trp4. The first CaM-binding site overlaps closely with one of the IP$_3$-binding site. Common binding sites for CaM and IP$_3$Rs also exist in other Trp proteins. In functional studies, we show that currents are activated in inside-out membrane patches excised from Trp4-expressing HEK293 cells by calmodiazidom (CMZ), an antigen of CaM, and by a peptide representing one of the Trp-binding domains of IP$_3$R3.

**MATERIALS AND METHODS**

**DNA Constructs**—Fragments of IP$_3$Rs and TrpS were generated by polymerase chain reaction (PCR). All sense primers contain an NcoI recognition site at the 5’ with ATG in frame with the codon for the first amino acid. The antisense primers start with an A nucleotide followed by the antisense codon for the last amino acid. PCR products were subcloned into pCRII (Invitrogen), and nucleotide compositions were confirmed by DNA sequencing. Glutathione S-transferase (GST) fusion constructs were made by subcloning NcoI/EcoRI fragments into a modified pGEX4T-1 vector (Amersham Pharmacia Biotech), in which an NcoI site overlaps closely with one of the IP$_3$R binding site. By design, the insert in each fusion protein starts with a Met and ends with a “TAA” stop codon (Tfollowed by inserts, of which the cDNAs were subcloned into pCRII vectors). Complementary DNA for enhanced blue fluorescence fusion protein starts with a Met and ends with a “TAA” stop codon (Tfollowed by inserts, of which the cDNAs were subcloned into 3′-endMBP fusion proteins contain the first 322 residues of MBP existing restriction sites on mouse trp4 at the 5′- and XbaI at the 3′-end. MBP fusion proteins contain the first 322 residues of MBP followed by inserts, of which the cDNAs were subcloned into NcoI/EcoRI sites.

**In Vitro Binding Experiments**—Preparation of GST fusion proteins; 3$5$-Labeled CaM, EBFP, and MBP fusion proteins; and procedures for pulling experiments are as described (30). For interaction with CaM, CaM-Sepharose (Amersham Pharmacia Biotech) was used. The binding buffer used for IP$_3$R-Trp interaction contains 100 mM KCl, 2 mM MgCl$_2$, 0.5% Lubrol, and 20 mM Tris-HCl, pH 7.5. The buffer used for CaM-Trp interaction generally contains 120 mM KCl, 0.5% Lubrol, 20 mM Tris-HCl, 10 mM EGTA, 9.96 mM CaCl$_2$, pH was adjusted to 7.5. The estimated dissociation constant for MBP fusion is 10$^{-4}$ M, but because EGTA is a poor buffer for Ca$^{2+}$, the micromolar range, the actual free Ca$^{2+}$ concentration of this buffer is about 70 μM as determined by spectrophotometric measurements using Furaz2FF (TEP Laboratories, Austin, TX) as a low affinity Ca$^{2+}$ indicator and HEK2-buffered solutions as standards.

For the determination of Ca$^{2+}$-dependence of Trp binding to CaM, HEK2A or nitrotriocetic acid instead of EGTA was used, and CaCl$_2$ was added according to the MaxChelator program (C. Patton, Stanford University) to give rise to desired free Ca$^{2+}$ concentrations. 3$5$-Labeled MBP fusion proteins containing the CaM-binding sites were incubated with CaM-Sepharose at room temperature for 30 min in varying free Ca$^{2+}$ concentrations. Each sample was washed twice with the same binding buffer that was used for the incubation; thus, the free Ca$^{2+}$ concentration was kept unchanged. Bound proteins were subjected to SDS-polyacrylamide gel electrophoresis. The radioactivity of $[^{35}S]$MBP fusion proteins retained was quantified by phosphorimaging analysis. The percentage of maximal increase over the value obtained in 10 mM EGTA with no added Ca$^{2+}$ was fitted with the Hill equation \[ H(x) = \frac{C(A + (K_i/n))}{1 + (K_i/n)}, \] where \( H(x) \) represents the relative binding, \( C \) is the Ca$^{2+}$ concentration, \( n \) is the Hill coefficient, and \( K_i \), is the Ca$^{2+}$ concentration that gives rise to half-maximal binding.

For competition studies, recombinant human CaM was prepared from bacterial lysate using phenyl-Sepharose (Sigma) as described (31). Peptide F2v (EYLSEYSEEEVWLTTWD) was synthesized by Research Genetics. CaM or peptide F2v, in the desired final concentrations, was included in the binding and washing buffers containing 50 or 70 μM free Ca$^{2+}$.

**Affinity Measurement for Trp-CaM Interaction**—All fluorescence measurements were performed on a PerkinElmer Life Sciences LS55 Spectrophotometer at 22 °C. Peptides for CaM-binding sites of Trp-1–7 were synthesized by the University of Toronto, Canada. Peptide for the second CaM-binding site of mTrp4 was synthesized by the University of Toronto, Canada. Phosphodiesterase activity was assayed by monitoring the hydrolysis of fluorescent 2′-methylthymidinyl cGMP (8 μM) in 1 ml of solution containing 200 mM MOPS, pH 7.0, 90 mM KCl, 3 mM MgCl$_2$, 2 mM EGTA, 100 μM free Ca$^{2+}$, 25 mM CaM, and the desired amount of peptide. Samples were excited at 330 nm, and emission at 450 nm was measured. The dissociation constant (Kd) with Ca$^{2+}$/CaM was calculated from the activation curves of phosphodiesterase by CaM in the absence and the presence of the peptide as described (32).

**Cell Lines and Electrophysiology**—HEK293 cells stably expressing mTrp4 (T4-1 and T4-60 cells) and culture conditions were as described (33). Cells were seeded in 35-mm dishes 2 days prior to patch clamp recordings. Conditions for recording from inside-out patches were essentially as described (34). The pipette solution contained 140 mM Na-Hepes, 5 mM NaCl, and 2 mM CaCl$_2$, pH 7.5. Inside-out patches were excised from the Trp4 cells into a Ca$^{2+}$-free intracellular solution containing 140 mM potassium gluconate, 5 mM NaCl, 1 mM MgCl$_2$, 5 mM EGTA, 10 mM Hepes, pH 7.5. CMZ, CaM, and peptide F2v were diluted to the final concentration in the intracellular solution containing no Ca$^{2+}$ (for F2v) or 18 μM free Ca$^{2+}$ (for CMZ) and were applied to the cytoplasmatic side of excised patches through perfusion. A T3C8 bound containing continuous alternating steps of 40 and –40 mV, each for 1 s, from holding at 0 mV was applied throughout the experiments. Currents were recorded at sampling frequency of 5 kHz and filtered at 1 kHz. Currents recorded at –40 mV from 100 episodes under each condition were averaged using pCLAMP 8 (Axon Instruments) after digital filtering at 500 Hz and base-line adjustment.

**RESULTS**

The C Terminus of Trp4 Binds to IP$_3$R3 and CaM—A previous study showed that a GST fusion protein containing the F2q fragment of human IP$_3$R3 (Glu$^{669}$–Asp$^{698}$) interacted with the C terminus of hTrp3 (14). The Trp3 peptides showed to interact with IP$_3$R3 were T3C7 (Met$^{632}$–Glu$^{680}$) and T3C8 (Gln$^{777}$–Asp$^{797}$). T3C8 bound to IP$_3$R3 more weakly than T3C7 and thus represents a partial IP$_3$R-binding domain of Trp3. Because the sequence homology at the regions that align to T3C7 is relatively low (13, 15, and 17% identical between Trp3 and Trp1, Trp2, and Trp4, respectively), it was difficult to predict whether the IP$_3$R-binding domain is conserved among all Trp homologues. Therefore, we tested the binding of GST-IP$_3$R3F2q to mTrp4. 3$5$-Labeled MBP and EBFP fusion proteins containing the N and C terminus of mTrp4, respectively, were synthesized by Waterloo Peptide Synthesis (University of Waterloo, Ontario, Canada). Peptide for the second CaM-binding site of mTrp4 was synthesized by the University of Toronto, Canada.

**Determination of CaM and IP$_3$R Binding Sites on Trp Channels**

For the determination of Ca$^{2+}$-dependence of Trp binding to CaM, HEK2A or nitrotriocetic acid instead of EGTA was used, and CaCl$_2$ was added according to the MaxChelator program (C. Patton, Stanford University) to give rise to desired free Ca$^{2+}$ concentrations. 3$5$-Labeled MBP fusion proteins containing the CaM-binding sites were incubated with CaM-Sepharose at room temperature for 30 min in varying free Ca$^{2+}$ concentrations. Each sample was washed twice with the same binding buffer that was used for the incubation; thus, the free Ca$^{2+}$ concentration was kept unchanged. Bound proteins were subjected to SDS-polyacrylamide gel electrophoresis. The radioactivity of $[^{35}S]$MBP fusion proteins retained was quantified by phosphorimaging analysis. The percentage of maximal increase over the value obtained in 10 mM EGTA with no added Ca$^{2+}$ was fitted with the Hill equation \[ H(x) = \frac{C(A + (K_i/n))}{1 + (K_i/n)}, \] where \( H(x) \) represents the relative binding, \( C \) is the Ca$^{2+}$ concentration, \( n \) is the Hill coefficient, and \( K_i \), is the Ca$^{2+}$ concentration that gives rise to half-maximal binding.

For competition studies, recombinant human CaM was prepared from bacterial lysate using phenyl-Sepharose (Sigma) as described (31). Peptide F2v (EYLSEYSEEEVWLTTWD) was synthesized by Research Genetics. CaM or peptide F2v, in the desired final concentrations, was included in the binding and washing buffers containing 50 or 70 μM free Ca$^{2+}$.
struct that contains mostly the sequence equivalent to T3C7 but not T4Cf (Cc) interacted with both IP₃R3F2q and Ca²⁺/CaM. These results indicate that just as T3C7, a similar region at the C terminus of Trp4 also binds to IP₃R3. In addition, there is at least one additional IP₃R-binding site more C-terminal to the previously defined site. Moreover, both IP₃R-binding regions bind to Ca²⁺/CaM.

The First CaM-binding Site of Trp4 Closely Overlaps with an IP₃R-binding Site—To determine how close the first CaM-binding site of Trp4 is to the site that binds to IP₃R3F2q, we produced MBP- and EBFP-fusion proteins containing smaller fragments of T4Cc and tested their binding to GST-IP₃R3F2q and CaM. A picture of a Coomassie Blue-stained gel displayed below the second panel shows the amount of GST and GST fusion protein used. C and D, additional binding results showing that two C-terminal regions of mTrp4a (CT1 and CT2) (C) and Trp4Cc (D) bind to both IP₃R3F2q and Ca²⁺/CaM.

FIG. 1. Localization of IP₃R-binding domain and CaM-binding sites at the C terminus of mTrp4a. A, diagram of mTrp4a and its fragments included in the EBFP or MBP fusion proteins. Numbers in parentheses indicate the positions of the fragments. Thick and thin lines denote that the binding to CaM is positive and negative, respectively. Shaded boxes in the full-length mTrp4a indicate the locations of transmembrane (TM) segments. B, representative binding results showing the autoradiograms of input 35S-labeled EBFP-Trp4 C-terminal (CT), MBP-Trp4 N-terminal (NT) fusion proteins, EBF (E), and MBP (M) and those retained by GST, GST-IP₃R3F2q, and CaM. A picture of a Coomassie Blue-stained gel displayed below the second panel shows the amount of GST and GST fusion protein used. C and D, additional binding results showing that two C-terminal regions of mTrp4a (CT1 and CT2) (C) and Trp4Cc (D) bind to both IP₃R3F2q and Ca²⁺/CaM.

The Second CaM-binding Site Is Present in Trp4a but Not Trp4b—The minimal domains that bind to CaM and IP₃R3F2q at the CT2 region were sought in a similar manner (Fig. 3). However, it appears that the CT2 IP₃R-binding domain is not confined to a short sequence like the CIRB domain. When CT2 was divided into CT2a and CT2b, both retained weak interaction with IP₃R3F2q (Fig. 3B). Further analyses suggested that the region from Gly751 to Ser864 retains significant binding to IP₃R3F2q (Fig. 3C). Interestingly, molecular cloning and immunoblot analysis have revealed the presence of two major isoforms of Trp4, Trp4a and Trp4b (33). The β form lacks the 84 amino acids corresponding to Gly⁷⁵¹–Ser⁸⁶⁴ of the α form. Thus, CT2β binds very weakly to IP₃R3F2q (Fig. 3B).

In contrast to IP₃R3F2q, CaM binds to a short, confined sequence in CT2, CT2p (Arg⁷⁸⁷–Asn⁸¹², Fig. 3, C and D). Removing two residues from its N terminus (CT2r) nearly abolished the binding, whereas removing two residues from its C terminus (CT2q) reduced the binding by 45%, as determined by phoshorimaging analysis from two independent experiments. Although CT2p is contained within the CT2 IP₃R3F2q-binding region, it does not bind to the IP₃R fragment. Therefore, the binding sites for CaM and IP₃R3F2q in the CT2 region do not overlap as closely as the CIRB sequence. Because the CaM binding site is not present in Trp4B, CT2β does not bind to Ca²⁺/CaM (Fig. 3B).

CIRB Domain Is Conserved among Trp Proteins—As shown in Fig. 4, regions equivalent to T4Cf from hTrp1, mTrp2, hTrp3, mTrp5, mTrp6, mTrp7, and Drosophila Trp and TrpL also interacted with both GST-IP₃R3F2q and Ca²⁺/CaM. For TrpL, the CIRB domain is in addition to the two CaM-binding sites (710–725, 854–875) identified previously (26, 27). For Drosophila Trp, the CIRB domain is within a previously reported CaM-binding region (685–976) (28) for Trp3, the CIRB domain is the only CaM-binding domain, because deletion of this site eliminated the binding and the regulation of Trp3 activity by Ca²⁺/CaM (34). This may also be the case for Trp6 and Trp7, since they are very similar to Trp3. Trp1 terminates soon after the CIRB sequence and therefore is unlikely to have a second binding site at the C terminus. The C-terminal sequence of mTrp2 (residues 944–1072) and of mTrp5 (residues 762–975) interacted with both Ca²⁺/CaM and GST-IP₃R3F2q
mTrp4

Trp1–7 and the second CaM-binding site of Trp4, we determined the affinities to range from 10 nM for mTrp2 to 290 nM for mTrp6 (Table I). The second CaM-binding site of Trp4 has lower affinity (600 nM) than the CIRB domains of all Trp proteins. The apparent affinities for Ca$^{2+}$, as measured by in vitro binding assays using $[^{35}S]$MBP-Trp fusion proteins and CaM-Sepharose, differed greatly from 1.0 μM for the second CaM-binding site of Trp4 to 44.2 μM for the CIRB domain of Trp5 (Table I). The Hill coefficients of Ca$^{2+}$ dependence for the CaM-Trp interactions were about 1.5–3.5.

All IP$_3$Rs Interact with Trp3—Just as the CIRB domain is conserved in all Trp homologues, the F2q Trp-binding site is also conserved in the three known mammalian IP$_3$Rs. Fig. 5A shows that regions homologous to IP$_3$R3F2q from IP$_3$R1 and IP$_3$R2 also interacted with the C terminus of Trp3. The alignment of the three sequences indicates that only the C-terminal halves are conserved. Further experimentation using smaller segments of IP$_3$R3F2q fused to GST showed that the C-terminal half of F2q (represented by F2v) is the minimal domain of IP$_3$R3 that interacts with Trp3 (Fig. 5B).

Competition between CaM and IP$_3$R3 for Binding to Trps—In vitro binding assays, we have shown that for T3C7, the interaction with IP$_3$R3F2q was inhibited by purified recombinant human CaM and the binding to CaM was blocked by a synthetic peptide, composed of the sequence of F2v (34). Peptide F2v appears to be a potent inhibitor for CaM binding to Trp3, because the competition was observed in a binding buffer that contained Ca$^{2+}$, a condition under which CaM has a high affinity for Trp3. In order to learn the relative abilities of CaM to compete with the IP$_3$R for binding to different Trps, we tested the competition between CaM and IP$_3$R3F2q for binding to the CIRB domains of other mammalian Trp homologues. The addition of CaM reduced the binding of $[^{35}S]$-labeled MBP-T4C to the CIRB domains of Trp1, -2, -5, -6, and -7 to various degrees (Fig. 6A). The effect of CaM was Ca$^{2+}$ dependent, since the binding of CaM was not observed in a Ca$^{2+}$-free binding buffer (Fig. 6B). These results indicate that at high Ca$^{2+}$ concentrations, CaM competes with IP$_3$Rs for binding to the CIRB domains of all Trp proteins. However, the extent of inhibition varies from Trp to Trp, which is probably related to the differences in their affinities to CaM. Although the CT2 IP$_3$R-binding site does not overlap as closely to the CIRB domains of all Trp proteins, the extent of inhibition by CaM was not observed (Fig. 6C).

Peptide F2v blocked the interaction between CaM and the CIRB domain of Trp1–7 with IC$_{50}$ values ranging from 1.7 to 90 μM (Fig. 6D). The peptide is more effective in inhibiting the interaction between CaM and Trp3, -6, and -7 than between CaM and Trp1, -2, -4, and -5. The effect of the peptide is specific because the binding of CaM to Trp4CT2k (residues 781–814), which does not interact with IP$_3$R3F2q (not shown), was not inhibited (Fig. 6D).

Peptide F2v and a CaM Antagonist Activate Trp4 in Excised Inside-out Patches—CaM bound to the CIRB domain has a general inhibitory function on Trp3, because in inside-out patches excised from HEK293 cells expressing Trp3, removing or inactivating CaM led to a large increase in Trp3 activity (34). In the absence of Ca$^{2+}$ and at 5 μM, peptide F2v activated Trp3 by competing with CaM for binding to the CIRB domain. The activated channel was blocked by CaM (34). Consistent with the finding that F2v was 10 times less effective in competing

**Fig. 3. Determination of binding sites for Ca$^{2+}$/CaM and IP$_3$R3F2q on Trp4CT2a.** A, diagrams of mTrp4a and mTrp4b and compositions of MBP fusion proteins containing subfragments of Trp4CT2. Positions in respect to the full-length mTrp4a are shown in parentheses. CT2β contains the CT2 fragment of mTrp4a and lacks the 84-amino acid region (shown as a dashed line in MBP-CT2β) and black box in mTrp4a. Thick and thin lines denote that the binding to CaM is positive and negative, respectively. B–D show binding results from representative experiments. Like Trp4CT2p, Trp4CT2h–o did not bind to GST-IP$_3$R3F2q (not shown).

**Fig. 4. The common CaM/IP$_3$R binding site is present in all Trp proteins.** A, amino acid compositions of mammalian Trp1–7, Drosophila Trp (DmTrp), and TrpL (DmTrpL) present in the MBP fusion proteins. Positions for these sequences in the full-length proteins are shown in parentheses. $[^{35}S]$-Labeled MBP fusion proteins were tested for binding to GST-IP$_3$R3F2q and to Ca$^{2+}$/CaM as described under “Materials and Methods.” Representative binding results are shown in B. (not shown), suggesting that the second binding sites for the two modulators are conserved in Trp2, Trp4a, and Trp5, despite the fact that the homology among the three Trps is very low at these regions.

Using synthetic peptides representing the CIRB domains of Trp1–7 and the second CaM-binding site of Trp4, we determined the affinities to range from 10 nM for mTrp2 to 290 nM for mTrp6 (Table I). The second CaM-binding site of Trp4 has lower affinity (600 nM) than the CIRB domains of all Trp proteins. The apparent affinities for Ca$^{2+}$, as measured by in vitro binding assays using $[^{35}S]$MBP-Trp fusion proteins and CaM-Sepharose, differed greatly from 1.0 μM for the second CaM-binding site of Trp4 to 44.2 μM for the CIRB domain of Trp5 (Table I). The Hill coefficients of Ca$^{2+}$ dependence for the CaM-Trp interactions were about 1.5–3.5.

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with CaM for binding to Trp4 than to Trp3 (Fig. 6D), we found that in inside-out patches, F2v was also less potent in activating Trp4 than Trp3 (Fig. 7A). At 5 \( \mu \text{M} \), F2v only caused a small increase in Trp4 activity, which is significantly different (\( p < 0.05 \)) from the basal activity in one Trp4 cell line (T4-1) but not in the other one (T4-60). When the concentration of F2v was increased to 50 \( \mu \text{M} \), both cell lines showed significant increase in activity (\( p < 0.01 \)), which is more than 6 times higher than that stimulated by 5 \( \mu \text{M} \) F2v. Additionally, patches excised from Trp4 cells were activated by 1 and 10 \( \mu \text{M} \) CMZ (Fig. 7B). Under the same conditions, untransfected HEK293 cells did not show any significant increase in activity after treatment with peptide F2v or CMZ (Fig. 7, A and B).

**DISCUSSION**

The mechanism of activation for SOCs remains mysterious. Three major hypotheses have been proposed. The first assumes that a small diffusible soluble factor capable of stimulating SOCs appears in the cytosol upon store depletion. Although it has been shown that acid extracts from activated Jurkat cells and platelets or a Ca\(^{2+}\) store-depleted mutant yeast strain contain such a factor, which when applied to naive cells could stimulate Ca\(^{2+}\) influx (35, 36) or cation conductance (37), the identity of the Ca\(^{2+}\) influx factor has not been determined.

The second hypothesis claims that a secretion-like process involving the insertion of channel-containing vesicles into the plasma membrane is required for activating SOCs (38). In agreement with this is the finding that actin redistribution affected capacitative Ca\(^{2+}\) entry (39). The third hypothesis is called conformational coupling and is modeled after the well known mechanism of excitation-contraction coupling between the L-type Ca\(^{2+}\) channel and the ryanodine receptor in skeletal muscle (40). In this case, IP\(_3\)Rs are thought to serve not only as channels for Ca\(^{2+}\) release but also as sensors for store depletion. The signal of store depletion is sent to the plasma membrane Ca\(^{2+}\) entry channels via a direct protein-protein interaction (2, 41). Consistent with this hypothesis, it has been demonstrated that IP\(_3\) activates cation channels on the plasma membranes of endothelial cells, macrophages and A431 epithelial cells (42–44) and that the activity of IP\(_3\)R1 purified from rat cerebellum is modulated by luminal Ca\(^{2+}\) (45).

Despite the controversy about whether or not Trp proteins form SOCs, accumulating evidence has indicated that IP\(_3\) and IP\(_3\)Rs are involved in the activation of SOCs as well as Trp3 (12–14, 46). Also, just like the native SOCs, Trp3 activation was prevented by actin filament condensation induced by cytochalasin A, a phosphatase inhibitor (46). Therefore, the conformational coupling mechanism, and perhaps the secretion-like coupling mechanism as well, are applicable to Trp-based channels. Using molecular and biochemical approaches, Boulay et al. (14) identified the interacting domains of Trp3 and IP\(_3\)R3 and showed that they are involved in the regulation of native SOCs. In the current study, we further demonstrate that the interactions between Trp and IP\(_3\)R are common for all Trp proteins and for all IP\(_3\)Rs. Thus, conformational coupling involving direct physical interaction with activated IP\(_3\)Rs may be a common mechanism for the activation of Trp-based channels. Moreover, we show that the IP\(_3\)-binding domains of the Trp proteins also bind to Ca\(^{2+}\)/CaM and that the binding by CaM inhibits the association between Trp and IP\(_3\)Rs. Therefore, the competition between CaM and IP\(_3\)Rs for binding to a common site may play a key role in controlling the gating of Trp-based channels. Based on the functional study of Trp3 using inside-out membrane patches and a non-Ca\(^{2+}\)-binding CaM mutant, we concluded that at rest, CaM is tethered to the channel and prevents it from being spontaneously active (34). Maneuvers that displaced CaM from Trp3 strongly activated the channel (34). Here, we show that Trp4 is also strongly activated by inactivating CaM with CMZ, indicating that CaM probably plays the same inhibitory role in all Trp-based channels. Consistent with this, CMZ also activates current in patches excised from cells expressing Trp1 or Trp6 (not shown). Thus, binding to CaM is essential to prevent the spontaneous activity of Trp channels.

Analysis of genomic sequences revealed that for hTrp1, hTrp4, hTrp5, Drosophila Trp, and TrpL, the coding sequence for the N-terminal end of the CIRD domain coincides with the
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**FIG. 6. Competition between CaM and IP₃R for binding to the Trp CIRB domains.** A, CaM inhibits Trp4 binding to IP₃R. Varying concentrations of CaM were included in the binding reactions for 35S-labeled MBP-T4C₄ and GST-IP₃R-F2q. The binding buffer contained 70 μM free Ca²⁺. After washing, bound [35S]MBP-T4C₄ was separated by SDS-polyacrylamide gel electrophoresis. The left panel shows an autoradiogram from a representative experiment, while the right panel shows averages of results of phosphorimaging analysis of the relative amount of [35S]MBP-T4C₄ retained from three experiments. The curve is the least-square fit of the equation, y = 1/(1 + [P]/IC₅₀), where y is relative binding, [P] is CaM concentration, and IC₅₀ = 2.1 μM is the concentration that causes 50% inhibition. B, representative experiments show that 20 μM CaM inhibits the binding of IP₃R-F2q to the CIRB domain of Trp1, -2, -5, and -7 in a Ca²⁺-dependent manner. 10 mM HEDTA and EGTA were used to buffer Ca²⁺ to 50 μM and 0 (≤10 mM), respectively. C, binding of Trp4CT2C to IP₃R-F2q was inhibited by 20 μM CaM. IC₅₀ was 50 μM. D, peptide F2v inhibits binding of CaM to the CIRB domain of Trp1–7. Varying concentrations of peptide F2v were included in the binding reaction for 35S-labeled CaM and GST fusion proteins containing the CIRB sequence of Trp1–7 or the second CaM-binding site of Trp4 (CT2C). Positions of peptide F2v were included in the GST fusion proteins as indicated in parentheses. The binding buffer contained 70 μM free Ca²⁺. After washing, bound [35S]CaM was separated by SDS-polyacrylamide gel electrophoresis and revealed by autoradiography. IC₅₀ values were determined from results of phosphorimaging analysis of the relative amount of [35S]CaM retained by each Trp fragment from two or three experiments. The percentage of inhibitions for different concentrations of F2v were fitted with the equation as in A, except that [P] is the concentration of the peptide.

**FIG. 7. Peptide F2v and CMZ stimulate channel activity in inside-out patches excised from Trp4.** Inside-out patches were excised from untransfected HEK293 cells (control) or two stable cell lines expressing mTrp4a (T4–60 and T4–1) to a bath solution that contained either no Ca²⁺ (A) or 18 μM Ca²⁺ (B) as described under “Materials and Methods.” Peptide F2v at 5 or 50 μM (A) and CMZ at 1 or 10 μM (B) were applied to the cytoplasmic side of the membrane by perfusion. Bar graphs show averages ± S.E. of the mean current (sampled from periods of 400 s) at −40 mV from the numbers of patches indicated in parentheses. Representative traces for control and T4–60 cells at basal level and 50 μM F2v (A) or 10 μM CMZ (B) are shown on the right. Dashed lines indicate closed level. *, p < 0.01; **, p < 0.05 different from basal level by Student’s t test.

* M. X. Zhu, unpublished observation.
region affect the function of Trp4a and how they differ from
bindings to the more upstream CIRB domain.

Diversity also arises from the differences in the affinities of
CIRB domains of different Trp homologues for CaM and for
IP$_3$Rs. The affinities for CaM differ by as much as 29-fold
between the CIRB domains of Trp2 and Trp6 (Table I). Because
peptide F2v is more effective in displacing CaM from Trp3 and
the closely related Trp6 and Trp7 than from the CIRB domain
of other Trps, the affinities of the CIRB domains for the
Trp-binding site of IP$_3$R3 may also be very different. In the context
of conformational coupling, one important step accomplished
by IP$_3$Rs is to displace the inhibitory CaM from the CIRB
domain. This may be accomplished mainly by the binding of the
F2v domain of IP$_3$R3 and homologous regions of IP$_3$R1 and
IP$_3$R2. The effectiveness of the F2v may be dependent on local
Ca$^{2+}$ concentrations, since Trp binding to CaM is greatly
enhanced by Ca$^{2+}$. In the binding assay shown in Fig. 6D, 70 µM
free Ca$^{2+}$ was used to facilitate the detection of CaM binding.

Under resting conditions when the cytosolic Ca$^{2+}$
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Identification of Common Binding Sites for Calmodulin and Inositol 1,4,5-Trisphosphate Receptors on the Carboxyl Termini of Trp Channels
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