Calmodulin and Calcium Interplay in the Modulation of TRPC5 Channel Activity

IDENTIFICATION OF A NOVEL C-TERMINALDOMAIN FOR CALCIUM/CALMODULIN-MEDIATED FACILITATION*

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TRPC5 forms Ca^{2+}-permeable nonselective cation channels important for neurite outgrowth and growth cone morphology of hippocampal neurons. Here we studied the activation of mouse TRPC5 expressed in Chinese hamster ovary and human embryonic kidney 293 cells by agonist stimulation of several receptors that couple to the phosphoinositide signaling cascade and the role of calmodulin (CaM) on the activation. We showed that exogenous application of 10 μM CaM through patch pipette accelerated the agonist-induced channel activation by 2.8-fold, with the time constant for half-activation reduced from 4.25 ± 0.4 to 1.56 ± 0.85 min. We identified a novel CaM-binding site located at the C terminus of TRPC5, 95 amino acids downstream from the previously determined common CaM/IP3R-binding (CIRB) domain for all TRPC proteins. Deletion of the novel CaM-binding site attenuated the acceleration in channel activation induced by CaM. However, disruption of the CIRB domain from TRPC5 rendered the channel irreversible to agonist stimulation without affecting the cell surface expression of the channel protein. Furthermore, we showed that high (>5 μM) intracellular free Ca^{2+} inhibited the current density without affecting the time course of TRPC5 activation by receptor agonists. These results demonstrated that intracellular Ca^{2+} has dual and opposite effects on the activation of TRPC5. The novel CaM-binding site is important for the Ca^{2+}/CaM-mediated facilitation, whereas the CIRB domain is critical for the overall response of receptor-induced TRPC5 channel activation.

In 1994, we demonstrated (1, 2) that the transient receptor potential (Trp) gene and its homologue, Trp-like (Trpl), from Drosophila melanogaster encoded calcium-permeable cationic channels activated either by store depletion or by stimulation of G_{q}\text{1,-}-coupled receptors. These initial findings prompted the search for mammalian homologues, leading to the identification of seven TRP genes with different degrees of sequence similarity to the original insect Trp gene (3). These genes are now designated TRP-Canonical or TRPC, symbolizing their close similarity to the original Drosophila Trp. Many recently discovered cation channels are found to share some limited homology with the TRPCs. These include TRPVs (similar to the vanilloid receptor), TRPMs (named after the first identified member, melastatin), and TRPs (named after PRD2 for polycystic kidney disease), etc. Together, there are at least 28 non-allelic TRP genes in the mammalian genome. The TRP channels serve diverse functions in many tissues from somatosensory to cardiovascular systems (4).

TRPC5 is a member of the TRP family of Ca^{2+}-permeable nonselective cationic channels. It has drawn attention recently because of its role in modulating hippocampal growth cone motility and neurite elongation in the mammalian brain (5). The TRPC5 channel activity is induced upon stimulation of the phosphoinositide signaling cascade by receptors that stimulate phospholipase C; however, the exact mechanism of channel activation remains controversial (6). The activation of TRPC5 is dependent on the presence of Ca^{2+} at both the extracellular and the intracellular sides of the plasma membrane (7–10). Although the extracellular effect of Ca^{2+} has been shown to be mediated by the acidic residues, Gh543, Gh595, and Gh598, located at the putative pore loop of the TRPC5 protein (7), the mechanism for the intracellular Ca^{2+} dependence of TRPC5 remains to be elucidated.

Calmodulin (CaM) is a common intracellular mediator of many Ca^{2+}-dependent regulations. All TRPC proteins possess a C-terminal CaM-binding domain that also interacts with an N-terminal sequence of the inositol 1,4,5-trisphosphate receptor (IP_{3}R) (6). We have demonstrated that IP_{3}R and CaM compete with each other for binding to the common CaM/IP_{3}R-binding (CIRB) site of the TRPC. In functional studies, the TRPC-binding region of the IP_{3}R activated and Ca^{2+}/CaM inhibited the activation of TRPC3 and TRPC4. Moreover, TRPC channels were activated by removing or inactivating CaM from binding; H1R, histamine receptor type 1; HEK, human embryonic kidney; IP_{3}R, inositol 1,4,5-trisphosphate receptor; MD, malate-binding protein; mTRPC5, mouse canonical transient receptor potential 5; TRPL, TRP-like; W7, N-aminohexyl-5-chloro-1-naphthalenesulfonamide; TFP, trifluoperazine dimaleate; RT, reverse transcription; HEDTA, N-(2-hydroxyethyl)ethylenediaminetetraacetic acid.
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Excised inside-out membrane patches, indicating that displacement of the inhibitory CaM from the common CIRB site is sufficient for the activation of TRPC channels (11). Consistent with this, we have shown that CaM increased the delay between the release of Ca$^{2+}$ from internal storage compartments and the activation of Ca$^{2+}$ influx via endogenous TRPC1 channels in Chinese hamster ovary (CHO) cells, which were subjected to the regulation by IP$_x$R and CaM in a similar fashion as the exogenously expressed TRPC3 and TRPC4 in human embryonic kidney (HEK) 293 cells (12). Additional C-terminal CaM-binding domains outside of the CIRB sites have been found on TRPC proteins (6, 13). Unlike the CIRB sites, these sites are not conserved among all TRPC channels, and they do not bind to IP$_x$Rs. The second CaM-binding (CBII) site of TRPC1 has been shown to be involved in the slow Ca$^{2+}$-induced channel inactivation (13).

In the present study we have identified the CBII site from mouse TRPC5 (mTRPC5). We have explored the specific roles of the CIRB and CBII sites in the modulation of channel activity after activation of receptors that stimulate the phosphoinositide signaling cascade by selectively disrupting each CaM-binding site from mTRPC5 and heterologous expression of the wild type and mutant channels in CHO and HEK293 cells. We show for the first time that intracellular application of CaM accelerated the activation of mTRPC5 by receptor agonists. Although mutations in the CIRB site rendered the channel inactive, deletion of the CBII site attenuated the Ca$^{2+}$-induced acceleration of receptor-evoked mTRPC5 activation.

**EXPERIMENTAL PROCEDURES**

**Reagents and Solutions—**All salts were of analytical grade purchased from Sigma. GTP and ATP were also obtained from Sigma. Bovine brain calmodulin, bradykinin, human thombin, the CaM inhibitors N-amoinehoxy-5-chloro-1-naphthalensulfonamide (WL) and trifluoperazine dimaleate (TFP), and histamine were purchased from Calbiochem. The pipette (intracellular) solution contained (mM) the following: 130 NaCl, 5 KCl, 1 CaCl$_2$, 1.7 Mg$_2$SO$_4$, 5 glucose, 10 HEPES, pH 7.2. The extracellular (bath) solution contained (mM) the following: 120 NaCl, 5 KCl, 1 CaCl$_2$, 1.7 Mg$_2$SO$_4$, 5 glucose, 10 HEPES, pH 7.2.

**DNA Constructs and Mutagenesis—**Complementary DNA for the open reading frame of mTRPC5 was subcloned in pIRESneo (Clontech, Mountain View, CA) vector. Mutageneses were performed using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Mutations were confirmed by DNA sequencing. Constructs for maltose binding protein (MBP) fusion proteins that contained different fragments of mTRPC5 were made in the pGAG3 vector as described previously (14). cDNA for the guinea pig histamine type 1 receptor (H1R) in pcDNA3 was kindly provided by Dr. Michael Schaefer (Charite ´-Universitatsmedizin Berlin) and that for the human type 2 bradykinin receptor (Bk2R) was a generous gift from Dr. William W. Schilling (Case Western Reserve University).

**Analysis of the Expression of mTRPC5 mRNA by RT-PCR—**Total RNA was extracted from wild type cells and cells transfected with mTRPC5 and the mTRPC5 mutants using the Trizol reagent (Invitrogen). Approximately 500 ng of total RNA was used as the template for RT-PCR, which was carried out using the OneStep system with Superscript II (Invitrogen) following the manufacturer’s protocols. The reaction was performed on a TC-512 thermal cycler (TECHNE, Cambridge, UK) using 30 cycles of the following PCR protocol: 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. The primers used for mTRPC5 were 5′-CTATGAGACGACGTTGATG (forward) and 5′-CCTAGGGGATCGTTGATG (reverse), which amplify a 236-bp-long product. The 5′-GACATCAAGAAGCTGGTGAAGC (forward) and 5′-TACTCGTGAGGCGGATGTA (reverse) were used for glyceraldehyde-3-phosphate dehydrogenase, the primers used for mTRPC5 were 5′-CTGACGAGGCGGGTTGATG (forward) and 5′-TACTCGTTGGAGGCGGATGTA (reverse), which amplify a 236-bp-long product. The PCR products were sequenced to identify the products, run on a 2% agarose gel, and stained with ethidium bromide. The gel was analyzed with a Typhoon 8600 Imager (Amer sham Biosciences).

**Western Blotting—**Whole-cell lysate and streptavidin precipitated portion represents 12 and 355 μg, respectively, of total proteins in cell lysates. Identical exposure time was used to reveal the chemiluminescent signals for the mTRPC5 proteins in crude lysates and streptavidin-precipitated samples.

**Fluorescence-based Membrane Potential Measurements—**HEK293 cells were transfected with mTRPC5 and H1R in wells of a 96-well plate as described previously (16). One day after the transfection, cells were washed once with Hanks’ balanced salt solution and then incubated for 30 min with 80 μl of FLIPR membrane potential dye (Molecular Devices, Sunnyvale, CA) diluted in the Hanks’ solution. Changes in membrane potential were measured at 32 °C using a fluid handling and integrated fluorescence plate reader, FlexStation (Molecular Devices). Histamine was diluted in Hanks’ solution at 300 μM, and 40 μl were delivered to the sample plate by the integrated robotic 8-channel pipettor at 20 s after readings began. Samples were excited at 530 nm, and emission of 565 nm was collected from the bottom of the plate at 0.67 Hz.

**Cell Surface Biotinylation Assay—**Transfection, biotinylation, and streptavidin precipitation were performed as described previously (15). Immunoblotting was performed using anti-mTRPC5 antibodies (Alomone Labs, Jerusalem, Israel). Sample loading for the crude cell lysate and streptavidin precipitated portion represents 12 and 355 μg, respectively, of total proteins in cell lysates. Identical exposure time was used to reveal the chemiluminescent signals for the mTRPC5 proteins in crude lysates and streptavidin-precipitated samples.

**Whole-cell Measurements of mTRPC5 Currents—**The whole-cell configuration of the patch clamp technique was utilized to study mTRPC5-mediated currents as described previously (12). Briefly, cells were plated on glass coverslips and mounted on the stage of an inverted microscope (Nikon Instruments, Japan). The amplifier used was the Axopatch 200A (Axon Instruments, Union City, CA). Pipette resistance was 10–12 meghoms when tested with pipette and bath solutions. Whole-cell resistances were in the range of 1–2 gigohms, and cell capacitance ranged from 10 to 12 picofarads for CHO and 12 to 16 picofarads for HEK293 cells. Cells were held at the holding potential of ~80 mV and repetitively stepped to ~120 through ~60 mV for 500 ms each in a 20-mV increment once every second. Receptor agonists were applied through bath perfusion, and CaM was introduced intracellularly by dialysis through the patch pipette.

**Data Analysis and Curve Fitting—**All data were plotted and fitted using Sigmaplot 8 (SYSTAT, Point Richmond, CA). To obtain activation time constants, the mean outward current was plotted over time for the duration of the experiments under the different experimental conditions indicated in each figure legend. Data were fitted to sigmoidal Equation 1 as follows:

$$ f = a/(1 + \exp(-(x - x_0)/b)) $$

where $a$ indicates the maximum outward current value (top asymptote); $b$ indicates the minimum outward current value (bottom asymptote); $x_0$ indicates time constant for half-activation, and $x$ indicates the time explored for each data point.

**RESULTS**

**Time Course of the Activation of mTRPC5 by Agonists—**Agonist stimulation of CHO cells stably expressing the wild
type mTRPC5 channel results in a time-dependent activation of outwardly rectifying currents (Fig. 1). We followed current activation in the presence of agonists for 15 min (only the first 8 min are shown). The current reached a steady-state level after 7 min in the continuous presence of the agonists. After this period of time, 30% of the cells showed a small (about 5%) reduction of current amplitude. This phenomenon was not further studied in the present work.

![Figure 1](image)

**Fig. 1.** Activation time course of the wild type mTRPC5 channels in response to agonists. A, family of currents evoked by the voltage protocol illustrated in the inset (“Experimental Procedures”). Each letter (a–d) corresponds to the time position shown in B. B, activation time course of mTRPC5 currents in response to 1 unit of thrombin. The panel shows the current-voltage relationships as lines. The gray bar shows the range of currents obtained from untransfected (control) CHO cells. Time 0 corresponds to the time of thrombin application. The inset shows results of RT-PCR experiments with CHO cells illustrating that only cells transfected with mTRPC5 show a PCR product of 220 bp. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as control of the RT-PCR conditions and was amplified in transfected and untransfected cells. Molecular weight markers are indicated as MW.

To confirm the expression of mTRPC5, we performed RT-PCR experiments with total RNA obtained from control cells (wild type CHO cells) and cells expressing mTRPC5. The inset in Fig. 1B shows that that only mTRPC5-transfected cells contained the mTRPC5 transcript.

The time course of mTRPC5 current activation was independent of the agonist and receptor utilized. Fig. 1 only illustrates the response to thrombin. We have compared the responses to thrombin and bradykinin (Bk) at concentrations that gave the maximal response (1 unit and 100 nM, respectively) in CHO cells stably expressing mTRPC5, and we obtained similar time constant values for half-activation of the outward current of 4.25 ± 0.4 and 4.01 ± 0.7 min, respectively.

**Calmodulin Accelerates the Time Course of the Activation of mTRPC5**—In order to examine how CaM affects the activation of mTRPC5, we introduced CaM into mTRPC5-expressing cells by including the Ca2+-binding protein in the patch pipette. As illustrated in Fig. 2A, addition of CaM (10 µM) to the pipette resulted in a faster activation of mTRPC5 currents. Plotting the activation time courses for the outward currents in the absence (Fig. 2B, gray circles) and presence (black circles) of the exogenously applied CaM yielded activation curves that can be fitted with the sigmoidal equation as described under “Experimental Procedures.” The time constant for half-activation was
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Identification of Two CaM-binding Sites at the C Terminus of mTRPC5—The above results suggest that CaM plays an important role in facilitating the receptor-induced activation of mTRPC5 channels. We have reported previously (14) the presence of a conserved common CIRB site at the near C-terminal regions of all TRPC proteins. For mTRPC5, the CIRB site lies in between Glu²⁰¹ and Lys²¹⁸ and requires higher Ca²⁺ concentrations (apparent \( K_{1/2} = 44.2 \mu M \)) for binding to CaM than the CIRB sites of other TRPC proteins. We also reported that the rest of the mTRPC5 C terminus downstream from the CIRB site (Gly⁷⁰²–Leu⁷⁰⁵, clone J1170 in Fig. 3A) bound to CaM as well. To narrow down the CBI site of mTRPC5, we have tested a large number of smaller fragments generated from J1170 by using an in vitro pull-down assay. As shown in Fig. 3, the minimal binding site for CaM is confined to a stretch of 27 residues, Pro⁶²⁸–Asn⁶⁵⁴, 95 amino acids downstream from the CIRB site. The isolated mTRPC5 CBI fragment bound to CaM in a Ca²⁺-dependent manner (Fig. 3D) with an apparent \( K_{1/2} \) for Ca²⁺ of 3.1 ± 0.2 \( \mu M \) (n = 3). This value is lower than the previously determined \( K_{1/2} \) value (44.2 \( \mu M \)) of Ca²⁺ for CaM binding to the mTRPC5 CIRB site but within the range of those values (1.6–12.9 \( \mu M \)) for the CIRB sites of other TRPC isoforms (14).

Mutations on CaM-binding SitesAlter mTRPC5 Channel Activation—To investigate the function for each of the CaM-binding sites of mTRPC5, we destroyed the CIRB and CBI sites by substituting Arg⁷¹⁸, Lys⁷²², and Arg⁷²³ with alanines (CIRBm1) and deleting Pro⁶²⁸–Asn⁶⁵⁴ (ΔCBI), respectively (Fig. 4A). The loss of CaM binding in these mutants was confirmed by in vitro binding studies (Fig. 4B). Receptor-mediated activation of mTRPC5 currents was studied after coexpression of the full-length clones of the mutated mTRPC5 with Bk2R or by stimulation of the endogenous thrombin receptor in CHO. As shown in Fig. 4C, without CaM in the pipette, the activation of mTRPC5ΔCBI was slightly delayed as compared with the wild type mTRPC5. Inclusion of 10 \( \mu M \) CaM in the pipette only weakly accelerated the activation of the mutant channel, indicating that CBI is important for the Ca²⁺/CaM-mediated facilitation of mTRPC5 activation. After fitting the outward current, the time constants for mTRPC5ΔCBI of 6.04 ± 0.5 (control) and 5.48 ± 0.7 min (with 10 \( \mu M \) CaM) were obtained.

In contrast, the mTRPC5-CIRBm1 mutant failed to respond to the stimulation by thrombin and Bk in both CHO and HEK293 cells. In a fluorescence-based membrane potential assay using the FLIPR membrane potential dye, coexpression of mTRPC5 with H1R in HEK293 cells resulted in a histamine-evoked membrane depolarization (Fig. 4D). Although cells expressing mTRPC5ΔCBI showed a slightly reduced depolarization as compared with those expressing the wild type mTRPC5, cells expressing mTRPC5-CIRBm1 failed to show any difference from those expressing H1R alone. This result confirms that mTRPC5-CIRBm1 is a loss-of-function mutant.

In order to determine whether mTRPC5-CIRBm1 is sufficiently expressed on the plasma membrane, we performed surface biotinylation assays for mTRPC5 and its mutants transiently transfected in the HEK293 cells. Two days after

**Table I**

| Condition | CHO   | HEK293 |
|-----------|-------|--------|
| Thrombin – CaM | 4.25 ± 0.4 | NT² |
| Bradykinin – CaM | 4.01 ± 0.7 | 4.29 ± 0.81 |
| Thrombin + CaM | 1.56 ± 0.85 | NT |
| Bradykinin + CaM | 1.75 ± 0.62 | 1.47 ± 0.74 |

² NT indicates not tested.
null
These time constants were not statistically different.

At 4 °C all vesicle transport and plasma membrane translocation of vesicles were inhibited, and therefore if the slow activation induced by agonists was the result of more mTRPC5 reaching the plasma membrane, it was expected to observe differences in time constants between 4 and 27 °C. Furthermore, if CaM via the CBII domain was affecting the translocation of mTRPC5 to the plasma membrane, it would be expected to observe differences in time constants between 4 and 27 °C. As illustrated in Fig. 6B, this was not the case.

To explore further any possible role of channel translocation to the plasma membrane induced by agonist stimulation, we performed biotinylation assays with mTRPC5-expressing cells under resting conditions and after 1 min Bk. The outward current was measured by a voltage pulse to +60 mV as described under "Experimental Procedures." D, histamine-induced membrane depolarization in HEK293 cells that coexpressed H1R and mTRPC5 or its mutant. 100 μM histamine was added as indicated. Fluorescence values shown are 1/100,000 of those displayed by the instrument. E, biotinylation experiments illustrating that all mutants reached the plasma membrane similarly to wild type mTRPC5. Actin was used as loading control on each lane. kD illustrates the molecular mass markers (in kilodaltons).

discussion

Ca²⁺ / CaM-mediated Facilitation of mTRPC5 Activation by Receptor Agonists—Stimulation of many cell surface receptors triggers intracellular Ca²⁺ signaling through activation of phospholipase C and breakdown of phosphoinositides. Members of the TRPC family of Ca²⁺-permeable nonselective cation channels have emerged as important players for the receptor-induced Ca²⁺ signaling because they are activated downstream from phospholipase C activation and mediate Ca²⁺ entry into the cells. Exactly which step(s) or component(s) of the phosphoinositide signaling cascade is involved in gating the TRPC channels remains to be elucidated. In the present study, we have expressed mTRPC5 in two cell lines (CHO and HEK293), and we studied kinetics and the magnitudes of its activation after agonist stimulation of two different receptors (thrombin receptor and Bk2R). For both receptors, the activation of the mTRPC5 channel was slow, requiring ~7 min to reach steady-state values in the continuous presence of the agonists. The two different receptor agonists induced channel activity with similar time constants (Table I), indicating that the slow activation kinetics is a property of the mTRPC5 channels independent of receptor and cell types.

Most interestingly, rat TRPC5 expressed in HEK293 cells showed a slow initial activation phase that was clearly distinguishable from the later fast activation in response to the simulation of coexpressed H1R (18). In our study, there was no clear transition from the slow to fast activation, and the rate of activation for mTRPC5 was much slower than that for the rat
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The activation of TRPC5 causes Na+ and Ca2+ influxes, which in turn lead to membrane depolarization and intracellular Ca2+ increase, respectively. In unclamped cells, the positive feedback mechanism should be more important in the initial phase of channel activation before membrane depolarization may be related to the amount of protein expressed/transported to the membrane, due to clonal differences.

These data suggest that the CBII site has a modulatory role in controlling the rate of channel recruitment following the activation of G-protein-coupled receptors. This positive feedback modulation by Ca2+ through CaM is a novel mechanism for TRPC channels and may have important physiological implications. The rate of mTRPC5 activation is thus controlled by how fast and how high the Ca2+ concentration near the cytoplasmic side of the channel can increase. This explains why the activation of TRPC5 is heavily influenced by the Ca2+-buffering capacity of intracellular solutions (7, 9).

Biotinylation and electrophysiological experiments carried out at 4 °C strongly suggest that the effects of CaM via CBII do not involve significant changes in mTRPC5 channel translocation to the plasma membrane. Although we observed a rapid increment in biotinylated mTRPC5 in response to Bk, no significant differences were observed at the time periods when we observed the acceleration of mTRPC5 current activation in response to agonists.

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Figure 5. Dual regulation of mTRPC5 by Ca2+ and CaM. A, mean ± S.D. of current-voltage relationships obtained from CHO cells expressing mTRPC5. Closed circles represent currents before the addition of thrombin, and open circles indicate those 10 min after addition of 1 unit of thrombin for the same cells with 0.4 μM Ca2+ in the pipette. B, mean ± S.D. of current-voltage relationships obtained from CHO cells expressing mTRPC5 with 5 μM in the patch pipette (n = 20). C, mean ± S.D. of maximal current amplitudes at +60 mV (positive bars) and −120 mV (negative bars) from wild type mTRPC5 (black) and mTRPC5/CBII (gray) with different Ca2+ concentrations in the presence (+) or absence (−) of 1 unit of thrombin and CaM. The CaM inhibitors W7 and TFP were tested at 500 nM and 5 μM, respectively. Statistics obtained from at least 20 independent cell measurements. Dotted line shows the zero current level. Positive current represents outward current and negative inward current. D, time constants of half-activation induced by 1 unit of thrombin for the wild type and TRPC5/CBII with pipettes containing 0.4 or 5 μM free Ca2+ with (black) or without (white) 10 μM CaM. *, p < 0.05 by Student’s t test.
becomes predominant, in which case the reduced driving force for Ca\(^{2+}\) influx would prevent further intracellular Ca\(^{2+}\) elevation through TRPC5. However, membrane depolarization in excitable cells would lead to the activation of voltage-gated Ca\(^{2+}\) channels, causing further increases in intracellular Ca\(^{2+}\).

Because TRPC5 activation is also inhibited by high concentrations of intracellular Ca\(^{2+}\), the Ca\(^{2+}\) increases mediated by the voltage-gated channels could cause either further potentiation or inhibition of the TRPC5 channel depending on the relative density of each channel type and the level of receptor activation and CaM present. The relative activity of TRPC5 would again affect the voltage-gated channels through membrane potential regulation. Such an intricate interplay among receptors, TRPC5, and voltage-gated Ca\(^{2+}\) channels provides a mechanism to fine tune Ca\(^{2+}\) signal in the growth cones of hippocampal neurons, in which both TRPC5 and voltage-gated Ca\(^{2+}\) channels are abundantly expressed (5).

**Ca\(^{2+}\)**-mediated Inhibition of TRPC5 Activation—The regulation of TRPC5 by Ca\(^{2+}\) is further complicated by the finding that at constantly high intracellular Ca\(^{2+}\) concentrations, the receptor-induced activation of TRPC5 is inhibited. Different from the Ca\(^{2+}\)/(CaM-mediated facilitation discussed above, the Ca\(^{2+}\)-mediated inhibition did not affect the rate of channel activation but rather the maximal current amplitudes. Introduction of exogenous CaM did not overcome the inhibitory effect of high Ca\(^{2+}\) on the current amplitudes but still facilitated the rate of channel activation by receptor agonist. Furthermore, the mTRPC5ΔCBII mutant was inhibited to a similar degree by high Ca\(^{2+}\) as the wild type channel, indicating that the CBII site is not involved in the Ca\(^{2+}\)-mediated inhibition. Therefore, different sites are involved in the Ca\(^{2+}\)/CaM-mediated facilitation and Ca\(^{2+}\)-mediated inhibition of the receptor-induced activation of TRPC5 channels. Given that the CIRB site is important for the Ca\(^{2+}\)-dependent inhibition of TRPC3 activation by IP\(_{3}\)Rs (11), it is possible that the TRPC5 CIRB site is responsible for channel inhibition by high concentrations of intracellular Ca\(^{2+}\). This is supported by the finding that the Ca\(^{2+}\) affinity for CaM binding to the CIRB site is more than 10 times lower than to the CBII site of TRPC5. Thus, much higher Ca\(^{2+}\) concentrations are needed for CaM to affect the CIRB than the CBII site. Unfortunately, the role of the CIRB site on this effect could not be determined because mutations introduced at this domain rendered the channel inactive.

Dual regulations by Ca\(^{2+}\) have been shown for the Drosophila TRPL channel (19, 20), which was originally identified in a CaM binding assay (21). Low intracellular Ca\(^{2+}\) enhanced the activation of the TRPL channel with an EC\(_{50}\) value of 0.45 \(\mu\)M (20), whereas higher (micromolar) intracellular Ca\(^{2+}\) inhibited the channel activity (20, 22). Whether or not CaM is involved in the Ca\(^{2+}\)-mediated regulation of TRPL has not been completely resolved, although at least two CaM-binding sites (23), in addition to the CIRB domain (14), are present at its C terminus. Thus, for TRPC5 whether or not the Ca\(^{2+}\)-mediated inhibition on current density is mediated by CaM and the critical site(s) involved in this regulation warrant further investigation.

**The Critical Role of the CIRB Site in the Receptor-induced Activation of TRPC5**—We have also found that the common
CIRB site is essential for the function of TRPC5 channels. Mutations at the CIRB site rendered the mTRPC5 channel inactive. However, the biotinylation experiments showed that a similar amount of the mTRPC5-CIRB mutants reached the plasma membrane as the wild type mTRPC5, ruling out the possibility that the lack of function was because of a defect in channel protein synthesis, stability, or trafficking and translocation. Thus it appears that an intact CIRB site is required for the activation of mTRPC5 channels via receptor agonists. This is in contrast to the finding that deletion of the CIRB site impaired the plasma membrane translocation of TRPC3 (24), arguing against the claim that trafficking-related defect is the cause for the lack of TRPC5 channel activity resulting from the disruption of CaM and/or IP3R binding at the CIRB domain.

It has been shown previously (25) that activation of IP3R is required for the opening of the mTRPC5 channels. The results presented here are in agreement with this early finding, while highlighting the key role of the CIRB domain during agonist-induced channel activation. Because this domain is conserved in all TRPC members, it is important to determine to what extent a functional CIRB domain, with perhaps a separable binding capability to IP3R and CaM, is required for channel activation in the TRPC family.

In summary, the current study demonstrates that CaM binding to a C-terminal 95 residues downstream from the previously identified CIRB domain is critical for the Ca2+/CaM-mediated facilitation of receptor-induced activation of TRPC5 channels. It shows that in addition to facilitating the rate of channel opening, intracellular Ca2+ also blocks the receptor-induced channel activation at high concentrations. This effect appears to be independent of CaM, as indicated by the use of two potent CaM antagonists. Moreover, it provides a further support for a critical role of the CIRB site on the activation of TRPC5 channels by receptor agonists. Together, these results reveal a complex modulation of TRPC5 channel function, which provides cells with sophisticated means to exquisitely control the rate of cell membrane depolarization and Ca2+ influx in response to agonist stimulation of cell surface receptors. The role of TRPC5 in modulating hippocampal growth cone motility and neurite elongation has been established recently (5). Most interestingly, it is well known that CaM is also important during neuron growth and regeneration (26). Future experiments using mTRPC5ΔCBIII or similar mutant channels could provide insightful information about the role of CaM and TRPC5 in controlling neurite elongation in the mammalian brain.

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