Regulation of the Transient Receptor Potential Channel TRPM2 by the Ca\(^{2+}\) Sensor Calmodulin*

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TRPM2, a member of the transient receptor potential (TRP) superfamily, is a Ca\(^{2+}\)-permeable channel activated by oxidative stress or tumor necrosis factor \(\alpha\) involved in susceptibility to cell death. TRPM2 activation is dependent on the level of intracellular Ca\(^{2+}\). We explored whether calmodulin (CaM) is the Ca\(^{2+}\) sensor for TRPM2. HEK 293T cells were transfected with TRPM2 and wild type CaM or mutant CaM (CaM\(_{\text{MUT}}\)) with substitutions of all four EF hands. Treatment of cells expressing TRPM2 with \(H_2O_2\) or tumor necrosis factor \(\alpha\) resulted in a significant increase in intracellular calcium ([Ca\(^{2+}\)]. This was not affected by coexpression of CaM, suggesting that endogenous CaM levels are sufficient for maximal response. Cotransfection of CaM\(_{\text{MUT}}\) with TRPM2 dramatically inhibited the increase in [Ca\(^{2+}\)], demonstrating the requirement for CaM in TRPM2 activation. Immunoprecipitation confirmed direct interaction of CaM and CaM\(_{\text{MUT}}\) with TRPM2, and the Ca\(^{2+}\) dependence of this association. CaM bound strongly to the TRPM2 N terminus (amino acids 1–730), but weakly to the C terminus (amino acids 1060–1503). CaM binding to an IQ-like motif (amino acids 406–416) in the TRPM2 N terminus was demonstrated utilizing gel shift, immunoprecipitation, biotinylated CaM overlay, and pull-down assays. A substitution mutant of the IQ-like motif of TRPM2 (TRPM2-IQ\(_{\text{MUT}}\)) reduced but did not eliminate CaM binding to TRPM2, suggesting the presence of at least one other CaM binding site. The functional importance of the TRPM2 IQ-like motif was demonstrated by treatment of TRPM2-IQ\(_{\text{MUT}}\)-expressing cells with \(H_2O_2\). The increase in [Ca\(^{2+}\)], observed with wild type TRPM2 was absent and cell viability was preserved. These data demonstrate the requirement for CaM in TRPM2 activation. They suggest that Ca\(^{2+}\) entering through TRPM2 enhances interaction of CaM with TRPM2 at the IQ-like motif in the N terminus, providing crucial positive feedback for channel activation.

The transient receptor potential (TRP)\(^2\) channel superfamily is a diverse group of voltage-independent calcium-permeable channels expressed in mammalian cells and related to the archetypal Drosophila TRP (1–3). The TRP superfamily has been divided into seven subfamilies (TRPC, TRPV, TRPM, TRPA, TRPP, TRPML, and TRPN) including at least 28 genes, which are involved in many important physiological processes including vasoactivation, sensation, fertility, and cell proliferation (1, 3). Mammalian isoforms share several characteristics including six transmembrane segments, voltage independence, and a proposed tetrameric structure (3). The TRPM subfamily was named after the first described member, melastatin (TRPM1), a putative tumor suppressor protein (4, 5), and members of this subfamily have been shown to have important roles in cell proliferation and survival (4, 6–9). TRPM2, also called LTRPC-2, was the second member of the TRPM subfamily to be described (10–14), and is expressed in many cell types including brain, hematopoietic cells, and the gastrointestinal tract (10). Extracellular signals that activate TRPM2 include oxidant stress and TNF\(\alpha\) (14–16). TRPM2 activation mediates susceptibility to cell death in a number of cell types (11, 14, 17), and therefore elucidating the mechanism of TRPM2 activation is of critical importance in understanding how extracellular signals such as oxidative stress affects cell viability.

TRPM2 channels are permeable to calcium, sodium, and potassium, and are activated by the second messenger adenosine diphosphoribose (ADPR). ADPR activates TRPM2 by binding to the TRPM2 C-terminal NUDT9-H domain, which has homology with NUDT9 ADPR hydrolase (15, 16, 18–20). NAD can also activate TRPM2, but the predominance of evidence suggests that this is through conversion to ADPR (15, 20). In addition, cyclic adenosine diphosphoribose (cADPR) can gate the TRPM2 channel by itself at high concentrations and, at lower concentrations, potentiates the effects of ADPR (21). TRPM2 channel currents activated by ADPR have a strong requirement for Ca\(^{2+}\) at the intracellular surface of the membrane (22). Low level activation was seen at 100 nM [Ca\(^{2+}\)], and maximal activation at 600 nM. Whether the mechanism of this effect is because of a direct action of Ca\(^{2+}\) on the channel through interaction with an EF hand or via a Ca\(^{2+}\)-sensing protein such as calmodulin (CaM) is not known.

Calmodulin is a small ubiquitously conserved protein (16.7 kDa) that modulates calcium-dependent processes in all mammalian cell types (23, 24). CaM has four Ca\(^{2+}\) binding domains called EF hands, each of which bind a single Ca\(^{2+}\) ion with a high affinity. The conformation of Ca\(^{2+}\)-saturated CaM is different from the Ca\(^{2+}\)-free form, one factor responsible for different binding characteristics. I on channels are a recognized target of CaM (25), and CaM has been shown to be the Ca\(^{2+}\) sensor for activation or inactivation of different channels (26). TRPC channels have a common binding site for CaM and the inositol 1,4,5-trisphosphate receptor largely activates and Ca\(^{2+}\)/CaM inhibits channel activation (27, 28). Additional CaM binding sites have been found in the C terminus of TRPC proteins, and these either inhibit (29) or facilitate (30) channel activation. TRPV6 activity is positively regulated by CaM binding to TRPV6 transmembrane domains in a Ca\(^{2+}\)-dependent manner (31). For TRPM channels, regulation by CaM has not been described, although TRPM4b activation shows Ca\(^{2+}\) sensitivity similar to TRPM2 (32).

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Here, we demonstrate that CaM is the Ca\(^{2+}\) sensor responsible for Ca\(^{2+}\)-dependent activation of TRPM2. A Ca\(^{2+}\)-insensitive mutant of CaM with aspartic acid substitutions in all four Ca\(^{2+}\)-binding EF hand motifs inhibited cation influx through TRPM2, blocking the rise in [Ca\(^{2+}\)]\(_i\) observed in response to H\(_2\)O\(_2\); treatment. Immunoprecipitation demonstrated that CaM directly associates with TRPM2, and that this association increases in a Ca\(^{2+}\)-dependent manner. Furthermore, an IQ-like motif in the TRPM2 N terminus was identified that binds CaM and is of critical importance in TRPM2 activation and function. These data demonstrate Ca\(^{2+}\)-dependent CaM binding to the TRPM2 IQ-like motif and the requirement for this association for sustained Ca\(^{2+}\) influx and effects on cell viability mediated by oxidative stress.

**EXPERIMENTAL PROCEDURES**

**Cells Lines, cDNAs, and Transfection Methods**—HEK 293T cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. The following constructs of TRPM2 (11) were prepared: a construct consisting of aa 1–730 of the N terminus of TRPM2 (TRPM2-N); a construct consisting of aa 1060–1503 of the C terminus (TRPM2-C); and a mutant of the IQ-like motif of full-length TRPM2, changing the putative “IQ” sequence at aa 406–416 IQDIVRRRQLL to AADI-

**Experimental Methods**—The fluores-

**Measurement of [Ca\(^{2+}\)]\(_i\), with Digital Video Imaging**—The fluores-

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**Immunoblotting of Whole Cell Lysates**—For Western blotting, whole cell lysates were separated on either 8% polyacrylamide gels or on discontinuous polyacrylamide gradient gels with the lower third being 16%, the next third 12%, and the top third 8% polyacrylamide. This was followed by trans-

**Biotinylated Calmodulin Overlay**—HEK 293T cells were transfected with V5-TRPM2 or V5-TRPM2-IQMUT, V5-TRPM2-N, or V5-TRPM2-C, in pcDNA3.1/V5-His TOPO; CaM or CaMMUT in pcDNA3.1; or combinations of these vectors. Experiments performed with defined concentrations of Ca\(^{2+}\) and EGTA used lysis buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, and 1× protease inhibitor mixture (without EDTA) in the presence of 100 μM CaCl\(_2\) or 5 mM EGTA. Protein lysates were incubated with anti-V5 (Invitrogen), or anti-CaM antibodies for 2 h at room temperature. Protein G-Sepharose 4B Fast Flow beads (Sigma) were then added for 2 h at 4°C with mixing, and immunoprecipitates were washed three times. Sample buffer (3×) was added to the pellets and the samples were boiled. Western blotting was performed as described above and blots were probed with anti-V5 or anti-CaM antibodies, followed by the appropriate HRP-conjugated secondary antibodies and ECL. Quantitation of band intensity was performed with the Bio-Rad GS800 Calibrated Densitometer using Quantity One software.

**Gel Shift**—Three hundred picomoles of purified CaM (Calbiochem) were incubated with different molar ratios of IQ peptide (0–10) in a buffer containing 10 mM Na-HEPES, pH 7.2, and 2 mM CaCl\(_2\) or 5 mM EGTA for 30 min at room temperature. Bound complexes and unbound CaM were then resolved by nondenaturing PAGE on a 15% polyacrylamide gel, which was run at 4°C to minimize peptide unbinding. Bound and unbound CaM bands were visualized with Coomassie Blue staining. IQ peptides used in gel shift assays were synthesized by the Macromolecular Core Facility of the Pennsylvania State University College of Medicine. Densitometry of bands was performed with the Kodak IS440CF imaging system using Kodak One-dimensional Image Analysis software.

**Biotinylated Calmodulin Pull-down with Streptavidin Beads**—HEK 293T cells were transfected with V5-TRPM2 or V5-TRPM2-IQMUT, in pcDNA3.1/V5-His TOPO, lysates were prepared, and immunoprecipitation was performed with anti-V5 antibody. Immunoprecipitates were resolved on a 10% polyacrylamide gel and transferred to Hybond-C. Membranes were blocked with 1% bovine serum albumin in TBST buffer overnight at 4°C, and probed with 300 ng/ml biotinylated calmodulin (Calbiochem) in buffer containing 1 mM CaCl\(_2\) or 5 mM EGTA for 2 h. Membranes were then washed and incubated for 1 h with 1:5000 streptavidin-HRP (Pierce) at room temperature. Bound CaM was detected by ECL. Blots were stripped and reprobed with anti-V5-HRP as described above.

**Biotinylated CaM Pull-down with Streptavidin Beads**—HEK 293T cells were transfected with V5-TRPM2 or TRPM2-IQMUT, in pcDNA3.1/V5-His TOPO. Cell lysates were prepared, and 1 mg of protein was mixed with 5 μg of biotinylated calmodulin in the presence of 1 mM CaCl\(_2\) or 5 mM EGTA at 4°C for 3 h. Streptavidin-agarose beads (Calbiochem) in a 50% slurry were added, and mixing was continued at room temperature for 30 min. Samples were then spun and washed four times. The agarose bead pellets were boiled with 2× sample buffer, spun, and
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the supernatant loaded on a 10% polyacrylamide gel. Following transfer, blots were probed with anti-V5 and anti-CaM antibodies as described above, followed by ECL.

**Biotinylation of Cell Surface Proteins**—HEK 293T cells were transfected with V5-TRPM2, V5-TRPM2-IQMUT, empty pcDNA3.1/V5-His TOPO vector, BFP-TRPM2, BFP-TRPM2-IQMUT, or empty pQBI50 vector for 48 h. Cells were then washed three times with ice-cold PBS, and incubated for 30 min at 4 °C with 2 mg/ml Sulfo-NHS-LC-Biotin (Pierce) (38). The biotinylation reaction was terminated by washing cells three times with ice-cold PBS. Cells were lysed, and immunoprecipitation was performed with anti-V5 or anti-TRPM2-C antibodies. Western blotting was performed with immunoprecipitation pellets, and blots were probed with streptavidin-HRP, or anti-V5-HRP or anti-TRPM2-C followed by the HRP goat anti-rabbit antibody. ECL was used for detection of signal.

**Statistics**—All results are mean ± S.E. Statistical significance was determined by unpaired Student’s t test or by one-way analysis of variance.

**RESULTS**

*Mutant CaM Exerts a Dominant Negative Knock-out of Ca\(^{2+}\)-Dependent Activation of TRPM2*—The role of calmodulin as a regulatory subunit of TRPM2 was explored utilizing a system we established to quantitate [Ca\(^{2+}\)](i) in single cells (34). HEK 293T cells, which express low levels of endogenous TRPM2, were transfected with empty vector, wild type CaM, or CaMMUT in pTracer-CMV, with or without TRPM2 in pQBI50. CaMMUT was made insensitive to Ca\(^{2+}\) by substituting an aspartate from all four EF hands with alanine (26). Successfully transfected individual cells were identified by detection of GFP and BFP. [Ca\(^{2+}\)](i) was measured in transfected, Fura Red-loaded cells over 20 min after treatment with vehicle (PBS), 1 mM H\(_2\)O\(_2\), or 100 ng/ml TNF\(\alpha\). A much higher increase in [Ca\(^{2+}\)](i) was observed in cells transfected with TRPM2 following treatment with 1 mM H\(_2\)O\(_2\), or 100 ng/ml TNF\(\alpha\), compared with nontransfected cells or cells expressing empty pQBI50 vector (Fig. 1A, p < 0.0001), Coexpression of CaM in TRPM2-expressing cells did not significantly affect the increase in [Ca\(^{2+}\)](i), suggesting that CaM is not the Ca\(^{2+}\) sensor for TRPM2 or that endogenous CaM levels are sufficient for maximal effect. In contrast, coexpression of CaMMUT significantly inhibited the rate of rise and peak increase of [Ca\(^{2+}\)](i) in TRPM2-expressing cells in response to H\(_2\)O\(_2\), or TNF\(\alpha\) (p < 0.0001), demonstrating that CaM is the Ca\(^{2+}\) sensor for TRPM2 and suggesting that CaMMUT competes with endogenous CaM for binding to TRPM2. Western blotting was performed to establish that TRPM2 expression was not decreased in cells expressing TRPM2 and CaMMUT compared with cells expressing TRPM2 and CaM, which could explain differences in [Ca\(^{2+}\)](i) between these two groups after treatment. A representative experiment is shown on Fig. 1B. In three experiments, the mean densitometric measurement of the TRPM2 band in cells doubly transfected with TRPM2 and CaM was outer diameter \(\times\) mm = 0.75 ± 0.20, compared with outer diameter \(\times\) mm = 0.90 ± 0.10 in cells expressing TRPM2 and CaMMUT. The ratio of TRPM2 in cells expressing wild type CaM to TRPM2 in cells expressing CaMMUT was 0.69 ± 0.15, demonstrating that the amount of TRPM2 expressed in CaM-cotransfected cells was not greater than that expressed in CaMMUT-cotransfected cells. The higher molecular mass of TRPM2 in Fig. 1B (200 kDa) compared with that observed in other experiments (171 kDa) is secondary to linkage of TRPM2 to BFP. CaMMUT migrated faster than CaM, possibly because its mass is 176 daltons lighter than CaM as a result of substitution of aspartic acids with alanines in the mutant. A small amount of endogenous CaM was detected in longer exposures of cells transfected with TRPM2 alone (Fig. 1B).

![Figure 1. CaMMUT blocks the increase in [Ca\(^{2+}\)](i) in TRPM2-expressing cells. A, modification of [Ca\(^{2+}\)](i) in cells expressing TRPM2, CaM, and CaMMUT. HEK 293T cells were untransfected (NonTxD), or transfected with empty pQBI50 vector (BFP-V), TRPM2 in pQBI50, CaM, or CaMMUT in pTracer-CMV, or combinations of these vectors. At 48 h, single transfected cells were identified by GFP or BFP fluorescence with digital video imaging. [Ca\(^{2+}\)](i) was measured in Fura Red-loaded cells during 20 min of treatment with PBS, 1 mM H\(_2\)O\(_2\), or 100 ng/ml TNF\(\alpha\). The mean baseline [Ca\(^{2+}\)](i) in all groups was 38 ± 1 nM, with a range of 35 to 41 nM in individual groups. Nine experiments were performed and 19 to 46 individual cells were studied in each group. The mean ± S.E. % increase of [Ca\(^{2+}\)](i) above baseline is shown. ** indicates significant difference between specified groups (p < 0.01). B, Western blot (WB) of HEK 293T cells transfected with TRPM2 alone with BFP-tagged TRPM2, CaM, or CaMMUT. One hundred \(\mu\)g of protein was loaded in each lane. Western blotting was performed with anti-TRPM2-C or anti-CaM antibodies, followed by ECL. A representative result of three experiments is shown. C, immunoprecipitation of TRPM2 with CaM and CaMMUT. TRPM2, CaM, or CaMMUT were expressed individually or together in HEK 293T cells. Immunoprecipitation (IP) was performed on lysates prepared in buffer without calcium chelator, with anti-V5 or anti-CaM antibodies, followed by Western blotting. This experiment was performed four times with similar results.

To examine whether CaM directly interacts with TRPM2, immunoprecipitation experiments were performed on lysates from cells transfected with vectors expressing V5-TRPM2, CaM, CaMMUT, or combinations of these proteins, using anti-V5 or anti-CaM antibodies. These experiments were performed with lysis buffer to which no calcium chelators or additional CaCl\(_2\) was added. CaM and CaMMUT immunoprecipitated reciprocally with TRPM2 (Fig. 1C), demonstrating that TRPM2, and CaM or CaMMUT, directly interact. The apparently greater immunoprecipitation of CaMMUT than CaM with anti-V5
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To characterize the influence of CaM on the rise in [Ca\textsuperscript{2+}], stimulated by H\textsubscript{2}O\textsubscript{2} or TNF\textalpha through TRPM2, cation influx was examined in the presence of extracellular Mn\textsuperscript{2+} rather than Ca\textsuperscript{2+}. HEK 293T cells were transfected with empty pQBI50 vector, TRPM2 in pQBI50, CaM or CaMMUT in pTracer-CMV, or combinations of these vectors, and treated with 1 mM H\textsubscript{2}O\textsubscript{2} or 100 ng/ml TNF\textalpha. Influx of Mn\textsuperscript{2+} was monitored by the decrease in fluorescence near the Fura Red Ca\textsuperscript{2+}-insensitive isosbestic point (458 nm) (35, 40). The time course of changes in

antibody may be secondary to the greater affinity of the anti-CaM antibody for Ca\textsuperscript{2+}-free calmodulin than for Ca\textsuperscript{2+}-calmodulin (39).

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**FIGURE 2.** H\textsubscript{2}O\textsubscript{2} and TNF\textalpha modulate Mn\textsuperscript{2+} influx through TRPM2. HEK 293T cells transfected with empty pQBI50 (BFP-vector), or vectors expressing TRPM2, CaM, or CaMMUT were treated with 1 mM H\textsubscript{2}O\textsubscript{2} or 100 ng/ml TNF\textalpha. Cation influx was monitored by Mn\textsuperscript{2+} quenching of Fura Red fluorescence. The fluorescence intensity at 450 nm (F\textsubscript{450}) was measured in Fura Red-loaded cells with 0.68 mM Mn\textsubscript{Cl}\textsubscript{2} in the extracellular, nominally Ca\textsuperscript{2+}-free buffer. Fluorescence was measured at baseline and at 2.5 to 5-min intervals for 20 min after treatment. The mean ± S.E. of F\textsubscript{450} intensity during this time course is shown for cells transfected with BFP-vector (A, 21 cells, E, 15 cells), TRPM2 (B, 28 cells, F, 12 cells), TRPM2 with CaM (C, 30 cells, G, 29 cells), or TRPM2 with CaMMUT (D, 30 cells, H, 32 cells).

**FIGURE 3.** TRPM2 association with CaM is Ca\textsuperscript{2+}-dependent. TRPM2 or CaM were expressed individually or together in HEK 293T cells. Immunoprecipitation was performed with anti-V5 or anti-CaM antibodies in the presence of 100 \mu M Ca\textsubscript{Cl}\textsubscript{2} or 5 mM EGTA, followed by Western blotting (WB) with anti-V5 and anti-CaM antibodies. This experiment was performed five times with similar results.

$F_{450}$ intensity, indicating Mn\textsuperscript{2+} entry and quenching of Fura Red fluorescence, is shown in Fig. 2. The rate of cation influx was calculated by linear regression of the $F_{450}$ value at each time point. H\textsubscript{2}O\textsubscript{2} treatment of 293T cells expressing TRPM2 (Fig. 2B; $-2.22$ ± 0.19 units/min) resulted in Mn\textsuperscript{2+} influx significantly faster than that observed with vector alone (Fig. 2A; $-1.28$ ± 0.21 units/min; $p < 0.01$). After 20 min of exposure, the mean percentage Fura Red fluorescence remaining (fluorescence at 20 min ($F_{20}$)/baseline fluorescence ($F_{0}$) × 100%) was also significantly different: 75 ± 3% for cells transfected with vector alone (Fig. 2A) and 56 ± 3% for cells transfected with TRPM2 (Fig. 2B; $p < 0.0001$). To examine the ability of calmodulin to modulate Mn\textsuperscript{2+} influx, HEK 293T cells transfected with TRPM2 and CaM or CaMMUT were stimulated with H\textsubscript{2}O\textsubscript{2}. Cells expressing TRPM2 and CaMMUT demonstrated significantly slower Mn\textsuperscript{2+} quenching of Fura Red fluorescence ($-1.46$ ± 0.12 units/min; Fig. 2D) compared with cells expressing TRPM2 and CaM ($-3.06$ ± 0.17 units/min; Fig. 2C; $p < 0.0001$) or TRPM2 alone ($-2.22$ ± 0.19 units/min; Fig. 2B; $p < 0.002$). Mn\textsuperscript{2+} quenching of fluorescence in cells expressing TRPM2 and CaMMUT was not different in cells expressing pQBI50 empty vector. Similar results were obtained for TNF\textalpha. Cotransfection of cells with TRPM2 and CaMMUT demonstrated slower Mn\textsuperscript{2+} quenching of Fura Red ($-1.24$ ± 0.28 units/min; Fig. 2H) compared with cells transfected with TRPM2 alone ($-1.97$ ± 0.15 units/min; Fig. 2F; $p < 0.05$) or cells expressing TRPM2 and CaM ($-2.67$ ± 0.15 units/min; Fig. 2G; $p < 0.001$), but no difference from cells transfected with empty pQBI50 vector ($-1.41$ ± 0.17 units/min; Fig. 2E). Cells cotransfected with TRPM2 and CaM showed faster quenching than cells transfected with TRPM2 alone ($p < 0.01$). These results demonstrate that CaM affects the rate of Mn\textsuperscript{2+} influx through TRPM2. This contrasts with the rates of [Ca\textsuperscript{2+}], increase derived from the data in Fig. 1A, in which CaM expression did not affect the rate of [Ca\textsuperscript{2+}], increase (9.04 ± 0.55 versus 9.56 ± 0.42 nM/min). This may result from differences between Mn\textsuperscript{2+} and Ca\textsuperscript{2+} permeability. In addition, Fura Red quenching (Fig. 2) measures primarily Mn\textsuperscript{2+} influx. In contrast, the rate of [Ca\textsuperscript{2+}], increase is dependent not only on Ca\textsuperscript{2+} influx, but also on cytoplasmic and organellar Ca\textsuperscript{2+} buffering, potential Ca\textsuperscript{2+} release from intracellular stores, and Ca\textsuperscript{2+} efflux through the plasma membrane Ca\textsuperscript{2+}-ATPase. The important result derived from these experiments (Figs. 1 and 2) is that mutant calmodulin significantly inhibited the rate of Ca\textsuperscript{2+} and Mn\textsuperscript{2+} entry and reduced the peak concentrations achieved.

CaM Binding to TRPM2—To characterize the Ca\textsuperscript{2+} dependence of CaM binding to TRPM2, immunoprecipitation with anti-V5 and anti-CaM antibodies was performed with lysates of HEK 293T cells transfected with vectors expressing V5-TRPM2, CaM, or both. Immunoprecipitation was performed in the presence of 100 \mu M Ca\textsubscript{Cl}\textsubscript{2} or 5 mM EGTA. Anti-V5 antibody immunoprecipitated CaM in the presence of V5-TRPM2 (Fig. 3), and in reciprocal experiments, anti-CaM antibody
immunoprecipitated V5-TRPM2 in the presence of CaM. This association was much stronger in the presence of Ca²⁺/H₁₁₀₀₁ than with EGTA (Fig. 3). These experiments, performed five times, confirm that TRPM2 and CaM directly associate. They demonstrate both Ca²⁺/H₁₁₀₀₁-dependent and Ca²⁺/H₁₁₀₀₁-independent binding of CaM to TRPM2.

The above results suggest that CaM plays an important role in facilitating activation of TRPM2 channels through direct association. To begin to identify sites of interaction, we prepared constructs consisting of the TRPM2 N-terminus (TRPM2-N, aa 1–730, 80 kDa) or C terminus (TRPM2-C, aa 1060–1503, 49 kDa) in pcDNA3.1/V5-His TOPO, shown in Fig. 4A. Western blotting performed with anti-V5 antibody on lysates of HEK 293T cells transfected with vectors expressing V5-TRPM2, V5-TRPM2-N, or V5-TRPM2-C. Western blots of lysates were probed with anti-V5 antibody. Immunoprecipitation (IP) of TRPM2-N and TRPM2-C with CaM. Lysates from HEK 293T cells transfected with V5-TRPM2-N or V5-TRPM2-C and CaM were immunoprecipitated with anti-V5 or anti-CaM antibodies in the presence of 100 μM CaCl₂ or 5 mM EGTA. Western blotting was performed with anti-V5 or anti-CaM antibodies. Representative results of three experiments are shown.

**Figure 4. CaM interacts with the N terminus of TRPM2.**

A, diagram of TRPM2. The N- and C-terminal fragments (dark lines), the IQ-like motif (residues 406–416), the NUDT9 ADPR hydrolase domain (residues 1236–1503), and the six transmembrane domains (S1–S6) are shown. B, Western blot (WB) demonstrating specific expression of TRPM2-N and TRPM2-C. HEK 293T cells were transfected to express V5-TRPM2, V5-TRPM2-N, or V5-TRPM2-C. Western blots of lysates were probed with anti-V5 antibody. C, immunoprecipitation (IP) of TRPM2-N and TRPM2-C with CaM. Lysates from HEK 293T cells transfected with V5-TRPM2-N or V5-TRPM2-C and CaM were immunoprecipitated with anti-V5 or anti-CaM antibodies in the presence of 100 μM CaCl₂ or 5 mM EGTA. Western blotting was performed with anti-V5 or anti-CaM antibodies. Representative results of three experiments are shown.

CaM Binds to an "IQ-like Motif" in the TRPM2 N Terminus—A number of specific calmodulin binding domains have been identified on target proteins, and one of these is the "IQ motif," corresponding to an IQXXXRGXXX consensus sequence (23). TRPM2 has a putative IQ-like sequence at aa 406–416 (IQDIVRRRQLL, Fig. 4A). This IQ-like domain was confirmed with four 21-amino acid peptides (Fig. 5): the IQ-like motif of the L-type calcium channel α₁C subunit, known to bind CaM, selected as a positive control (26); the N-terminal sequence surrounding the putative TRPM2 IQ-like motif; a mutant of the TRPM2 IQ-like motif in which the IQ domain was changed to AADIVAAAAQLA (TRPM2 IQMUT1); and a mutant of the TRPM2 IQ-like motif in which the IQ domain was changed to AADIVRRRQLL (TRPM2 IQMUT2). In the later two peptides, amino acids were deleted that have been shown to be critical for CaM binding (41). Gel shift assays demonstrated Ca²⁺/H₁₁₀₀₁-dependent binding of CaM to the IQ-like domain of TRPM2, which was abolished or significantly reduced with mutant peptides (Fig. 5). A representative Coomassie Blue-stained gel for each peptide, showing its ability to complex with CaM at different molar ratios, is shown on the left hand side of Fig. 5. Densitometry analysis of the CaM band with increasing concentrations of each peptide in three independent exper-
Immunoblotting is shown on the right hand side. These studies demonstrate that mutation of two amino acids in the TRPM2 IQ-like motif is sufficient to abolish significant CaM binding to this site. Three experiments showed similar results. A shift in the CaM band was observed with the L-type channel IQ peptide and the TRPM2 IQ peptide, but not the TRPM2 IQMUT1 or IQMUT2 peptides, in the presence of EGTA at high peptide ratios. Of note, because these gel shift assays were performed on non-denaturing gels, the smear observed at high peptide concentrations with EGTA may result from multiple conformations or charge densities of the CaM complex. The specificity of this interaction is uncertain.

**FIGURE 5.** Gel shift analysis of CaM binding to a 21-amino acid peptide corresponding to the TRPM2 IQ-like motif. The upper figure shows alignments of IQ-like regions from the L-type calcium channel (a1c residues 1647–1667), TRPM2 (residues 399 – 419), and substitution mutants of the TRPM2 IQ-like motif (IQMUT1, IQMUT2). The canonical IQ motif is shown at the top (26), where X corresponds to any amino acid. Gel shift assays were performed by reacting purified CaM and varying molar ratios (0.25:10) of IQ-like peptides from L-type calcium channels, TRPM2, TRPM2-IQMUT1, and TRPM2-IQMUT2 in the presence of 2 mM CaCl2 or 5 mM EGTA. The reaction mixture was resolved by 12% nondenaturing PAGE. Representative Coomassie Blue-stained gels are shown on the left. The lower band corresponds to free CaM, whereas the higher band corresponds to a CaM-peptide complex. On the right, the intensity of the Coomassie-stained CaM band in the presence of increasing peptide was determined by densitometry and normalized to the intensity of the CaM band in the absence of peptide. The mean ± S.E. % of control is shown, and was calculated from three independent experiments for each peptide at each [peptide]/[CaM] ratio. * indicates a significant decrease in the density of the CaM band compared with the density of the band in the absence of peptide (p < 0.05).
Although high concentrations of peptide may cause a problem with peptide solubility, this is unlikely to be the case here because all samples were loaded into the gel equally.

To further examine the role of the IQ-like motif in the association of CaM with TRPM2, we prepared a mutant of full-length TRPM2 cDNA (TRPM2-IQMUT) with the six amino acid substitutions in the IQ-like motif used in gel shift assays (AADIVAAQLA, Fig. S5), and subcloned it into pcDNA3.1/V5-His TOPO. HEK 293T cells were cotransfected with vectors expressing V5-TRPM2 or V5-TRPM2-IQMUT and CaM. Immunoprecipitation of lysates was performed with anti-V5 or anti-CaM antibodies in the presence of 100 μM CaCl₂ or 5 mM EGTA. Western blots of immunoprecipitates probed with anti-V5 or anti-CaM antibodies confirmed that CaM interacted with TRPM2 and TRPM2-IQMUT in a Ca²⁺-dependent manner (Fig. 6A). The Ca²⁺-dependent association of CaM with TRPM2 was significantly stronger than with TRPM2-IQMUT. This experiment was repeated three times, and bands were analyzed by densitometry. In three experiments, following immunoprecipitation of V5-TRPM2, the amount of CaM that immunoprecipitated with TRPM2-IQMUT was 62 ± 8% (p < 0.01) of the amount that precipitated with TRPM2 in the presence of calcium and 98 ± 35% of that which precipitated in the presence of EGTA. Nearly equivalent immunoprecipitation of TRPM2 and TRPM2-IQMUT with anti-V5 was demonstrated by reprobing blots with anti-V5 HRP. Following immunoprecipitation of CaM, the amount of V5-TRPM2-IQMUT that precipitated was 53 ± 16% (p < 0.05) of the amount of V5-TRPM2 that precipitated with CaM in the presence of calcium and 89 ± 20% in the presence of EGTA. Binding of CaM was also assessed in immunoprecipitates of V5-TRPM2 or TRPM2-IQMUT by using the biotinylated CaM overlay. Western blotting demonstrated that biotinylated CaM interacted with ~171-kDa TRPM2, and that this interaction was Ca²⁺-dependent (Fig. 6B). Results were analyzed by densitometry and confirmed that the interaction of wild type TRPM2 with CaM was stronger than that of TRPM2-IQMUT. The amount of biotinylated CaM that bound to TRPM2-IQMUT was 64% of the amount that bound to TRPM2 in the presence of CaCl₂ and 100% of the amount that bound in the presence of EGTA. Nearly equivalent expression of TRPM2 and TRPM2-IQMUT was demonstrated by reprobing blots with anti-V5 HRP. This experiment was performed twice, with similar results. To examine the interaction of TRPM2 and TRPM2-IQMUT with CaM from another approach, V5-TRPM2 or V5-TRPM2-IQMUT were mixed with biotinylated CaM in the presence of 100 μM CaCl₂ or 5 mM EGTA. Biotinylated CaM was pulled down with streptavidin-agarose beads. Results demonstrated that biotinylated CaM is able to precipitate TRPM2 in a Ca²⁺-dependent manner (Fig. 6C). Analysis of bands by densitometry confirmed the stronger association of CaM with TRPM2 than with TRPM2-IQMUT. The amount of V5-TRPM2-IQMUT pulled down with CaM was only 23% of the amount of TRPM2 that was precipitated. Equivalent loading of streptavidin beads with TRPM2 was confirmed by Western blotting of lysates with anti-V5 (Fig. 6C). This experiment was performed twice with similar results. Taken together, these three experimental approaches demonstrate Ca²⁺-dependent CaM binding to TRPM2. They confirm that the IQ-like motif of TRPM2 is an important CaM binding site because the association of TRPM2 with CaM is reduced when this site is mutated. However, they also suggest that more than one CaM binding site is present on TRPM2, because in the presence of this mutation, binding of CaM to full-length TRPM2 is still observed.

**Functional Role of the TRPM2 IQ-like Motif**—To determine whether the TRPM2 IQ-like motif is important in channel activation, HEK 293T cells were transfected with empty pQBI50 vector, or pQBI50 expressing TRPM2 or TRPM2-IQMUT. [Ca²⁺], was measured in Fura Red-loaded cells before and after treatment with PBS or 1 mM H₂O₂. Results, shown in Table 1, demonstrate that whereas treatment with H₂O₂ results in a significant increase in [Ca²⁺], in all groups, a statistically larger increase in [Ca²⁺], was observed in cells transfected with TRPM2, compared

![FIGURE 6. CaM binding to an IQ-like motif in the TRPM2 N terminus. A, Immunoprecipitation (IP) of CaM with TRPM2 or TRPM2-IQMUT. Lysates from HEK 293T cells transfected with wild type TRPM2 or TRPM2-IQMUT, and CaM were immunoprecipitated with anti-V5 or anti-CaM antibodies in the presence of 100 μM CaCl₂ or 5 mM EGTA. Blots were probed with anti-V5 or anti-CaM. Analysis of bands by densitometry is shown (OD × mm). This experiment was performed three times, showing that Ca²⁺-dependent binding of CaM is stronger to TRPM2 than TRPM2-IQMUT. B, Biotinylated CaM overlay. Lysates from HEK 293T cells transfected with wild type TRPM2 or TRPM2-IQMUT were immunoprecipitated with anti-V5 antibody, and immunoprecipitates were resolved by polyacrylamide gel electrophoresis. Blots were probed with biotinylated CaM in the presence of 1 mM CaCl₂ or 5 mM EGTA, followed by streptavidin-HRP and ECL. Blots were stripped and reprobed with anti-V5 antibody. Band strength was quantitated by densitometry (outer diameter × mm). This experiment was performed twice with similar results. C, Biotinylated CaM pull-down. Cell lysates were prepared from HEK 293T cells transfected with V5-TRPM2 or V5-TRPM2-IQMUT. Lysates were mixed with biotinylated CaM for 3 hr in the presence of 1 mM CaCl₂ or 5 mM EGTA, followed by addition of streptavidin-agarose beads and precipitation was performed as described under “Experimental Procedures.” Supernatant proteins were separated by polyacrylamide gel electrophoresis. Lysates were loaded as a control to demonstrate equivalent TRPM2 amounts. Blots were probed with anti-V5 or anti-CaM antibodies, followed by ECL. Two independent experiments were performed with similar results and a representative blot is shown. Bands were analyzed by densitometry (outer diameter × mm). WB, Western blot.]
TABLE 1

| Treatment | [Ca\(^{2+}\)](i) Baseline | Increase | n  |
|-----------|-----------------|----------|----|
|           | µM              | %        |    |
| BFP-V     | 36 ± 2          | 52 ± 3   | 46 ± 6 | 40  |
| H2O\(_2\) | 36 ± 1          | 103 ± 5  | 189 ± 12 | 38  |
| TRPM2     | 35 ± 3          | 45 ± 4   | 34 ± 9 | 11  |
| IQ\(_{MUT1}\)| 37 ± 1   | 165 ± 5\(^*\) | 363 ± 18\(^*\)| 65 |
| H2O\(_2\) | 33 ± 1          | 94 ± 4\(^*\) | 187 ± 13 | 46  |

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Endogenous CaM Associates with TRPM2—To examine the physiological relevance of these results, HEK 293T cells transfected with empty pcDNA3.1/V5-His TOPO vector, vector expressing V5-TRPM2 or V5-TRPM2-IQ\(_{MUT1}\), and at 48 h treated with 1 mM H2O\(_2\). Cell viability was assessed by trypan blue exclusion. Representative results of seven experiments are shown in Fig. 8A. At all time points after 2 h of treatment, cell viability was significantly less in TRPM2-expressing cells compared with cells expressing empty vector or V5-TRPM2-IQ\(_{MUT1}\) (p < 0.005). In contrast, viability of cells expressing V5-TRPM2-IQ\(_{MUT1}\) was not different from cells expressing empty vector. These results demonstrate the requirement for the IQ-like motif in both calcium influx and susceptibility of cells expressing TRPM2 to death.

Endogenous CaM Associates with TRPM2—To examine the physiological relevance of these results, HEK 293T cells transfected with vector expressing V5-TRPM2 were treated with 1 mM H2O\(_2\) and V5-TRPM2 was immunoprecipitated with anti-V5 antibody. The association of endogenous CaM with V5-TRPM2 was examined by Western blotting. Endogenous CaM associated with V5-TRPM2 (Fig. 8B), and this association increased after H2O\(_2\) treatment, peaking at 10 min. A long (2 h) exposure of the blot was required to see endogenous CaM association. In three independent repeats of this experiment, the mean ± S.E. intensity of the CaM band (outer diameter × mm) in untreated TRPM2-transfected cells was 0.21 ± 0.06 and after 10 min of H2O\(_2\) was 0.49 ± 0.05, a significant difference (p < 0.05). The mean ± S.E. intensity of the TRPM2 band in untreated TRPM2-transfected cells was 2.28 ± 0.50 and after 10 min of H2O\(_2\) was 2.21 ± 0.55, not significantly different (p > 0.9). These results confirm the physiological association of CaM with TRPM2 and the enhancement of this interaction following the increase in [Ca\(^{2+}\)](i), stimulated by H2O\(_2\).

with cells transfected with empty vector (p < 0.002) or TRPM2-IQ\(_{MUT1}\) (p < 0.001). In contrast, the peak increase in [Ca\(^{2+}\)](i), in cells transfected with TRPM2-IQ\(_{MUT1}\), was not different from cells expressing empty vector. These results demonstrate that the TRPM2 IQ-like motif is functionally important.

To confirm that the mutation in the IQ-like motif does not affect the insertion of TRPM2 into the plasma membrane, HEK 293T cells were transfected with empty pcDNA3.1/V5-His TOPO vector, vector expressing V5-TRPM2 or V5-TRPM2-IQ\(_{MUT1}\), empty pQBI50 vector, or vector expressing V5-TRPM2 or V5-TRPM2-IQ\(_{MUT1}\). Externalization of TRPM2 and TRPM2-IQ\(_{MUT1}\) was assessed by biotinylation of cell surface proteins, followed by immunoprecipitation with anti-V5 or anti-TRPM2-C (for BFP-TRPM2) antibodies. Results, shown on Fig. 7, demonstrate cell surface expression of V5-tagged and BFP-tagged TRPM2 and TRPM2-IQ\(_{MUT1}\). The higher molecular mass of TRPM2 in Fig. 7B (200 kDa) compared with that observed in Fig. 7A (171 kDa) is secondary to linkage of TRPM2 to BFP. These data demonstrate that the reduced increase in [Ca\(^{2+}\)](i) in H2O\(_2\)-treated TRPM2-IQ\(_{MUT1}\)-expressing cells as compared with H2O\(_2\)-treated TRPM2-expressing cells (Table 1) is not secondary to the inability of TRPM2-IQ\(_{MUT1}\) to externalize.

To further assess the functional importance of the TRPM2 IQ-like motif, HEK 293T cells were transfected with empty pcDNA3.1/V5-His TOPO vector, vector expressing V5-TRPM2 or V5-TRPM2-IQ\(_{MUT1}\), empty pQBI50 vector, or vector expressing V5-TRPM2 or V5-TRPM2-IQ\(_{MUT1}\). Lysates were prepared, and immunoprecipitation (IP) was performed with anti-V5 (A) or anti-TRPM2-C (B) antibodies. Western blotting (WB) was performed with streptavidin-HRP to detect biotinylated TRPM2 or TRPM2-IQ\(_{MUT1}\), and anti-V5 or anti-TRPM2-C antibody to detect total V5-tagged or BFP-tagged TRPM2 or TRPM2-IQ\(_{MUT1}\) protein, respectively.

FIGURE 7. Functional role of the TRPM2 IQ-like motif. A, viability of TRPM2 or TRPM2-IQ\(_{MUT1}\)-expressing cells after H2O\(_2\) treatment. HEK 293T cells were transfected with empty pcDNA3.1/V5-His TOPO vector, vector expressing V5-TRPM2 or V5-TRPM2-IQ\(_{MUT1}\). Cells were treated with 1 mM H2O\(_2\), and cell viability was assessed by trypan blue exclusion at 0–6 h. The asterisk indicates a significant difference from other groups of trans-
**Calmodulin Is the Ca^{2+} Sensor for TRPM2**

**DISCUSSION**

TRPM2 is a voltage-independent ion channel known to be regulated by the second messengers ADP-ribose (14, 15) and cADPR (21). In addition, TRPM2 currents are critically dependent on and positively regulated by intracellular Ca^{2+} (22), but the mechanism for the Ca^{2+} sensitivity is unknown. Because TRPM2 is involved in the regulation of cell viability following oxidative stress (11, 14, 17), understanding mechanisms involved in TRPM2 activation is of great physiological importance. Here, the role of CaM as an intracellular Ca^{2+} sensor required for TRPM2 activation was examined. We determined that TRPM2 activity and susceptibility of TRPM2-expressing cells to death is dependent on Ca^{2+}-sensitive CaM binding to an IQ-like motif in the TRPM2 N terminus.

The first major finding of this report is that TRPM2 is directly modulated by CaM. Cotransfection of CaM with TRPM2 did not affect the increase in [Ca^{2+}]_i, suggesting either that CaM was not the Ca^{2+} sensor or that endogenous CaM levels were sufficient for maximal effect. In contrast, a mutant CaM with substitutions in all four Ca^{2+}-binding EF motifs significantly reduced calcium influx through TRPM2. When extracellular Ca^{2+} was substituted with Mn^{2+}, a progressive quenching of Fura Red fluorescence was observed in TRPM2-expressing cells following H_2O_2 or TNFα treatment, indicating Mn^{2+} influx through TRPM2, which was also inhibited by CaM_MUT. Together, these studies provide significant support for the conclusion that CaM is required for TRPM2 activation.

The second major finding of this report is that CaM associates with TRPM2. Binding of CaM to full-length TRPM2, TRPM2-N, and TRPM2-IQ_MUT was Ca^{2+}-dependent, because significantly reduced interactions with CaM were observed in the presence of EGTA. In contrast, weak Ca^{2+}-independent binding of CaM to the TRPM2 C terminus was observed (Fig. 4C). Using gel shift assays, we determined that CaM interacts strongly with the N-terminal IQ-like motif, and that this interaction is Ca^{2+}-dependent. Whereas mutations in the TRPM2 IQ-like motif peptide used in gel shift assays abolished CaM binding, the same substitutions of the IQ-like motif in the context of full-length TRPM2 resulted in constitutive Ca^{2+}-independent and Ca^{2+}-dependent binding of CaM to TRPM2 using three different approaches. These experiments suggest that, whereas the IQ-like motif is critical for TRPM2 activation and function, TRPM2 and CaM interact at least at two sites, one in the N terminus and one in the C terminus, and that Ca^{2+}-dependent and Ca^{2+}-independent interactions occur. Definition of the number and location of CaM binding sites in TRPM2 will require further analysis. In addition, multiple determinants may exist for a single CaM binding site. Understanding the structural basis responsible for channel opening following Ca^{2+}-dependent CaM interaction with TRPM2 will require elucidation of these sites and determinants.

Because both CaM and CaM_MUT associate with TRPM2, these results suggest that CaM_MUT inhibits TRPM2 activation by competing with endogenous CaM for the same TRPM2 binding site. A similar mechanism has been reported for L-type calcium channels (42–47). CaM is constitutively reported to a segment at the N terminus of the IQ-motif of the main L-type calcium channel subunit (α_1C). Ca^{2+} entry results in tighter, Ca^{2+}-dependent binding of the tethered CaM to the region more proximal to the IQ motif, and the subsequent structural rearrangements of this ternary complex ultimately lead to channel inactivation. Our data and that of others (22) suggest that Ca^{2+}-dependent activation of TRPM2 may occur via a similar mechanism. Apo-CaM binds weakly to TRPM2 in resting cells. Whether Ca^{2+}-free CaM is tethered constitutively to TRPM2 near the IQ-like motif, the TRPM2 C terminus, or a yet unidentified site needs to be determined. Exposure to oxidative stress or TNFα results in enhancement of ADP-ribose, and the interaction of ADP-ribose with TRPM2 in the presence of physiological levels of [Ca^{2+}], supports limited Ca^{2+} entry through TRPM2. Ca^{2+} binds to CaM as [Ca^{2+}]

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