Ca\(^{2+}\)-dependent Potentiation of the Nonselective Cation Channel TRPV4 Is Mediated by a C-terminal Calmodulin Binding Site*

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Most Ca\(^{2+}\)-permeable ion channels are inhibited by increases in the intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)), thus preventing potentially deleterious rises in [Ca\(^{2+}\)]. In this study, we demonstrate that currents through the osmo-, heat- and phorbol ester-sensitive, Ca\(^{2+}\)-permeable nonselective cation channel TRPV4 are potentiated by intracellular Ca\(^{2+}\). Spontaneous TRPV4 currents and currents stimulated by hypotonic solutions or phorbol esters were reduced strongly at all potentials in the absence of extracellular Ca\(^{2+}\). The other permeant divalent cations Ba\(^{2+}\) and Sr\(^{2+}\) were less effective than Ca\(^{2+}\) in supporting channel activity. An intracellular site of Ca\(^{2+}\) action was supported by the parallel decrease in spontaneous currents and [Ca\(^{2+}\)]\(_i\) on removal of extracellular Ca\(^{2+}\) and the ability of Ca\(^{2+}\) release from intracellular stores to restore TRPV4 activity in the absence of extracellular Ca\(^{2+}\). During TRPV4 activation by hypotonic solutions or phorbol esters, Ca\(^{2+}\) entry through the channel increased the rate and extent of channel activation. Currents were also potentiated by ionomycin in the presence of extracellular Ca\(^{2+}\). Ca\(^{2+}\)-dependent potentiation of TRPV4 was often followed by inhibition. By mutagenesis, we localized the structural determinant of Ca\(^{2+}\)-dependent potentiation to an intracellular, C-terminal calmodulin binding domain. This domain binds calmodulin in a Ca\(^{2+}\)-dependent manner. TRPV4 mutants that did not bind calmodulin lacked Ca\(^{2+}\)-dependent potentiation. We conclude that TRPV4 activity is tightly controlled by intracellular Ca\(^{2+}\). Ca\(^{2+}\)-entry increases both the rate and extent of channel activation by a calmodulin-dependent mechanism. Excessive increases in [Ca\(^{2+}\)]\(_i\), via TRPV4 are prevented by a Ca\(^{2+}\)-dependent negative feedback mechanism.

Channels of the TRP family can be divided on the basis of structural features into three subfamilies: classic or canonical (TRPC) channels, melastatin-like (TRPV) channels (1–4). The TRPV subfamily is composed of six members with very different functional properties. TRPV1–4 are Ca\(^{2+}\)-permeable, nonselective cation channels activated by a variety of stimuli but having in common sensitivities to different levels of heat. TRPV1 (VR1) is activated by heat, vanilloids, and protons and is involved in the transduction of noxious heat stimuli in primary sensory neurons (5). TRPV2 (VRL-1, GRC) is activated by vanilloids and is involved in the transduction of noxious heat stimuli in primary sensory neurons (6) and is probably involved in high threshold nociception. In addition, this channel is expressed in other tissues and, in some, is translocated to the cell membrane by stimulators of cell proliferation (7). Recent studies (8–10) have shown that TRPV3 responds to innocuous levels of heat and is expressed in primary sensory neurons and keratinocytes, suggesting a role of this channel in non-nociceptive thermoreception. TRPV4 was first shown to respond to changes in cell volume or extracellular osmolarity by an unknown mechanism (11–14). Recently (15), this channel has been shown to be activated by phorbol esters independently of activation of protein kinase C and by heat (16, 17). In contrast to TRPV1–4, the other members of this subfamily, TRPV5 and TRPV6, are highly Ca\(^{2+}\)-permeable channels and are involved in epithelial Ca\(^{2+}\) transport in the kidney and gut (18, 19).

The activity of many Ca\(^{2+}\)-permeable cation channel types, including voltage-gated Ca\(^{2+}\) channels, cyclic nucleotide-gated channels, and N-methyl-D-aspartate receptors, is regulated by the intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)). This regulation is usually inhibitory and often provides a feedback mechanism to prevent excessive increases in [Ca\(^{2+}\)]\(_i\). In most cases, Ca\(^{2+}\) does not act directly on the channel but through binding to the Ca\(^{2+}\)-binding protein calmodulin (CaM). A number of TRP channel isoforms are modulated by Ca\(^{2+}\), and some have CaM binding domains in their C termini. All TRPC isoforms have at least one CaM binding domain (20–22) and bind CaM in a Ca\(^{2+}\)-dependent manner. For TRPC1, CaM has been shown to regulate Ca\(^{2+}\)-dependent feedback inhibition (23). TRPM2 requires intracellular Ca\(^{2+}\) for its activation, although the mechanism of Ca\(^{2+}\) action is unknown (24). Effects of intracellular Ca\(^{2+}\) have also been reported for members of the TRPV subfamily. Rapid desensitization of TRPV1 is dependent on Ca\(^{2+}\) (5), acting at an intracellular site via a Ca\(^{2+}\)-CaM-dependent phosphatase (25). The epithelial Ca\(^{2+}\)-permeable channels TRPV5 and TRPV6 are inhibited by intracellular Ca\(^{2+}\) in the submicromolar range (26–29), and, at least for TRPV6, part of this Ca\(^{2+}\)-dependent inactivation is CaM-dependent (28). Raising [Ca\(^{2+}\)]\(_i\) has also been reported recently (15) to inhibit TRPV4, suggesting that this channel may also undergo Ca\(^{2+}\)-dependent feedback inhibition.

In this paper we show that, in addition to an inhibitory effect, intracellular Ca\(^{2+}\) potentiates currents through TRPV4. Ca\(^{2+}\)-entry through the channel is involved in the maintenance of spontaneous activity and increases both the TRPV4 current amplitude and the rate of current activation in response to channel stimulation with hypotonic solutions or phorbol esters. Using mutagenesis, we localized the structural determinant of

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1 The abbreviations used are: CaM, calmodulin; HEDTA, N-2-hydroxyethylendiaminetriacetic acid; HEK, human embryonic kidney; NMDG, N-methyl-D-glucamine; PMA, phorbol 12-myristate 13-acetate; wt, wild-type.
the stimulatory effect to an intracellular C-terminal domain. Binding experiments showed that the region involved is a Ca\textsuperscript{2+}-dependent CaM binding domain. Mutations that prevent CaM binding led to a loss of Ca\textsuperscript{2+}-dependent potentiation of TRPV4.

EXPERIMENTAL PROCEDURES

Cloning of Human TRPV4 and Generation of a Stably Transfected Cell Line—For the cloning of human TRPV4, total RNA was prepared from human kidney tissue samples using the TRIzol LS reagent (Invitrogen) according to the manufacturer’s protocol. Reverse transcription was performed from 1 µg of total RNA using 200 units of SuperScript II reverse transcriptase (Invitrogen) and 5 pmol of the primer (dT\textsubscript{R}6). The human TRPV4 coding sequence was amplified in 30 cycles of PCR under the following conditions: 55 °C annealing temperature, extension at 72 °C for 3 min. Expand HF polymerase (Roche Applied Science), primers GGAAGCTTCCACATGGCGATTCGAGCA (sense) and CCGGTTCGCGCGGCGGTCTGACTG (antisense). The PCR product was subcloned into the pcDNA3.1 vector (Invitrogen), and the cDNA sequence was confirmed by DNA sequencing of both strands. A C-terminal green fluorescent protein fusion construct was obtained by HindIII/BamHI digestion of the fragment and ligation into the pEGFP-N1 vector (Clontech). Point mutations were inserted by overlap extension PCR using appropriate sense and antisense primers.

C-terminal deletion mutants were amplified using a modified antisense primer

For the generation of a stably transfected cell line, the HindIII/BamHI fragment was cloned into the pcDNA4TO vector (Invitrogen), and the construct was transfected into the T-REx-293 cell line using the FuGENE 6 transfection reagent (Roche Applied Science). Clonal selection was performed according to the manufacturer’s protocol with Zeo-cin (250 µg/ml), and multiple clones were functionally tested for TRPV4 expression.

CaM Pull-down Assays—Human TRPV4 C-terminal fragments were amplified by PCR and subcloned into the pGEX-2TK vector (Amersham Biosciences). The sequences were verified by DNA sequencing, and the glutathione S-transferase fusion proteins were expressed from Escherichia coli BL21 cells. After purification of glutathione-Sepharose according to the standard protocol (Amersham Biosciences), the eluted peptides were subjected to interaction with calmodulin-Sepharose (Amersham Biosciences) for 60 min at room temperature in a buffer containing (in mM) 50 Tris-HCl, pH 7.5, 100 NaCl, 0.1% Triton X-100, and 2 CaCl\textsubscript{2} or 2 EGTA. For quantitative interaction assays, the free calcium concentration was buffered to the desired value using the appropriate chelators (EGTA, HEDTA, nitrilotriacetic acid, or EDTA). Pellets were washed three times with the respective buffers and subjected to SDS gel electrophoresis. Gels were stained with Coomassie Blue dye and dried, and the relative intensities of the bands were analyzed using AI software.

Cell Culture and Transfection—HEK293 cells were cultured in minimum essential medium with Earle’s salts (Biochrom, Berlin, Germany), supplemented with 10% (v/v) fetal calf serum (Biochrom) and 100 units/ml penicillin and 100 µg/ml streptomycin. Cells were grown on glass coverslips 24–48 h prior to transfection. The cells were transiently transfected with 1 µg of DNA and 6 µl of FuGENE 6 transfection reagent (Roche Diagnostics) in 94 µl of OptiMEM medium (Invitrogen) per 85-mm dish. Ca\textsuperscript{2+} measurements and electrophysiological studies were performed 24–36 h after transfection.

T-REx cells and T-REx cells stably transfected with human TRPV4 (T-REx-V4) were cultured in Dulbecco’s modified Eagle’s medium (4.5 g/liter glucose) supplemented with 10% (v/v) fetal bovine serum (Invitrogen), 4 mm calcium gluconate (Fluka, Taufkirchen, Germany), 100 units/ml penicillin, 100 µg/ml streptomycin (both Biochrom, Berlin, Germany). For cells containing TRPV4, 5 µg/ml blasticidin and 250 µg/ml Zeocin (both Invitrogen) were added to the culture medium. All experiments were performed 1–2 days after transient transfection and, in the case of T-REx-V4 cells, 1–2 days after induction with tetracyclin (1 µg/ml; Invitrogen).

Patch Clamp Recording—Recordings of whole cell currents from single cells were made with an EPC-7 amplifier using Pulse software (HEKA, Lambrecht, Germany) as described previously (12). Experiments were performed using the standard whole cell mode of the patch clamp technique or the perforated patch technique. For the latter, an injection of 5 mM KCl (Sigma) was used as the intracellular current. Measurements of currents in perforated patch recordings were started when the series resistance approached a plateau at values <30 megohms. To measure channel currents, cells were held at a potential of 0 or ~20 mV and ramps from –100 to +100 mV with a duration of 400 ms applied at a frequency of 0.2 or 0.1 Hz. Raw data were acquired at a frequency of 4 kHz after filtering at 1 kHz.

The standard pipette solution contained the following (in mM): 110 CH\textsubscript{3}3O\textsubscript{3}SCs (cesium methane sulfonate), 25 CsCl, 2 MgCl\textsubscript{2}, 0.362 CaCl\textsubscript{2}, 1 EGTA, 30 HEPES (pH 7.2 with CsOH). The standard extracellular solution contained the following (in mM): 140 NaCl, 5 CsCl, 2 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, 10 glucose, 10 HEPES (pH 7.4 with NaOH). For experiments to test the effects of hypotonic solutions, cells were initially bathed in a solution containing 100 NaCl, 2 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, 10 HEPES, 10 glucose, 100 mannitol (pH 7.4 with NaOH), and mannitol and then removed to reduce the osmolarity without changing the ion concentrations. In other experiments the normal extracellular solution was diluted to 2/3, and Ca\textsuperscript{2+} was adjusted to 2 mM. For Na\textsuperscript{+}–free solutions, Na\textsuperscript{+} was replaced by N-methylglucamine (NMDG\textsuperscript{+}). For nominally Ca\textsuperscript{2+}–free solutions, Ca\textsuperscript{2+} was omitted. The osmolarity of the solutions was measured using a freezing-point depression osmometer (Roehling, Berlin, Germany).

For measurements of divalent cation permeabilities, currents were fully activated in an NMDG\textsuperscript{+} solution containing the divalent cation (X\textsuperscript{2+}) at a concentration of 20 mM. The solution was then exchanged for a Na\textsuperscript{+} solution. The relative permeability was then calculated according to the following equation: $F_{Na}/F_{X}$ = ([Na\textsuperscript{+}]/[4]X\textsuperscript{2+}]$\cdot$exp([FRT][V\textsubscript{X} - V\textsubscript{Na}]/[1 + exp(FRT/V\textsubscript{X})]).

Combined Patch Clamp and Ca\textsuperscript{2+}—Measurements—In some experiments, perforated patch recordings were combined with fluorometric recordings of [Ca\textsuperscript{2+}]\textsubscript{i}. Cells were loaded with fura-2/AM (Molecular Probes, Leiden, Netherlands), and Ca\textsuperscript{2+} measurements were performed using a monochromator/photon multiplier-based system (TILL Photonics, Gräfelfing, Germany). Cells were viewed with a ×40 oil immersion objective. Fluorescence was excited alternately at 360 and 390 nm for fura-2, and light output was measured from an adjustable rectangular aperture containing the cell of interest. Background fluorescence was obtained from the same region without a cell. The output of the photomultiplier was recorded simultaneously with the current data using Pulse software (HEKA) and the fluorescence signals together with the calculated ratio ($F_{Na}/F_{Na}$) displayed online.

RESULTS

Properties of Human TRPV4—The properties of human TRPV4 closely resembled those of the murine ortholog, when studied either after induction of expression in the T-REx-V4 cell line or following transient transfection in HEK293 cells. In whole cell recordings of membrane currents, many cells displayed spontaneous current activity that decayed rapidly in the open whole cell recording mode (Fig. 1D) but was more stable in perforated patch recordings (Fig. 1A). TRPV4 responds both to decreases in the extracellular osmolarity (11–14) and to the application of phorbol esters (15). In perforated patch recordings, application of hypotonic solutions resulted within a few seconds in an increase in membrane current that reached a maximum and then decayed (Fig. 1A). Both spontaneous and osmosensitive currents displayed a characteristic outwardly rectifying current-voltage (IV) relation (Fig. 1B). Scaling the IV relation obtained at an osmolarity of 320 mosmol/liter in Fig. 1B reveals the similarity in shape of the IV relations before and during stimulation with the hypotonic solution. The currents differ only in the reversal potential, which is more negative because of the stronger influence of background currents at the lower amplitudes in 320 mosmol/liter. In the open whole cell mode, responses to changes in osmolarity were weak (data not shown). In contrast, responses to phorbol esters were large, even after run-down of spontaneous activity (Fig. 1D), and displayed an identical IV relation to spontaneous currents and currents activated by hypotonic solutions (Fig. 1E).

Divalent Cation Permeability of TRPV4—Previous studies have shown that TRPV4 is somewhat more permeable to Ca\textsuperscript{2+} than to Na\textsuperscript{+} (12, 15). To estimate the permeability of human TRPV4 to Ca\textsuperscript{2+}, Ba\textsuperscript{2+}, and Sr\textsuperscript{2+}, currents were activated by 4α-PMA (1 µM) in a Na\textsuperscript{+}–free (NMDG\textsuperscript{+}) solution containing the divalent cation at a concentration of 20 mM. After reaching a maximum, the extracellular solution was changed to an Na\textsuperscript{+} solution. As the influence of background currents became smaller, the reversal potential in the divalent cation solution...
Values during voltage ramps. Note the decrease in current during the \( \text{Ca}^{2+} \) replacement by an \( \text{Na}^{+} \) solution (with reversal potential close to 0 mV) and following \( \text{Ba}^{2+} \) removal on the IV relation. Currents were recorded before stimulation by hypotonic solutions in the T-REx-V4 cell line. For this, we combined perforated patch recordings of spontaneous whole cell currents with fluorometric recordings of [Ca\(^{2+}\)]\(_i\), using fura-2. Cells were clamped at 0 mV to reduce \( \text{Ca}^{2+} \) entry through the spontaneously active channels and prevent steady increases in \([\text{Ca}^{2+}]_i \), that occur at negative holding potentials. Currents were estimated by ramps applied at 5- or 10-s intervals. In cells that showed spontaneous TRPV4 activity, replacement of the standard extracellular solution with a nominally \( \text{Ca}^{2+} \)-free solution resulted in a complete loss of spontaneous channel activity and a parallel decrease in \([\text{Ca}^{2+}]_i \), (see Fig. 2, A and B and Fig. 3A). The rate of reduction of currents and \([\text{Ca}^{2+}]_i \), was much slower than the solution exchange, which was complete within a few seconds. Readdition of \( \text{Ca}^{2+} \) to the extracellular solution resulted in a transient increase in \([\text{Ca}^{2+}]_i \), and currents to levels higher than those before \( \text{Ca}^{2+} \) removal (Fig. 2A). Thereafter, both parameters returned to stable levels similar to those before \( \text{Ca}^{2+} \) removal. The similarity in the time course of the currents and the fura ratio upon removal of extracellular \( \text{Ca}^{2+} \) suggests that spontaneous TRPV4 activity is dependent on an elevated \([\text{Ca}^{2+}]_i \), resulting from \( \text{Ca}^{2+} \) entry through the channel.

We then tested the ability of other permeant divalent cations to substitute for \( \text{Ca}^{2+} \) in the maintenance of channel activity. Neither \( \text{Ba}^{2+} \) nor \( \text{Sr}^{2+} \) (2 mM) were effective replacements for \( \text{Ca}^{2+} \) at the same concentration. Replacement of \( \text{Ca}^{2+} \) by either of these divalent cations (Ba\(^{2+}\), \( n = 8 \); Sr\(^{2+}\), \( n = 5 \)) resulted in reductions of inward and outward currents similar to those seen on \( \text{Ca}^{2+} \) removal (Fig. 2, C, D, and E). However, as evidenced by the shape of the IV relation, small TRPV4 currents persisted in Sr\(^{2+}\) but not in Ba\(^{2+}\) (Fig. 2, D and E).

**Stimulation of TRPV4 by \( \text{Ca}^{2+} \) Release from Intracellular Stores Indicates That \( \text{Ca}^{2+} \) Acts at an Intracellular Site**—As a permeant cation, \( \text{Ca}^{2+} \) could influence channel activity at either an extracellular or an intracellular site. To test whether increases in intracellular \( \text{Ca}^{2+} \) can restore TRPV4 activity in the absence of extracellular \( \text{Ca}^{2+} \), we raised \([\text{Ca}^{2+}]_i \) by releasing \( \text{Ca}^{2+} \) from intracellular stores. As described above, removal of extracellular \( \text{Ca}^{2+} \) abolished channel activity. After \([\text{Ca}^{2+}]_i \) had reached a stable level, in eight from nine cells addition of thapsigargin (1 \( \mu \)M) or ionomycin (1 \( \mu \)M) resulted in an increase in \([\text{Ca}^{2+}]_i \), and stimulation of an outwardly rectifying current with an identical shape of IV relation to the spontaneous current (Fig. 3, A and B). In Fig. 3, A and B, the addition of ionomycin after thapsigargin resulted in a further increase in \([\text{Ca}^{2+}]_i \) and current. Control experiments in cells not treated with tetracyclin and, thus, not expressing the channel (Fig. 3C) indicated that the currents observed are unlikely to result from...
activation of an endogenous Ca\(^{2+}\)-activated cation current like TRPM4 (30). In five of these cells, no current was activated by Ca\(^{2+}\) release induced by either ionomycin or thapsigargin in the absence of extracellular Ca\(^{2+}\), and, in another one, a small current with different properties to those of TRPV4 was observed. The Ca\(^{2+}\) signals observed in response to thapsigargin and ionomycin in control cells were of similar amplitudes to those in TRPV4-expressing cells (e.g., Fig. 3C). Hence, currents through TRPV4 can be potentiated by increases in [Ca\(^{2+}\)].

**Stimulation of TRPV4 by Ionomycin in the Presence of Extracellular Ca\(^{2+}\)**—In the presence of extracellular Ca\(^{2+}\), application of ionomycin resulted in large increases in current (from 7.6 ± 2.2 picoamperes/picofarad to 45.3 ± 8.9 picoamperes/picofarad at −100 mV, n = 9) and large, often irreversible increases in [Ca\(^{2+}\)]\(_i\) (data not shown). Fig. 3, D and E shows the effect of application of ionomycin (1 μM) on a cell with large, spontaneous TRPV4 currents. Following addition of ionomycin to the extracellular solution, the current increased transiently before decaying to a level much lower than that of the spontaneous current (Fig. 3D). The IV relation of the ionomycin-activated current had a similar shape to spontaneous TRPV4 currents (Fig. 3E) and to currents activated by 4α-PMA or reductions in extracellular osmolarity. In control cells, ionomycin did not activate any current in the presence of extracellular Ca\(^{2+}\) (n = 4). Thus, the large increases in [Ca\(^{2+}\)]\(_i\), resulting from ionomycin application in the presence of extracellular Ca\(^{2+}\) revealed a dual effect of Ca\(^{2+}\), an initial potentiation followed by inhibition.

**Ca\(^{2+}\) Dependence of Current Activation by Hypotonic Solutions and Phorbol Esters**—To test the role of Ca\(^{2+}\) during current activation by hypotonic solutions or phorbol esters, the stimulus was applied in the absence of extracellular Ca\(^{2+}\), followed by Ca\(^{2+}\) readdition to the extracellular solution in the continued presence of the stimulus.

In a first series of experiments, we studied the activation of TRPV4 by hypotonic solutions in a Ca\(^{2+}\)-free, Ba\(^{2+}\)-containing solution (Fig. 4A). Reduction of the osmolarity from 300 to 200 mosmol/liter resulted in a slow increase in outward current and a comparatively small increase in inward current. Replacement of Ba\(^{2+}\) by Ca\(^{2+}\) when activation in Ba\(^{2+}\)-containing solution was near maximal led to an increase in both inward and outward currents (Fig. 4, A and B). The differences in the time course of current activation by a reduction in osmolarity from 300 to 200 mosmol/liter in Ba\(^{2+}\) and in Ca\(^{2+}\) solutions are illustrated in Fig. 4C. In Ba\(^{2+}\)-containing solution in the presence of extracellular Ca\(^{2+}\), currents increased more rapidly to a maximum and began to decay in the continued presence of the stimulus. Following the response, the current decreased to values below those of spontaneous activity before osmotic stimulation and then recovered slowly. The current response in Ca\(^{2+}\) resembles that to ionomycin in the presence of extracellular Ca\(^{2+}\).

Similar results to those in the presence of Ba\(^{2+}\) were ob-
response to hypotonic solutions in Ba$^{2+}$, that Ca$^{2+}$ previously (28) for another member of the TRPV subfamily, application bar 4 lar Ca$^{2+}$ of TRPV4 — Ca$^{2+}$ with TRPV4. Whole cell recordings were from HEK293 cells transiently transfected D 2m M to the extracellular solution resulted in a very large, mediated by the binding of Ca$^{2+}$-PMA in a Ca$^{2+}$-dependent Potentiation of TRPV4 currents by switching from Ba$^{2+}$ to Ca$^{2+}$, IV relations recorded following stimulation with a hypotonic solution in 2 m M Ba$^{2+}$—activated currents by extracellular Ca$^{2+}$-PMA prior to and tested for their interaction with Sepharose-bound CaM (Fig. 5B). Peptides that include the cluster of positively charged amino acids (C1, C3, C5, and C6) bind to CaM in a Ca$^{2+}$-independent manner (Fig. 5B). Peptides lacking this domain (C2 and C4) did not bind to CaM. The smallest of the peptides that binds to CaM (C6) limits the CaM binding domain to a region between amino acids 812 and 831. To investigate the Ca$^{2+}$ dependence of CaM binding, the interaction of the C5 peptide with CaM was measured at Ca$^{2+}$ concentrations from 0.1 μM to 100 mM (Fig. 6). The calmodulin-bound peptide fraction was quantified by measuring the relative band intensities of the SDS-PAGE gel. In 10 mM EGTA no pull-down was detectable. At a [Ca$^{2+}$] of 0.1 μM some peptide binding was already observed, and this increased with [Ca$^{2+}$] to a maximum at about 100 μM. Half-maximal CaM binding was observed at a [Ca$^{2+}$] of ~200 nM. As also described for the CaM binding site in TRPV6 (28), higher Ca$^{2+}$ concentrations resulted in a decrease in CaM binding.

To further define the structural features that are essential for the CaM interaction, point mutations were inserted into the C3 and C5 peptides. The corresponding peptides (Fig. 5A) bearing the deletion of central tryptophan (W822A), a RWSS to AASA exchange (821AASA) or the replacement of the five basic arginine residues with glutamate at position 816 (5R/E) were subjected to a similar pull-down experiment with CaM-Sepharose. All of these mutations led to a loss of Ca$^{2+}$-dependent CaM binding (Fig. 5C). Equal Ca$^{2+}$-CaM binding properties were observed for C3 and C5 fragments. Thus, the CaM binding site within the C5 peptide could be localized to the tryptophan at position 822 and its positively charged vicinity.

Ca$^{2+}$ Dependence of C-terminal TRPV4 Mutants—To investigate the functional role of the C terminus and, in particular, the C-terminal CaM binding site, we used two of the mutants of TRPV4 described above (TRPV4-5E/E and TRPV4-W822A) and two additional mutants. The latter were a deletion mutant lacking the last 70 amino acids including the CaM binding domain (TRPV4-K801term) and a mutant in which the two serines at positions 823 and 824 were mutated to alanine (TRPV4-S823A/S824A). These residues are part of a canonical phosphorylation motif for protein-serine/threonine kinases (32) that is contained within the CaM binding site and might be subject to phosphorylation as described for TRPV6 (28). Following transient transfection in HEK293 cells, all of the mutants, C-terminally fused to green fluorescent protein, displayed a subcellular expression pattern indistinguishable from that of the wild-type channel, with expression in the cell membrane and in intracellular compartments.
In whole cell recordings, cells expressing all of the mutants showed spontaneous TRPV4 currents which decayed during the course of the recording like wild-type TRPV4. Removal of extracellular Ca$^{2+}$/H$^{11001}$ led to a reduction of the remaining TRPV4 current in cells expressing TRPV4-wt and TRPV4-S823A/S824A but not in cells expressing TRPV4-K801term, TRPV4–5R/E, or TRPV4-W822A. We then compared the ability of Ca$^{2+}$/H$^{11001}$ to potentiate 4α-PMA-activated currents in the mutants with that for TRPV4-wt (see above). For all channels, application of 4α-PMA in Ca$^{2+}$/H$^{11001}$-free solutions resulted in a slow increase in current, as described above. For TRPV4-wt and TRPV4-S823A/S824A, addition of 0.5 mM Ca$^{2+}$/H$^{11001}$ resulted in about a 5-fold increase in current at both −100 and +100 mV (Fig. 7, A and C). In contrast, TRPV4-K801term, TRPV4–5R/E, and TRPV4–W822A showed no potentiation (Fig. 7B), and, indeed, TRPV4–5R/E showed a clear but poorly reversible inhibition upon addition of Ca$^{2+}$. Similar results were observed with 2 mM Ca$^{2+}$/H$^{11001}$ in the extracellular solution. The results are summarized in Fig. 7C. It should be noted that the current density for TRPV4–5R/E after 4α-PMA addition in Ca$^{2+}$/H$^{11001}$-free solutions was much higher than that for TRPV4-wt and the other mutants. For all isoforms, raising the extracellular Ca$^{2+}$/H$^{11001}$ concentration resulted in a change in the shape of the current voltage relationship increasing outward rectification as described recently by Voets et al. (33).

If Ca$^{2+}$ entry potentiates the current response of TRPV4 by a Ca$^{2+}$-CaM-mediated interaction with the C-terminal domain, mutations that prevent the interaction of CaM should slow the rate of channel activation. We therefore compared the time course of current activation by 4α-PMA in solutions containing 2 mM Ca$^{2+}$/H$^{11001}$ for two mutants that do not bind CaM with the response of TRPV4-wt. The mutants TRPV4-K801term and TRPV4–W822A both responded to 4α-PMA with a transient increase in current, but current activation was much slower than for TRPV4-wt (Fig. 7D). We quantified the rise time of the current by measuring the time required for the current to increase from 10 to 90% of the maximum. TRPV4-K801term and TRPV4–W822A had similar rise times, but these were significantly longer (on average about 3-fold) than that of TRPV4-wt (Fig. 7E). Interestingly, raising the Ca$^{2+}$ buffer capacity of the intracellular solution by increasing the EGTA concentration in the pipette solution from 1 to 10 mM led to a similar slowing of current activation to that in the two mutants (Fig. 7E).
Ca\textsuperscript{2+}-dependent Potentiation of TRPV4

In the presence of Ca\textsuperscript{2+}/H\textsubscript{11005} mutants TRPV4

tracellular solution containing 2 mM Ca\textsuperscript{2+} 0.5 mM Ca\textsuperscript{2+}

rents in the absence of extracellular Ca\textsuperscript{2+} TRPV4-W822A. Currents were activated by 4α-PMA (1 μM) in an extracellular solution containing 2 mM Ca\textsuperscript{2+}.

FIG. 7. TRPV4 mutants that no longer bind calmodulin lack Ca\textsuperscript{2+}-dependent potentiation and activate more slowly. A, potentiation of TRPV4-wt by Ca\textsuperscript{2+}. Currents were activated by 4α-PMA in the absence of Ca\textsuperscript{2+} followed by Ca\textsuperscript{2+} readoption at a concentration of 0.5 mM. B, mutant TRPV4-W822A lacks potentiation by Ca\textsuperscript{2+}. C, effect of addition of 0.5 mM Ca\textsuperscript{2+} on TRPV4 and TRPV4 mutants. Bars indicate the current at −100 mV (downward) and +100 mV (upward) in 0.5 mM Ca\textsuperscript{2+} relative to the absolute value in 4α-PMA in 0 Ca\textsuperscript{2+} immediately prior to Ca\textsuperscript{2+} addition for TRPV4-wt (n = 10) and the mutants TRPV4-5R/E (n = 16), TRPV4-S823A/S824A (n = 3), TRPV4-K801term (n = 8), and TRPV4-W822A (n = 5). D, activation of TRPV4 in the presence of Ca\textsuperscript{2+} is slowed in the mutants TRPV4-K801term and TRPV4-W822A. Currents were activated by 4α-PMA (1 μM) in an extracellular solution containing 2 mM Ca\textsuperscript{2+}.

DISCUSSION

In this study we have shown that, in addition to having a feedback inhibitory effect, intracellular Ca\textsuperscript{2+} potentiates TRPV4 currents and plays an important role in accelerating and amplifying the current response to hypotonic solutions and phorbol ester agonists. The potentiation effect of Ca\textsuperscript{2+} occurs through an action at an intracellular site in the C terminus of the channel protein and is mediated by CaM binding. Ca\textsuperscript{2+} regulates both the spontaneous activity of TRPV4 and the response of the channel to stimulation by phorbol esters and hypotonic solutions as evidenced by the reduction in current upon Ca\textsuperscript{2+} removal. Our data suggest that this effect of Ca\textsuperscript{2+} is mediated by an intracellular site and that Ca\textsuperscript{2+} entering the cell via the channel potentiates TRPV4 currents. The evidence for an intracellular site of action includes the ability of Ca\textsuperscript{2+} release from intracellular stores to restore TRPV4 currents in the absence of extracellular Ca\textsuperscript{2+}, the slowing of channel activation with intracellular solutions with stronger Ca\textsuperscript{2+} buffering, and the loss of potentiation following mutation of putative intracellular domains. Ba\textsuperscript{2+} was unable to potentiate TRPV4, and Sr\textsuperscript{2+} was much less effective than Ca\textsuperscript{2+} in supporting spontaneous channel activity, a sequence of effectiveness shared by a number of Ca\textsuperscript{2+}-binding proteins including CaM (34). For TRPV4 it is likely, though not proven, that heat and osmotic stimulation lead to the generation of a common messenger that activates the channel and that phorbol esters mimic this endogenous activator (17). Evidence that both osmotic stimuli and phorbol esters can increase TRPV4 currents without an increase in [Ca\textsuperscript{2+}], is an indication that the effect of Ca\textsuperscript{2+} is as a potentiator rather than a primary activator of the channel.

The C-terminal CaM binding domain involved in Ca\textsuperscript{2+}-dependent potentiation of TRPV4 is similar but not identical to the domain in TRPV6 that mediates slow channel inactivation (28). Mutations in the domain of TRPV4 like those that reduce inactivation of TRPV6 led to a loss of Ca\textsuperscript{2+}-dependent potentiation. These mutants also no longer bind CaM, suggesting that the effects of Ca\textsuperscript{2+} involve its binding to CaM and the subsequent interaction of Ca\textsuperscript{2+}-CaM with the C-terminal domain. Attempts to pharmacologically prevent the action of CaM with CaM antagonists were unsuccessful because of the instability of recordings in the presence of the substances used (calmidazolium, trifluoperazine, and W7; data not shown). For TRPV6, the interaction of CaM is competitively regulated by protein kinase C-mediated phosphorylation of a threonine residue in the CaM binding domain (28). TRPV4 also has a consensus sequence for protein-serine/threonine kinase phosphorylation within the CaM binding domain. However, mutation of two serines that are prospective phosphorylation sites within this domain in the mutant TRPV4-S823A/S824A were without effect on Ca\textsuperscript{2+}-dependent potentiation. Thus, the regulation of TRPV4 differs from that of TRPV6 in at least two important aspects. There is the surprising finding that CaM binding domains in similar regions of related proteins have opposite effects on channel function. In addition, for TRPV6, Ca\textsuperscript{2+}-CaM binding is modified by phosphorylation, but this is not the case for TRPV4.

Activation or potentiation of Ca\textsuperscript{2+}-permeable channels by Ca\textsuperscript{2+} is uncommon, particularly for channels in the plasma membrane, because of the positive feedback effect and danger of cellular Ca\textsuperscript{2+} overload. Other Ca\textsuperscript{2+}-permeable channels that are potentiated by Ca\textsuperscript{2+} are those involved in Ca\textsuperscript{2+} release from intracellular stores and includeryanodine receptors and inositol 1,4,5-trisphosphate receptors. As for TRPV6, the activity of these channels is tightly controlled by Ca\textsuperscript{2+}-dependent negative feedback mechanisms that involve CaM (35). Polycystin-2 and polycystin-L, cation channels that may be involved in Ca\textsuperscript{2+} release from intracellular stores or in Ca\textsuperscript{2+} entry through the plasma membrane, are also activated by increases in cytoplasmic Ca\textsuperscript{2+} (36). Among the TRP channels, the response of TRPC5, which is modestly Ca\textsuperscript{2+}-permeable, is potentiated by increases in [Ca\textsuperscript{2+}]\textsuperscript{i} (37, 38). It has also been shown recently (39, 40) that intracellular ADP-ribose-mediated activation of TRPM2, a Ca\textsuperscript{2+}-permeable nonelective cation channel, is also dependent on Ca\textsuperscript{2+} entry through the channel (24). Unlike TRPV4, TRPM2 does not appear to have an inhibitory mechanism, and, thus, uncontrolled positive feedback of Ca\textsuperscript{2+} entry leads to cell death. TRPM4, the other TRP channel that is activated by intracellular Ca\textsuperscript{2+}, is only poorly Ca\textsuperscript{2+}-permeable (30). More distantly related channels that, like TRPV4, are activated by a Ca\textsuperscript{2+}-CaM interaction with a C-terminal domain, include the small conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels (41).
It is likely that Ca\(^{2+}\)-CaM, by binding to the C-terminal domain, induces a conformational change in the TRPV4 channel protein resulting in increased channel activity. Similar mechanisms have been proposed for the activation of a number of channel types, including Ca\(^{2+}\)-activated K\(^+\) channels and cyclic nucleotide-gated channels, whose activity is controlled by intracellular mediator (Ca\(^{2+}\), Ca\(^{2+}\)-CaM, or cAMP/cGMP) binding in the C terminus. We could only demonstrate CaM binding to the C terminus of TRPV4 in the presence of Ca\(^{2+}\). In this respect, the channel differs from small conductance Ca\(^{2+}\)-activated K\(^+\) channels where Ca\(^{2+}\)-dependent and Ca\(^{2+}\)-independent CaM binding sites in their C termini are involved in Ca\(^{2+}\)-induced conformational changes (41).

The biphatic current responses following Ca\(^{2+}\) entry suggest that smaller increases in [Ca\(^{2+}\)]\(_i\) potentiate TRPV4, but larger increases are inhibitory. Data on the Ca\(^{2+}\)-dependence of CaM binding indicate that Ca\(^{2+}\)-CaM binds to TRPV4 at submicromolar concentrations that will readily be reached at the intracellular membrane surface close to the channel mouth. In a previous study (15), an inhibitory effect of intracellular Ca\(^{2+}\) on 4a-phorbol didecanoate-activated currents has been reported for TRPV4 with an IC\(_{50}\) in whole cell experiments of 406 nM. In this respect, the spontaneous activity before Ca\(^{2+}\) entry is prevented by Ca\(^{2+}\)-dependent inhibition to levels lower than those in expression systems. Without knowing the physiological role of the channel, it is too early to speculate further on the importance of Ca\(^{2+}\)-dependent potentiation.

Further studies are necessary to determine the physiological role and activation mechanism of this channel. However, TRPV4 is unusual among plasma membrane Ca\(^{2+}\)-permeable channels with moderate or high Ca\(^{2+}\) permeabilities in being potentiated by increases in intracellular Ca\(^{2+}\) concentration. Deleterious increases in intracellular Ca\(^{2+}\) resulting from Ca\(^{2+}\)-activated Ca\(^{2+}\) entry are prevented by Ca\(^{2+}\)-dependent negative feedback.

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Mediated by a C-terminal Calmodulin Binding Site

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