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# Regulation of the Mouse Epithelial $Ca^{2+}$ Channel TRPV6 by the $Ca^{2+}$-sensor Calmodulin*

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**TRPV5 and TRPV6 are members of the superfamily of transient receptor potential (TRP) channels and facilitate $Ca^{2+}$ influx in a variety of epithelial cells. The activity of these $Ca^{2+}$ channels is tightly controlled by the intracellular $Ca^{2+}$ concentration in close vicinity to the channel mouth. The molecular mechanism underlying the $Ca^{2+}$-dependent activity of TRPV5/TRPV6 is, however, still unknown. Here, the putative role of calmodulin (CaM) as the $Ca^{2+}$ sensor mediating the regulation of channel activity was investigated. Overexpression of $Ca^{2+}$-insensitive CaM mutants ($CaM_{1234}$ and $CaM_{34}$) significantly reduced the $Ca^{2+}$ as well as the $Na^+$ current of TRPV6- but not that of TRPV5-expressing HEK293 cells. By combining pull-down assays and co-immunoprecipitations, we demonstrated that CaM binds to both TRPV5 and TRPV6 in a $Ca^{2+}$-dependent fashion. The binding of CaM to TRPV6 was localized to the transmembrane domain ($TRPV6^{327–577}$) and consensus CaM-binding motifs located in the N (1–5-10 motif, $TRPV6^{88–97}$) and C termini (1–8-14 motif, $TRPV6^{643–656}$), suggesting a mechanism of regulation involving multiple interaction sites. Subsequently, chimeric TRPV6/TRPV5 proteins, in which the N and/or C termini of TRPV6 were substituted by that of TRPV5, were co-expressed with $CaM_{34}$ in HEK293 cells. Exchanging, the N and/or the C termini of TRPV6 by that of TRPV5 did not affect the $CaM_{34}$-induced reduction of the $Ca^{2+}$ and $Na^+$ currents. These results suggest that CaM positively affects TRPV6 activity upon $Ca^{2+}$ binding to EF-hands 3 and 4, located in the high $Ca^{2+}$ affinity CaM C terminus, which involves the N and C termini and the transmembrane domain of TRPV6.**

The superfamily of transient receptor potential (TRP)[1] channels is involved in diverse processes ranging from sensory activity to fertility and epithelial $Ca^{2+}$ transport (1). The subfamily members TRPV5 (formerly named ECaC1) and TRPV6 (formerly named CaT1) are postulated as gatekeepers facilitating epithelial $Ca^{2+}$ influx in kidney and small intestine (2, 3). An initial electrophysiological characterization of these channels revealed that TRPV5 and TRPV6 are homologous channels that display a $Ca^{2+}$-dependent feedback regulation of channel activity. Differences between these channels concern their divalent cation permeability, the kinetics of $Ca^{2+}$-dependent inactivation, and subsequent recovery (4). Furthermore, it was shown that TRPV5 and TRPV6 form functional homo- and heterotetrameric channels displaying intermediate phenotypes (5). At present, the molecular mechanism underlying these differences in $Ca^{2+}$-dependent activity is unknown.

$Ca^{2+}$-regulated channel activity is not restricted to TRPV5 and TRPV6 because a diverse range of voltage and non-voltage-dependent ion channels are regulated by the intracellular $Ca^{2+}$ concentration. $Ca^{2+}$-dependent regulation of the L-type and P/Q-type voltage-gated $Ca^{2+}$ channels (Cav) is mediated by the ubiquitously expressed $Ca^{2+}$-sensor calmodulin (CaM) (6, 7). CaM consists of four $Ca^{2+}$-binding EF-hand structures, which are localized in the N and C termini. $Ca^{2+}$ binding to CaM is highly cooperative with $Ca^{2+}$ binding, first to the C-terminal EF-hands, which have the highest affinity for $Ca^{2+}$, followed by $Ca^{2+}$ binding to lower affinity sites located in the N terminus (8). The CaM termini can differentially regulate ion channel activity, as shown by electrophysiological analysis of $Ca^{2+}$-dependent regulation of P/Q- and L-type Cav channels (6, 7). $Ca^{2+}$ binding to the high $Ca^{2+}$-affinity C terminus of CaM selectively induces P/Q-type Cav channel facilitation, whereas $Ca^{2+}$-sensing by the low $Ca^{2+}$-affinity N terminus induces channel inactivation. Thus, CaM acts as a $Ca^{2+}$-sensor translating the local $Ca^{2+}$ signals that modulate these channels (7).

Consensus CaM binding motifs have been identified in associated proteins (9). CaM binding to Cav is localized to an IQ-like motif in the $\alpha$1C (L-type Cav) and $\alpha$1A C termini (P/Q-type Cav). Although TRPV5 and TRPV6 lack an IQ-related motif, a previous study (4, 10) indicated that CaM binds to the TRPV6 C terminus in a $Ca^{2+}$-dependent manner. The described mechanism of competitive regulation of TRPV6 by protein kinase C and CaM is, however, restricted to human TRPV6, because this protein kinase C site in the CaM-binding motif is not conserved between different species (10). Recently, the corresponding region in mouse TRPV6 was shown to bind CaM. Detailed analysis of this region predicted a casein kinase motif, but no significant phosphorylation could be detected in this particular domain (11). Also, other members of the TRP family are possibly regulated by CaM. CaM binding to TRPV1

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[1] The abbreviations used are: TRP, transient receptor potential; Cav, voltage-gated $Ca^{2+}$ channels; CaM, $Ca^{2+}$-sensor calmodulin; IRES-GFP, internal ribosome entry site-green fluorescent protein; HEK, human embryonic kidney; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; GST, glutathione *S*-transferase.

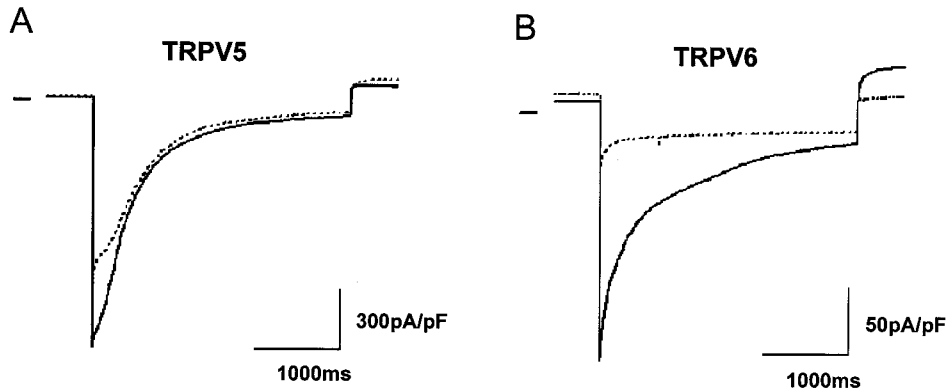


FIG. 1. **Effect of $Ca^{2+}$-insensitive CaM ($CaM_{1234}$) on TRPV5 and TRPV6 $Ca^{2+}$ current kinetics.** Mean currents through TRPV5-expressing (*A*) or TRPV6-expressing (*B*) HEK293 cells in response to a voltage step to −100 mV ($V_H$ = +70 mV) in the presence of 10 mM $[Ca^{2+}]_e$. Other cations were substituted by 150 mM $NMDG^+$. Cells were loaded with 10 mM BAPTA. Control traces are shown as *solid lines*; $CaM_{1234}$ traces are depicted as *dotted lines*. Zero current concentration is presented by a small horizontal *bar* at the *left* of each figure; $n > 11$ cells.

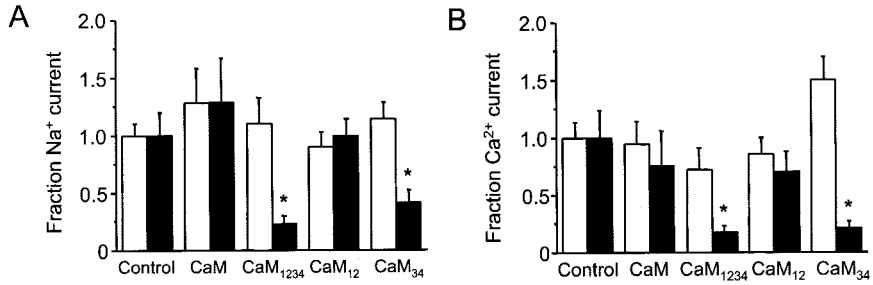


FIG. 2. **Effect of CaM mutants upon TRPV5 and TRPV6 activity.** Data obtained from linear ramps from +100 to −100 mV; holding potential was +20 mV. Currents were normalized to the density at −80 mV of controls. HEK293 cells heterologously expressing either TRPV5 or TRPV6 and CaM mutated in EF-hands in the N terminus ($CaM_{12}$), C terminus ($CaM_{34}$), or both ($CaM_{1234}$), as indicated on the *X*-axis. *A*, effect of CaM and CaM mutants on $Na^+$ current densities (100 $\mu$M EDTA in bath). *B*, effect of $CaM_{WT}$ and CaM mutants on $Ca^{2+}$ current densities in 10 mM $[Ca^{2+}]_e$. *White bars*, TRPV5; *black bars*, TRPV6. *, $p < 0.05$, significant difference from controls; $n$ = 7–23 cells.

was restricted to a 35 amino acid segment in the C terminus, and deletion of this CaM-binding segment prevented TRPV1 desensitization (12). Members of the TRPC family have been shown to bind CaM (13). Another study using CaM inhibitors described that TRPC6 can be regulated by CaM (14). Furthermore, CaM acts as a $Ca^{2+}$ sensor in the $Ca^{2+}$-dependent feedback inhibition of TRPC1, as demonstrated using $Ca^{2+}$-insensitive CaM mutants (15).

The aim of the present study was to investigate the potential role of CaM in $Ca^{2+}$-dependent regulation of TRPV5 and TRPV6 activity. To this end, we combined pull-down assays and co-immunoprecipitations with electrophysiological analysis of HEK293 cells heterologously expressing TRPV5, TRPV6, or TRPV5/6 chimeras in combination with $Ca^{2+}$-insensitive CaM mutants.

## EXPERIMENTAL PROCEDURES

*Construction of Mammalian Expression Vectors*—CaM, $CaM_{1234}$, $CaM_{12}$, and $CaM_{34}$ were mutated (D → A) in the EF-hand structures present in the N and C termini, resulting in (partial) $Ca^{2+}$-insensitive mutants (16). The CaM, $CaM_{1234}$, $CaM_{12}$, and $CaM_{34}$ (kindly provided by Dr. Adelman) (16) used in the patch-clamp analysis were cloned by PCR into the pCINeo/IRES-BFP vector (17) using the sense primer (5′-GGGACTAGTATGGCTGACCAACTGACTGAA-3′) and the antisense primer (5′-GGGCTGGAGTCACTTCGCTGTCATCATTTG-3′). $CaM_{34}$ was cloned by PCR into pGEX6p-2 (Amersham Pharmacia Biotech, Roosendaal, Netherlands) using the sense primer (5′-CGCGGATCCATGGCTGACCAACTGACTGAA-3′) and the antisense primer (5′-GGGGTCGACTCACTTCGCTGTCATCATTTG-3′). Mouse TRPV5, rabbit TRPV6, and TRPV5/6 chimeras-pCINeo/internal ribosome entry site-green fluorescent protein (IRES-GFP) were constructed as described previously (18). In short, TRPV5 and TRPV6 N termini were exchanged in pCINeo/IRES-GFP constructs via an introduced unique BspEI site at amino acid position $Ser^{221}$-$Tyr^{222}$, and C termini were exchanged by means of an introduced unique BsiWI site at amino acid position $Arg^{615}$-$Ser^{616}$. The fragment IQ-$\alpha$1C ($\alpha 1C^{1589-1708}$), containing the IQ-like motif from the L-type $Ca^{2+}$-channel $\alpha$-subunit C terminus (kindly provided by Dr. Birnbaumer) (19), TRPV5 N and C termini, and truncated forms of the TRPV6 C terminus were cloned into the oocyte-pT7Ts expression vector using PCR. Sense primers contained an EcoRV restriction site, a Kozak sequence, and ATG at the 5′ end, with ATG in-frame with the triplet encoding the first amino acid. Antisense primers contained an SpeI restriction site at the 3′ end, with a stop codon in-frame after the last amino acid. Truncated forms of the TRPV6 N terminus were cloned into the pT7Ts expression vector (using a sense primer as described above). The antisense primer contained a BstEII restriction site at the 3′ end, with a stop codon in-frame after the last amino acid. All pCINeo/IRES-BFP, pGEX6p-2, and pT7Ts constructs were verified by sequence analysis.

*Cell Culture and Transfection*—Human embryonic kidney (HEK293) cells were grown in Dulbecco's modified Eagle's medium (Bio Whittaker Europe, Vervier, Belgium) containing 10% (v/v) fetal calf serum (PAA, Linz, Austria), 13 mM $NaHCO_3$, 2 mM L-glutamine, 2 units/ml penicillin, and 2 mg/ml streptomycin at 37 °C in a humidity controlled incubator with 5% $CO_2$. The cells were transiently transfected with the pCINeo/IRES-GFP and pCINeo/IRES-blue fluorescent protein (BFP) vectors using Lipofectamine 2000 (Invitrogen-Life Technologies, Breda, Netherlands), as described previously (17). The GFP-containing vector was used for TRPV5, TRPV6, and TRPV5/6 chimeras, whereas the BFP-containing vector was used for CaM constructs. Transfected cells were identified visually by their green and/or blue appearance as described previously (17). GFP/BFP-negative cells from the same batch of cells were used as controls.

*Electrophysiology*—Electrophysiological methods have been described previously in detail (17, 20). Whole-cell currents were measured with an EPC-9 amplifier (HEKA, Lambrecht, Germany) using ruptured patches. Electrode resistances were 3–6 MΩ, and capacitance and access resistance were monitored continuously. A ramp protocol, consisting of linear voltage ramps from −100 to +100 mV (within 450 ms) was applied every 2 s from a holding potential of +20 mV. $Ca^{2+}$-dependent inactivation was studied using a 3-s voltage step to −100 mV from a holding potential of +70 mV. Current densities, expressed in units of

membrane capacitance, were calculated from the current at −80 mV during the ramp protocols (21).

*Solutions and Experimental Procedures*—The standard extracellular solution (Krebs) contained 150 mM NaCl, 6 mM CsCl, 1 mM $MgCl_2$, 10 mM HEPES/NaOH, pH 7.4, and 10 mM glucose. The concentration of $Ca^{2+}$ was varied between 1 and 10 mM. Divalent free solutions did not contain added divalent cations, whereas trace amounts of divalent cations were removed with 100 $\mu$M EDTA. To inhibit monovalent cation currents, 150 mM NaCl was replaced with an equimolar amount of *N*-methyl-D-glucamine-Cl. The standard internal (pipette) solution contained 20 mM CsCl, 100 mM Cs-aspartate, 1 mM $MgCl_2$, 4 mM $Na_2ATP$, 10 mM BAPTA, 10 mM HEPES/CsOH, pH 7.2. Cells were kept in a nominal $Ca^{2+}$-free medium to prevent $Ca^{2+}$ overload and exposed for a maximum of 5 min to a Krebs solution containing 1.5 mM $Ca^{2+}$ before sealing the patch pipette to the cell. All experiments were performed at room temperature.

*CaM-binding Assays*—pT7Ts constructs were linearized, and cRNA was synthesized *in vitro* using T7-RNA polymerase as described previously (21). [$^{35}$S]Methionine-labeled TRPV5 and TRPV6 proteins were prepared *in vitro* using a reticulocyte lysate system in the presence of canine microsomal membranes (Promega, Madison, WI). To investigate potential interactions, [$^{35}$S]methionine-labeled proteins were incubated for 2 h at room temperature either with CaM-coupled agarose beads or non-coupled agarose beads (Sigma) in Tris-buffered saline, pH 7.4 containing 1% (v/v) Triton X-100 and either 1 mM $CaCl_2$ or 5 mM EDTA. For pull-down experiments with $CaM_{34}$ (see Fig. 4*B*), glutathione *S*-transferase (GST)-$CaM_{34}$ and GST were expressed in and purified from transformed *Escherichia coli* BL21 according to the manufacturer's protocol (Amersham Pharmacia Biotech). For pull-down experiments (see Fig. 7), the indicated amount of free $Ca^{2+}$ was buffered with EGTA as calculated with SLIDERS version 2.10.[2] After extensive washing, bound proteins were subjected to SDS-PAGE, and binding was established by autoradiography.

*Xenopus laevis* oocytes were isolated as described previously (22) and subsequently co-injected with 5 ng of FLAG-tagged TRPV6 cRNA (23) and 15 ng of CaM cRNA. For each immunoprecipitation, 20 oocytes were lysed by incubation for 30 min on ice in buffer containing 150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 2 mM $CaCl_2$, 10% (v/v) glycerol, 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium desoxycholate, and the protease inhibitors leupeptin (0.01 mg/ml), pepstatin (0.05 mg/ml), and phenylmethylsulfonyl fluoride (1 mM). The lysates were centrifuged for 30 min at 16,000 × *g*, and supernatants were incubated with protein A beads (Kem-En-Tec A/S, Kopenhagen, Denmark), coated for 16 h at 4 °C with either rabbit anti-TRPV6 antiserum (23) (1:3000) or monoclonal anti-CaM (Campro-scientific, Veenendaal, The Netherlands) (1:2000). After extensive washing with lysis buffer, bound proteins were subjected to SDS-PAGE. After immunoblotting, co-immunoprecipitation was investigated by incubating either with anti-CaM or peroxidase-coupled anti-FLAG (Sigma).

*Statistical Analysis*—Data are expressed as mean ± S.E. Overall statistical significance was determined by analysis of variance. In case of significance ($p < 0.05$), individual groups were compared using a Student's *t* test.

## RESULTS

*Effect of CaM on TRPV5 and TRPV6 Activity*—To study the potential role of CaM in modulating TRPV5 and TRPV6, the activity of these channels was determined by whole-cell patch-clamp analysis in HEK293 cells transiently co-transfected with $Ca^{2+}$-insensitive CaM mutants and TRPV5 or TRPV6. The CaM mutant ($CaM_{1234}$), in which all four EF-hand structures were mutated, significantly reduced the inward $Ca^{2+}$ current in response to a hyperpolarizing voltage step in TRPV6-expressing HEK293 cells (Fig. 1*B*) ($p_{peak} < 0.05$, $n > 11$ cells), but had no significant effect upon TRPV5-expressing HEK293 cells (Fig. 1*A*) ($p_{peak} > 0.05$, $n > 11$ cells). Likewise, the $Na^+$ current, which was measured with a ramp protocol, was reduced in TRPV6- and $CaM_{1234}$-expressing cells, whereas in TRPV5-expressing cells, no effect was observed (Fig. 2). Subsequently, partially $Ca^{2+}$-insensitive CaM mutants were co-transfected with TRPV5 or TRPV6 in HEK293 cells (Fig. 2). CaM constructs were mutated (D → A) in either the low $Ca^{2+}$ affinity site present in the N terminus ($CaM_{12}$) or the high $Ca^{2+}$ affinity site present in the C terminus ($CaM_{34}$). Overexpression of $CaM_{1234}$ or $CaM_{34}$ significantly reduced the $Ca^{2+}$ as well as the $Na^+$ current density in TRPV6-expressing HEK293 cells, but did not affect the currents of TRPV5-expressing cells. In contrast, the expression of $CaM_{12}$ did not significantly affect the $Ca^{2+}$ or $Na^+$ currents of TRPV5- or TRPV6-expressing HEK293 cells. Thus, the high affinity EF-hand $Ca^{2+}$-binding sites contribute primarily to the observed CaM effect upon TRPV6 activity.

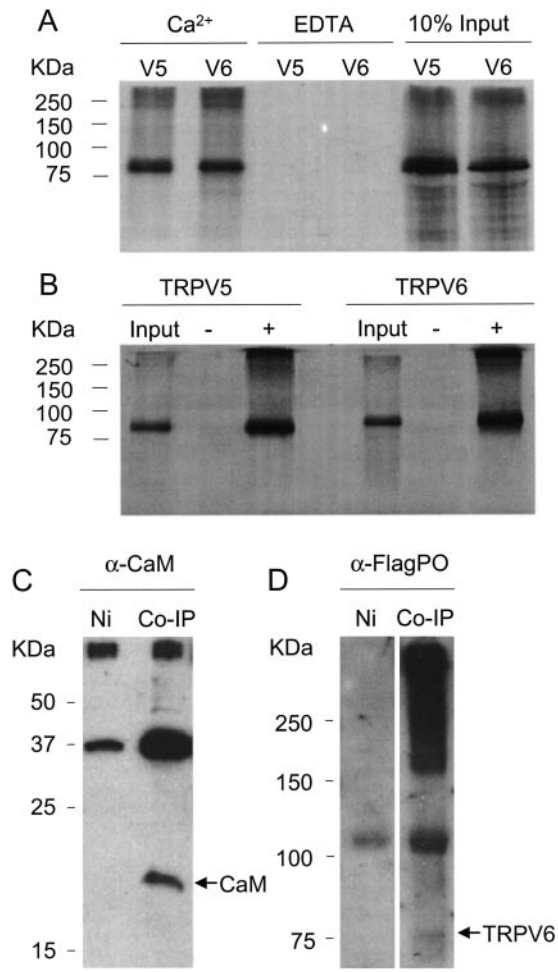


FIG. 3. **CaM binding to TRPV5 and TRPV6.** *A*, [$^{35}$S]Methionine-labeled, *in vitro* translated full-length TRPV5 or TRPV6 was incubated with CaM-coupled agarose beads either in the presence (1 mM $CaCl_2$) or absence (5 mM EDTA) of $Ca^{2+}$. *B*, [$^{35}$S]Methionine-labeled, *in vitro* translated full-length TRPV5 or TRPV6 was incubated with either CaM-coupled (+) or non-coupled (−) agarose beads in the presence (1 mM $CaCl_2$) of $Ca^{2+}$. Input control was 10% of the pull-down input. *X. laevis* oocytes were co-injected with 15 ng of $CaM_{WT}$ cRNA and 5 ng of FLAG-tagged TRPV6 cRNA. Oocyte lysates were loaded to protein A beads coated either with anti-TRPV6 (*C*) or monoclonal anti-CaM (*D*). Blots were incubated with monoclonal anti-CaM (*C*) or peroxidase-coupled monoclonal anti-FLAG (*D*).

*CaM Binding to TRPV5 and TRPV6*—Subsequently, CaM binding to TRPV5 and TRPV6 was investigated. Binding was determined by using CaM-coupled agarose beads and *in vitro* translated full-length TRPV5 or TRPV6. In the presence of $Ca^{2+}$ (1 mM $CaCl_2$), CaM bound to TRPV6, whereas in the absence of $Ca^{2+}$ (5 mM EDTA), binding was virtually abolished (Fig. 3*A*). Likewise, TRPV5 bound CaM as determined for TRPV6, although CaM mutants do not affect currents in TRPV5-expressing HEK293 cells. Binding experiments of *in vitro* translated full-length TRPV5 and TRPV6 to non-coupled

[2] Available on the World Wide Web at www.stanford.edu/~cpatton/maxc.html.

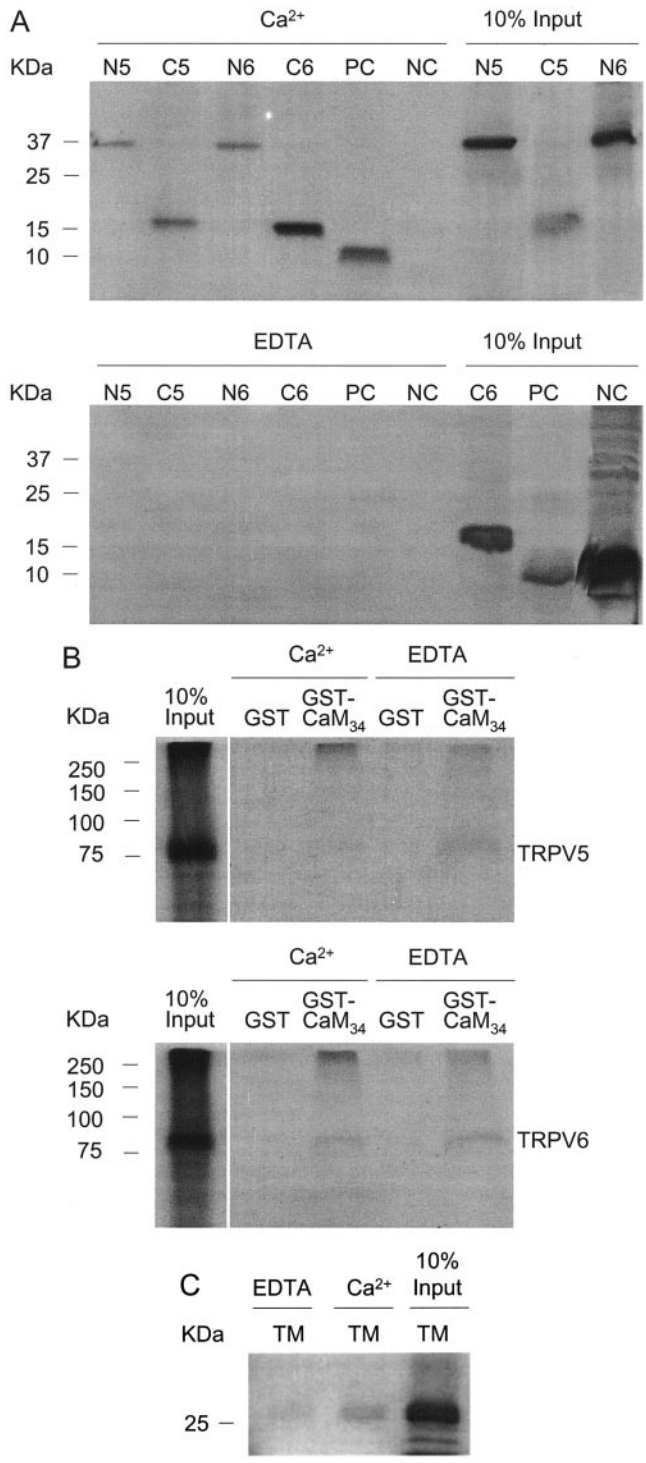


FIG. 4. **Localization of CaM interaction domains in TRPV5 and TRPV6.** *A*, [$^{35}$S]Methionine-labeled *in vitro* translated TRPV5 and TRPV6 N termini (TRPV5$^{1–327}$, TRPV6$^{1–326}$), C termini (TRPV5$^{578–730}$, TRPV6$^{577–727}$), positive (IQ-$\alpha$1C) and negative (TRPV5$^{421–512}$) controls were incubated with CaM-coupled agarose beads in the presence of 5 mM EDTA or 1 mM $CaCl_2$. *B*, [$^{35}$S]Methionine-labeled *in vitro* translated TRPV5 and TRPV6 were incubated with GST and GST-$CaM_{34}$ in the presence of 5 mM EDTA or 1 mM $CaCl_2$. *C*, [$^{35}$S]Methionine-labeled *in vitro* translated TRPV6 transmembrane domain (TRPV6$^{327–577}$) was incubated with CaM-coupled agarose beads in the presence of 5 mM EDTA or 1 mM $CaCl_2$. Input control was 10% of the pull-down input.

and CaM-coupled agarose beads in the presence of $Ca^{2+}$ indicated both TRPV5 and TRPV6 specifically bind CaM (Fig. 3*B*). Subsequently, we tested the association of TRPV6 and CaM by co-immunoprecipitation studies. To this end, CaM and FLAG-

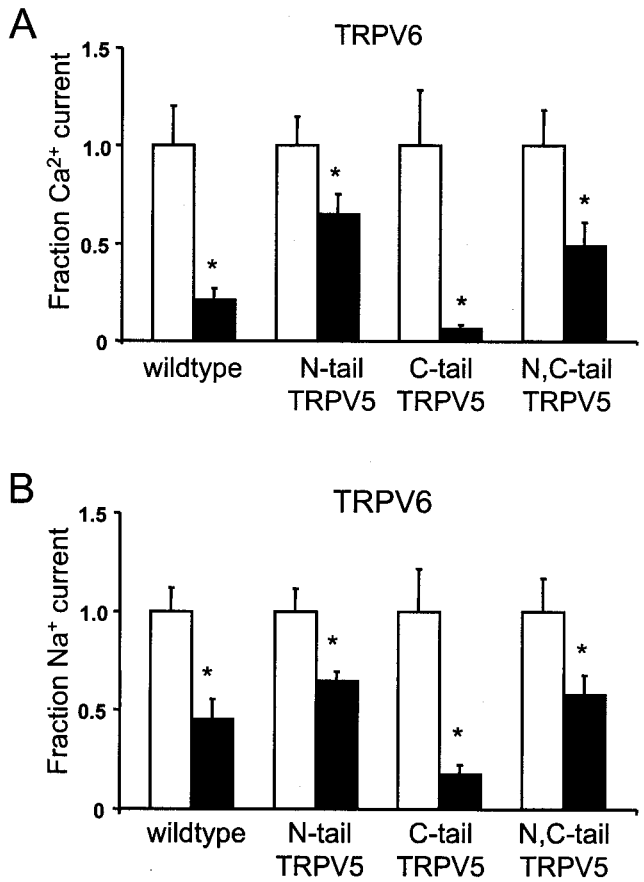


FIG. 5. **Effect of the N and C termini and transmembrane domain of TRPV6 on the inhibitory effect of $CaM_{34}$.** Effects of $CaM_{34}$ on $Na^+$ current density of TRPV6-expressing HEK293 cells with 100 $\mu$M EDTA in the bath solution. Data obtained from linear ramps from +100 to −100 mV; holding potential was +20 mV. Currents were normalized to the density at −80 mV of controls. TRPV6 chimeras (TRPV6 N and/or C termini replaced by that of TRPV5) were used for the experiments. *White bars* represent control currents; *black bars* represent co-transfection with $CaM_{34}$; $n > 12$ cells.

TRPV6 were co-expressed in *X. laevis* oocytes and immunoprecipitated with the CaM or TRPV6 antibodies. Immunoblots containing the precipitated complexes were probed with a peroxidase-coupled FLAG antibody or the CaM antibody, respectively. CaM (17 kDa) co-immunoprecipitated with the FLAG-TRPV6 and *vice versa*, confirming the association of CaM and TRPV6 (Fig. 3, *C* and *D*).

To further identify the CaM-binding site in TRPV5 and TRPV6, the N termini (TRPV5$^{1–327}$, TRPV6$^{1–326}$) and C termini (TRPV5$^{578–730}$, TRPV6$^{577–727}$) were *in vitro* translated, and subsequently CaM binding was determined (Fig. 4*A*). The described IQ-like CaM-binding motif in the C terminus of $\alpha$1C (24) was used as a positive control, whereas TRPV5 transmembrane region TRPV5$^{421–512}$ was used as a negative control. Besides the positive control, the N and C termini of both TRPV5 and TRPV6 bound CaM in the presence of $Ca^{2+}$, whereas no binding was observed with the negative control. In addition, it was demonstrated that both TRPV5 and TRPV6 bind to GST-$CaM_{34}$ in a $Ca^{2+}$-independent manner (Fig. 4*B*). Furthermore, the TRPV6 transmembrane domain (TRPV6$^{327–577}$) was *in vitro* translated, and subsequently CaM binding was determined (Fig. 4*C*). The TRPV6 transmembrane domain bound CaM only in the presence of $Ca^{2+}$, although binding seemed weaker than the CaM binding to both TRPV6 tails.

*Functional Effect of CaM-binding Domains in TRPV6*—Subsequently, TRPV5/6 chimeras were used to identify the critical

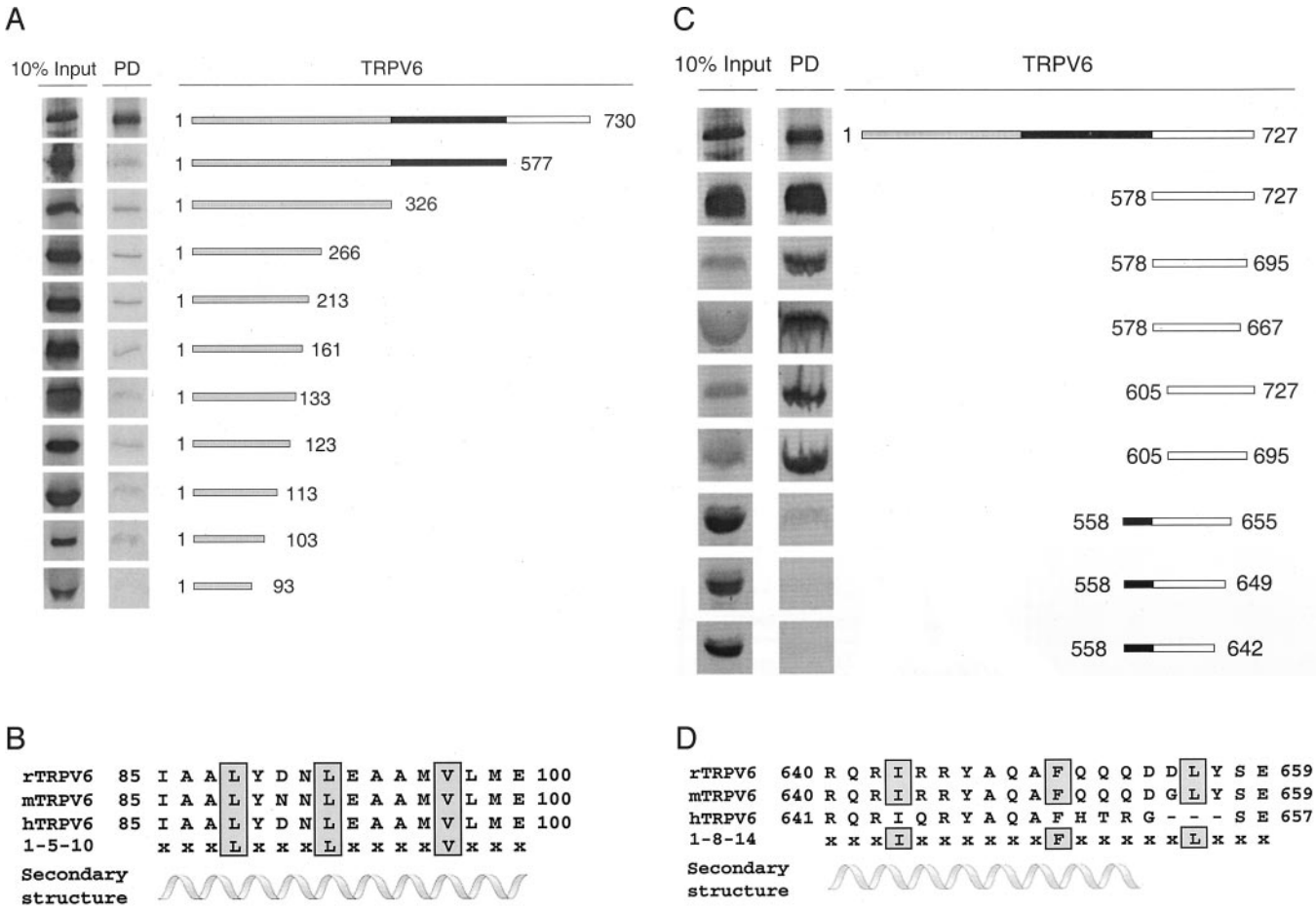


FIG. 6. **Mapping of the CaM-binding site in the N and C termini of TRPV6.** [$^{35}$S]Methionine-labeled *in vitro* translated N (*A*) or C (*C*) termini truncates were constructed and incubated with CaM-coupled agarose beads in the presence of 1 mM $Ca^{2+}$. Input control was 10% of the pull-down input. *B* and *D*, alignment of N- or C-terminal CaM-binding motif of different species with consensus CaM-binding motifs (*boxed*). From *top* to *bottom*: rat TRPV6 (AF160798); mouse TRPV6 (NM022413); human TRPV6 (NM018646). The structure prediction program GORIV was used to determine whether this region consisted of an $\alpha$-helix.

domains in TRPV6 for the inhibition by $CaM_{34}$. To this end, the effect of $CaM_{34}$ upon the $Ca^{2+}$ and $Na^+$ current densities was examined in HEK293 cells expressing the TRPV5/6 chimera, in which the N and/or C termini were exchanged (Fig. 5). Co-transfection of $CaM_{34}$ and TRPV6-C5, the chimera in which the TRPV6 C terminus was substituted by that of TRPV5, revealed a reduction of both the $Ca^{2+}$ and $Na^+$ currents, as observed with the co-transfections of $CaM_{34}$ and wild-type TRPV6. Co-transfection of $CaM_{34}$ with TRPV6-N5, the chimera in which the N terminus of TRPV6 was substituted by that of TRPV5, resulted in a reduction of the $Ca^{2+}$ and $Na^+$ currents, as compared with currents in TRPV6-N5-expressing cells. Co-transfection of $CaM_{34}$ and TRPV6-N5C5, the chimera in which both termini of TRPV6 were substituted by that of TRPV5, resulted in a reduction of the $Ca^{2+}$ and $Na^+$ currents, as compared with currents measured in TRPV6-N5C5-expressing cells.

*Elucidation of the CaM-binding Sites in TRPV6*—A series of TRPV6 N- and C-terminal deletion mutants were constructed as depicted in Fig. 6. Truncated forms of TRPV6 were *in vitro* translated, and binding was determined as described above. The interaction between TRPV6 N terminus and CaM was abolished when the N terminus was truncated at position 93, whereas truncations at positions 103 up to 266 had no effect upon the interaction with CaM (Fig. 6*A*). This result indicates that CaM binds the TRPV6 N terminus in a region at position 93–103. Detailed analysis according to the structural prediction program GORIV revealed that this putative CaM-binding region consists of an $\alpha$-helix.[3] A 1–5-10 motif with consensus sequence (FILVW)*XXX*(FAILVW)*XXXX*(FILVW) is present in this particular binding region (Fig. 6*B*). The interaction between TRPV6 C terminus and CaM was partially abolished when the C terminus was truncated at position 655, whereas truncations at position 649 and 642 completely abolished CaM binding (Fig. 6*C*). Truncation at position 667 up to 695 had no effect upon the interaction with CaM. These findings suggest that CaM binds the TRPV6 C terminus at position 649–667. This putative binding region contains a 1–8-14 motif with consensus sequence (FILVW)*XXXXXX*(FAILVW)*XXXXX*(FILVW) and consists of an $\alpha$-helix (Fig. 6*D*).

*CaM Binding to TRPV5 and TRPV6 at Low $Ca^{2+}$ Concentrations*—The $Ca^{2+}$-dependent binding of CaM to TRPV5 and TRPV6 was studied in detail using the TRPV5 and TRPV6 N termini ($TRPV5^{1–327}$, $TRPV6^{1–326}$) and C termini ($TRPV5^{578–730}$, $TRPV6^{577–727}$) in the absence or presence of increasing amounts of $Ca^{2+}$ (Fig. 7). Both the TRPV5 N and C termini bound CaM at a $Ca^{2+}$-concentration of 30 nM or higher, whereas binding is lost in the absence of $Ca^{2+}$ (Fig. 7*A*). Similar observations were made for TRPV6 (Fig. 7*B*); thus, CaM is bound to both TRPV5 and TRPV6 at $Ca^{2+}$-concentrations as low as 30 nM.

[3] Available on the World Wide Web at www.expasy.org.

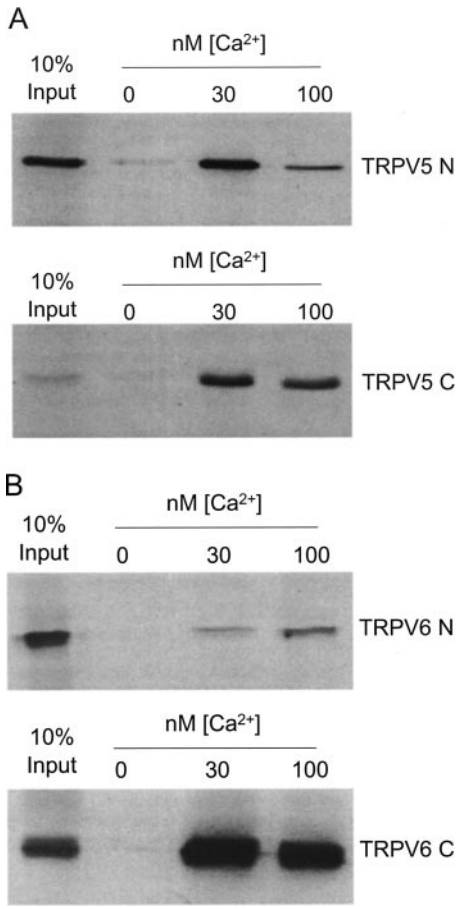


FIG. 7. **CaM binding to TRPV5 and TRPV6 at low $Ca^{2+}$ concentrations.** [$^{35}$S]Methionine-labeled *in vitro* translated TRPV5 (*A*) and TRPV6 (*B*) N and C termini were incubated with CaM-coupled agarose beads in the absence (5 mM EGTA) or presence of increasing concentrations of $Ca^{2+}$ (30 or 100 nm). Input control is 10% of the pull-down input. The figure shown is a representative blot of three independent experiments.

## DISCUSSION

The present study identified CaM as a $Ca^{2+}$-sensor specifically regulating TRPV6 activity. This finding is based upon the following observations. First, electrophysiological measurements of HEK293 cells heterologously expressing TRPV5 or TRPV6 and CaM mutants revealed that TRPV6, but not TRPV5, is regulated by CaM, although both channels bind CaM in a $Ca^{2+}$-dependent fashion. Second, the effect of CaM is mediated by the high $Ca^{2+}$-affinity EF-hand structures 3 and 4 present in the C terminus of CaM. Third, binding studies indicated that CaM binds in a $Ca^{2+}$-dependent fashion to both the TRPV5 and TRPV6 N termini ($TRPV5^{1–327}$, $TRPV6^{1–326}$) and C termini ($TRPV5^{578–730}$, $TRPV6^{577–727}$) and to the TRPV6 transmembrane domain ($TRPV6^{327–577}$). Fourth, co-transfection of $CaM_{34}$ and various TRPV5/6 chimeras revealed that the effect of CaM is mediated via the identified CaM-binding domains in TRPV6.

Previously, it has been shown that TRPV5 and TRPV6 activity are negatively regulated by the intracellular $Ca^{2+}$ concentration, and the ubiquitously expressed CaM could potentially play a regulatory role in this process (10, 11, 25). Interestingly, CaM is involved in the $Ca^{2+}$-dependent regulation of TRPV6 only, because $CaM_{1234}$ significantly reduced the $Ca^{2+}$ and $Na^+$ current densities in TRPV6-expressing HEK293 cells, whereas TRPV5-mediated currents were not significantly altered. This latter finding is remarkable, given the high homology between both channels, similar $Ca^{2+}$-dependent regulation of channel activity, and binding of CaM to both channels. The effect of $CaM_{1234}$ was localized to the high $Ca^{2+}$-affinity EF-hand structures in the CaM C terminus (EF-hand 3 and 4). Overexpression of $CaM_{34}$, the CaM mutant that lacks high-affinity $Ca^{2+}$ binding, reduced the TRPV6 current density to the same extent as $CaM_{1234}$, whereas $CaM_{12}$ did not effect TRPV6 currents. The effect of $CaM_{34}$ upon TRPV6 is not restricted to the $Ca^{2+}$ current because the $Na^+$ current density was also inhibited when $CaM_{34}$ was co-expressed. Together, these results imply that CaM positively affects TRPV6 activity at basal $Ca^{2+}$ concentrations by means of either structural changes in TRPV6 (leading to an increased open probability) or accumulation of active channels on the plasma membrane. However, to date, several studies on voltage-operated $Ca^{2+}$ channels (6, 7, 23, 26–28) have indicated that CaM can regulate channel activity on the plasma membrane, whereas little evidence has been presented for effects upon the routing of the channels.

The association of CaM with TRPV5 was localized to the N terminus ($TRPV5^{1–327}$) and C terminus ($TRPV5^{578–730}$). The association of CaM with TRPV6 was localized to consensus CaM-binding motifs in $\alpha$-helical structures present in the N and C termini and to the transmembrane part ($TRPV6^{327–577}$) of TRPV6, where CaM binds in a $Ca^{2+}$-dependent fashion. The N-terminal binding is localized to a consensus 1–5-10 motif (position 88–92-97) that is identical among different TRPV6 species. A 1–5-10 motif is a classical region for the binding of $Ca^{2+}$-CaM, which fits with the $Ca^{2+}$-dependent binding of CaM to the TRPV6 N terminus. The C-terminal binding is localized to a consensus 1–8-14 motif (position 643–650-656) that is identical between mouse and rat TRPV6. However, human TRPV6 does not contain a 1–8-14 motif at this particular position, which might explain the previous observation that CaM binding to human TRPV6 is localized to a different region (10). When mouse TRPV6 is truncated at the position corresponding to the described human binding region (10), it still binds CaM, whereas truncations in the identified 1–8-14 motif virtually abolished CaM binding (this study). The mouse TRPV6 peptide, corresponding to the described human binding region, binds CaM (11); however, these results are not in line with our results. The fact that only the TRPV6 activity was inhibited by $CaM_{34}$, despite binding of $CaM_{WT}$ and $CaM_{34}$ to both TRPV5 and TRPV6, suggests that the regulatory effect of CaM is restricted to TRPV6.

TRPV5 and TRPV6 chimeras were used to identify the essential domains of TRPV6 for the CaM effect because CaM mutants had no effect upon TRPV5-expressing HEK293 cells, and TRPV5/6 chimeras give functional channels (18). The TRPV6 domains that bind CaM all contribute to the $CaM_{34}$ effect, because chimeras in which the N and/or C termini of TRPV6 were substituted by that of TRPV5, revealed a significant decrease in both the $Ca^{2+}$ and $Na^+$ current densities when $CaM_{34}$ was co-expressed. Comparison of current amplitudes of the different chimeras suggests that the TRPV6 N terminus contributes predominantly to the inhibitory effect of $CaM_{34}$, because the $CaM_{34}$-induced decrease is maximally diminished when the TRPV6 N terminus is substituted by that of TRPV5.

The dominant-negative effect of CaM mutants upon TRPV6 activity suggests a constant tethering of CaM to the channel complex, because overexpression of $Ca^{2+}$-insensitive CaM would otherwise not interfere appreciably with currents of TRPV6-expressing HEK293 cells containing endogenous wild-type CaM. As described for Cav channels (23), we suggest a tight and constitutive interaction between TRPV6 and CaM. The fact that CaM inhibitors do not affect TRPV6 activity (data not shown) supports the tight and constitutive interaction, as

described previously for L-type Cav channels (23). The CaM binding to the TRPV6 N and C termini at a $Ca^{2+}$ concentration as low as 30 nM suggests that CaM is tethered to TRPV6 at $Ca^{2+}$-concentrations normally present in resting cells (60–100 nm). Thus, it is likely that CaM is constitutively tethered to the TRPV6 channel complex.

In conclusion, our data demonstrate a regulatory role of CaM in TRPV6-mediated $Ca^{2+}$ influx. The elucidated molecular mechanism possibly involves constitutive tethering of CaM to the TRPV6 channel complex. Upon $Ca^{2+}$ sensing of the CaM C terminus, TRPV6 activity is positively affected by means of the combined actions at the N and C termini and transmembrane domain of the channel.

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