

Ca²⁺/Calmodulin Modulates TRPV1 Activation by Capsaicin

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ABSTRACT TRPV1 ion channels mediate the response to painful heat, extracellular acidosis, and capsaicin, the pungent extract from plants in the *Capsicum* family (hot chili peppers) (Szallasi, A., and P.M. Blumberg. 1999. *Pharmacol. Rev.* 51:159–212; Caterina, M.J., and D. Julius. 2001. *Annu. Rev. Neurosci.* 24:487–517). The convergence of these stimuli on TRPV1 channels expressed in peripheral sensory nerves underlies the common perceptual experience of pain due to hot temperatures, tissue damage and exposure to capsaicin. TRPV1 channels are nonselective cation channels (Caterina, M.J., M.A. Schumacher, M. Tominaga, T.A. Rosen, J.D. Levine, and D. Julius. 1997. *Nature.* 389:816–824). When activated, they produce depolarization through the influx of Na⁺, but their high Ca²⁺ permeability is also important for mediating the response to pain. In particular, Ca²⁺ influx is thought to be required for the desensitization to painful sensations over time (Cholewinski, A., G.M. Burgess, and S. Bevan. 1993. *Neuroscience.* 55:1015–1023; Koplas, P.A., R.L. Rosenberg, and G.S. Oxford. 1997. *J. Neurosci.* 17:3525–3537). Here we show that in inside-out excised patches from TRPV1 expressed in *Xenopus* oocytes and HEK 293 cells, Ca²⁺/calmodulin decreased the capsaicin-activated current. This inhibition was not mimicked by Mg²⁺, reflected a decrease in open probability, and was slowly reversible. Furthermore, increasing the calmodulin concentration in our patches by coexpression of wild-type calmodulin with TRPV1 produced inhibition by Ca²⁺ alone. In contrast, patches excised from cells coexpressing TRPV1 with a mutant calmodulin did not respond to Ca²⁺. Using an in vitro calmodulin-binding assay, we found that TRPV1 in oocyte lysates bound calmodulin, although in a Ca²⁺-independent manner. Experiments with GST-fusion proteins corresponding to regions of the channel NH₂-terminal domain demonstrated that a stretch of ~30 amino acids adjacent to the first ankyrin repeat bound calmodulin in a Ca²⁺-dependent manner. The physiological response to pain involves an influx of Ca²⁺ through TRPV1. Our results indicate that this Ca²⁺ influx may feed back on the channels, inhibiting their gating. This type of feedback inhibition could play a role in the desensitization produced by capsaicin.

KEY WORDS: desensitization • capsaicin • TRPV1 • calcium • calmodulin

INTRODUCTION

The first TRP channel was identified in 1977 as a phototransduction mutant in *Drosophila* (Minke, 1977). Since that time the TRP channel family has grown to include at least 20 members (Clapham et al., 2001). The importance of TRP channels is demonstrated by the wide variety of their proposed functions: invertebrate phototransduction, responding to painful stimuli, responding to moderate temperature changes; repletion of intracellular calcium stores, receptor-mediated excitation, and modulation of the cell cycle (for reviews see Clapham et al., 2001; Montell, 2001; Montell et al., 2002).

Despite the clear physiological importance of TRP channels, little is known about what regulates their function. One type of TRP channel, TRPV1, is a polymodal receptor that integrates a number of painful stimuli. These stimuli include: noxious heat, with a threshold of ~42°C; extracellular acidification, with a pK_a of ~5.3 (Caterina et al., 1997; Jordt et al., 2000);

anandamide and other arachidonic acid metabolites (Smart et al., 2000); and capsaicin, a pungent extract from plants in the *Capsicum* family (Szallasi and Blumberg, 1999; Caterina and Julius, 2001). Although all the details of the capsaicin binding site are not known, it has been shown to bind from the intracellular side (Jung et al., 1999), though this result is controversial (Vyklícký et al., 2003).

With intracellular NH₂- and COOH-terminal domains, six putative membrane-spanning domains, and sequence similarity in the pore region, TRP channels fall within the superfamily of ion channels that includes voltage-gated Na⁺, K⁺, and Ca²⁺ channels and cyclic nucleotide-gated (CNG) channels (Jan and Jan, 1990, 1992). For those ion channels within this superfamily for which stoichiometry has been directly examined, all have been shown to be composed of four six transmembrane domain subunits or pseudosubunits, with auxiliary subunits sometimes present as well (Clapham et al., 2001).

TRPV1 channels are nonselective cation channels that do not discriminate among monovalent cations and are

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Abbreviations used in this paper: CaM, calmodulin; CNG, cyclic nucleotide-gated.

highly permeable to Ca^{2+} (Oh et al., 1996). Ca^{2+} is especially important for TRPV1 function, as extracellular Ca^{2+} mediates desensitization (Koplas et al., 1997), a process which enables a neuron to adapt to specific stimuli by diminishing its overall response to a sustained chemical or physical signal. Desensitization of TRPV1 ion channels is composed of at least two separate cellular mechanisms. One type of desensitization has been shown to depend on an increase in intracellular Ca^{2+} (Cholewinski et al., 1993; Docherty et al., 1996a; Liu and Simon, 1996; Koplas et al., 1997) and accounts for most of the acute (seconds) desensitization. There is also a slower (minutes) process of desensitization that can occur in the absence of extracellular Ca^{2+} (Docherty et al., 1996a; Liu and Simon, 1996; Koplas et al., 1997). There is good evidence that the rate at which acute Ca^{2+} -dependent desensitization develops is increased by dephosphorylation of the ion channels by the Ca^{2+} and calmodulin-dependent enzyme calcineurin (Docherty et al., 1996b).

Although it is known that desensitization of the TRPV1 channel depends on the presence of intracellular Ca^{2+} , the molecular mechanism of this regulation has remained elusive. It has recently been shown that a 35-amino acid region in the COOH-terminal region of TRPV1 (amino acids 767–801) binds calmodulin (CaM) in an in vitro assay in a Ca^{2+} -dependent manner (Numazaki et al., 2003). Mutant channels in which this region was deleted continued to show desensitization in whole-cell experiments, albeit with altered kinetics. We directly addressed whether CaM might act as a Ca^{2+} sensor, transducing changes in Ca^{2+} concentration into changes in channel behavior. We found that application of Ca^{2+} alone to the intracellular surface of excised patches produced a small decrease in capsaicin-activated current that could not be mimicked by Mg^{2+} . Application of Ca^{2+} with CaM produced a much larger reduction in current. Overexpression of CaM also potentiated the inhibitory effect of Ca^{2+} , whereas coexpression of a mutant CaM with TRPV1 did not produce a Ca^{2+} mediated inhibition. Using GST-fusion proteins corresponding to regions of the TRPV1 NH_2 terminus, we localized a CaM binding site to a region including amino acids 189–222. CaM binding to this NH_2 -terminal region was highly Ca^{2+} dependent. However, in vitro binding assays using full-length channels from cell lysates suggests a second, Ca^{2+} -independent binding site may be present as well. Our data indicate that CaM can mediate Ca^{2+} -dependent inhibition of capsaicin-activated currents, with the NH_2 -terminal region as a candidate binding site mediating this inhibitory action of Ca^{2+} /CaM.

MATERIALS AND METHODS

Oocyte Electrophysiological Recordings

Rat TRPV1 (a gift from Dr. D. Julius) was subcloned into either pGEMHE, a gift of Dr. E.R. Liman, or pCDNA3 modified to con-

tain the 5' and 3' untranslated regions of the *Xenopus* β -globin genes. A FLAG epitope tag was appended to the COOH-terminal end of the channel and did not affect measured functional properties (unpublished data). Rat wild-type calmodulin and mutant calmodulin_{1,2,3,4} in pGEMHE were a gift from Dr. J. Adelman. Inside-out patch clamp recordings were made using symmetrical solutions consisting of 130 mM NaCl, 3 mM HEPES (pH 7.2), and 200 μM EDTA for Ca^{2+} -free conditions. For solutions containing Ca^{2+} , EDTA was replaced with the given concentrations of Ca^{2+} , and the free Ca^{2+} concentrations were confirmed using a Ca^{2+} electrode (Qwick-Tip; World Precision Instruments). The measured free Ca^{2+} concentration in our bath (before addition of Ca^{2+}) was 64 nM. We originally used MaxChelator (<http://www.stanford.edu/~cpatton/maxc.html>) to determine appropriate buffer and Ca^{2+} concentrations for our experiments. Two lines of evidence suggested that this method did not appropriately control the Ca^{2+} concentration with either EGTA or HEDTA. First, the efficacy of Ca^{2+} in modulating channel function varied from one batch of solutions to the next. Second, the free Ca^{2+} concentration measured with the Ca^{2+} electrode similarly varied between batches of solution. Surprisingly, using unbuffered solution we observed consistent readings of both the free Ca^{2+} with the Ca^{2+} electrode (within a factor of two or less), as well as inhibition of the channels in the presence of calmodulin.

Calmodulin (Calbiochem) stocks were prepared in water and dilutions were made to obtain a final concentration of 500 nM. All solutions were applied to the intracellular surface of the patches with an RSC-200 rapid solution changer (Molecular Kinetics). Capsaicin (Sigma-Aldrich) stocks were prepared in ethanol and diluted to final concentrations of 4 or 20 μM , both saturating concentrations.

Currents were low pass-filtered at 2 kHz with an Axopatch 200B amplifier (Axon Instruments, Inc.) and sampled at 10 kHz. Data were acquired and analyzed with PULSE data acquisition software (HEKA, Inc.) and were plotted and fitted using Igor Pro (Wavemetrics, Inc.). For macroscopic recordings, the following voltage protocol was used. Patches were held at 0 mV and given a prepulse of -120 mV for 30 ms. The voltage was then stepped to voltages ranging from -150 to 110 mV in steps of 20 mV, for 60 ms. Currents in the absence of capsaicin were subtracted. For single-channel experiments, patches were held at 0 mV and stepped to $+60$ mV for 500 ms. In these experiments open probability (P_o) was calculated from fits to the data with two Gaussians. The area under the curve representing open channels relative to the total area was taken as P_o . Another way of estimating P_o for each experimental condition is to measure the instantaneous tail currents. Tail currents were measured using a protocol in which the patch was stepped to -120 mV for 10 ms, and then from -150 to 110 mV for 60 ms in increments of 20 mV, and then finally to -120 mV for 10 ms again to measure the tail currents. We assumed that $I_T(V) = P_o(V) \cdot N \cdot i(V)$, where $I_T(V)$ is the tail currents at a voltage V , $P_o(V)$ is the open probability as a function of voltage, N is the number of channels, and $i(V)$ is the unitary conductance at V . In this case, the values obtained from the tail currents are proportional to P_o since N and $i(V)$ did not vary (as seen from our single channel experiments). Student's t test (two-tailed, unpaired) was used as a statistical test in all cases where significance is discussed, with the threshold of significance set as $P < 0.05$.

Mammalian Cell Culture and Recording

HEK 293 cells expressing large T antigen were transfected with TRPV1-pCDNA3 and pIRES-GFP (BD Biosciences; to visualize successfully transfected cells) with Fugene (Roche Diagnostics) according to manufacturer's instructions. Two days after transfection had been initiated, cells were passaged onto coverslips. Cells were used for recording three days after passing. For coexpres-

sion with wild-type or mutant CaM, HEK 293 cells were transfected as described above using TRPV1 and either cYFP-CaM in pCDNA3 or CaM_{1,2,3,4} in pCDNA3 (both gifts of Dr. D. Yue) and pIRES-GFP. Recording solutions consisted of 130 mM NaCl and 3 mM HEPES (pH 7.2) either without added Ca²⁺ or with the addition of the stated concentrations of Ca²⁺. In every case, the free Ca²⁺ concentration was confirmed using a Ca²⁺ electrode as described above.

CaM Pull-down Assay

Oocytes were prepared as described (Rho et al., 2000; Rosenbaum et al., 2002). 2 mM Ca²⁺ or 2 mM EGTA were added to the lysis buffer as indicated in the text and figures. Cell lysates were incubated at 4°C overnight with 50 μl of a 50% CaM-agarose suspension (Sigma-Aldrich). The material was spun and washed with 1 ml of frog Ringer's solution with either 2 mM Ca²⁺ or 2 mM EGTA. After 15 washes, protein adhering to the beads was eluted with SDS sample buffer (with β-mercaptoethanol, final concentration of 0.71 M) and run on a Nupage 3–8% Tris-Acetate gel (Invitrogen). Western blots were performed as described previously (Rosenbaum et al., 2002) with anti-FLAG antibody (Sigma-Aldrich). Densitometry was performed using FlourChem software (Alpha Innotech) directly from chemiluminescent blots.

For the CaM pulldown assay with GST-fusion proteins, the fusion proteins purified from bacteria (see below) were incubated with CaM-agarose beads overnight in the presence of either 2 mM Ca²⁺ or 2 mM EGTA. The beads were then washed five times with 1 ml of the lysis buffer described above (Rho et al., 2000; Rosenbaum et al., 2002), also supplemented with either Ca²⁺ or EGTA. The proteins were then eluted using SDS sample buffer (with β-mercaptoethanol, final concentration of 0.71 M). Western blots for these experiments were done using either anti-GST or anti-FLAG antibodies (Santa Cruz Biotechnology, Inc.).

GST Fusion Protein Expression and Purification

Fragments of the NH₂-terminal region of TRPV1 (accession no. AF029310) were amplified using PCR primers with EcoRI and XhoI restriction sites with the addition of a FLAG epitope to the

COOH terminus of some of the constructs, and cloned into the multicloning site of pGEX-6P-1. BL21-competent cells were transformed with the GST-TRPV1 clones. 250 ml of LB with ampicillin (50 mg/L) were inoculated 1:10 with an overnight culture of bacteria and incubated at 37°C until OD₆₀₀ ~0.4. The cells were induced with 0.1 mM IPTG for 1.5 h, sonicated in 5 ml of PBS with protease inhibitors for 60 s and Triton X-100 was added to reach a final concentration of 0.1%. This mixture was then agitated at 4°C for 30 min. The proteins were purified using a batch method by incubation with 800 μl of a 50% GSH-agarose slurry for 30 min at room temperature, and then washed extensively with PBS. The proteins were eluted with 50 mM Tris pH 8, 15 mM GSH, 0.1% Triton X-100 for 30 min. For some experiments, a final concentration of 1 mM DTT was used in all steps starting with sonication.

RESULTS

To investigate the molecular basis for Ca²⁺ regulation of TRPV1 activity, we expressed TRPV1 channels in *Xenopus* oocytes and studied their activity using the inside-out configuration of the patch-clamp technique. This approach allowed us to control the solution bathing the intracellular surface of the channels, so that the capsaicin concentration activating the channels and the Ca²⁺ concentration at the intracellular surface could be controlled independently. Activation of TRPV1, and the subsequent entry of extracellular Ca²⁺ through the channels into cells, is believed to initiate a negative feedback loop that results in decreased TRPV1 current. This negative feedback is known as desensitization. We hypothesized that CaM may act as a Ca²⁺ sensor for TRPV1 channels, modulating channel activity in response to increases in intracellular Ca²⁺ concentration. To test whether Ca²⁺/CaM could alter TRPV1 ac-

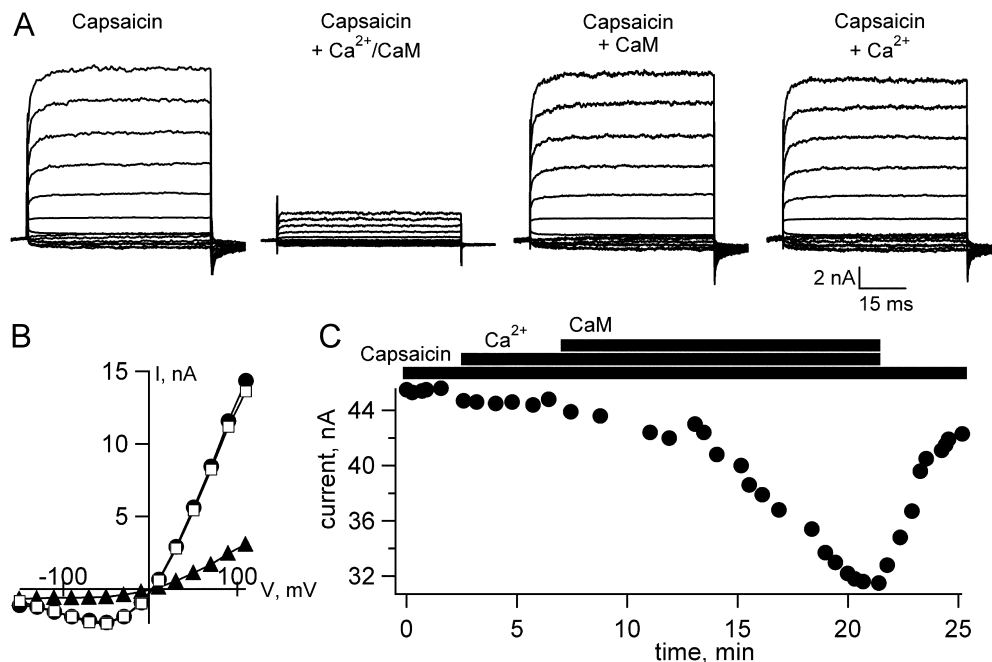


FIGURE 1. CaM mediates Ca²⁺ inhibition of TRPV1 channels expressed in *Xenopus* oocytes. (A) Current families obtained in the presence of 20 μM capsaicin, 20 μM capsaicin + 500 nM CaM + 50 μM free Ca²⁺, 20 μM capsaicin + 500 nM CaM, or 20 μM capsaicin + 50 μM free Ca²⁺, as indicated by the labels. (B) IV relation for currents shown in (A). Circles, capsaicin; squares, capsaicin + Ca²⁺; and triangles, capsaicin + Ca²⁺/CaM. (C) Time course of inhibition by and recovery from Ca²⁺/CaM. The intracellular (bath) solution contained 20 μM capsaicin, 50 μM free Ca²⁺, and/or 500 nM CaM, as indicated by the bars above the graph. The bar labels correspond to the bar immediately beneath each label.

tivity in our patches, we applied purified CaM along with 50 μM free Ca^{2+} to the inside surface of the patches. We found that application of $\text{Ca}^{2+}/\text{CaM}$ produced a large decrease in the capsaicin-activated current ($57.0\% \pm 9.7\%$, $n = 8$; Figs. 1 and 3 A). This decrease did not occur with CaM alone (Fig. 1 A), was rather slow, and reversed over several minutes in the absence of Ca^{2+} (Fig. 1 C).

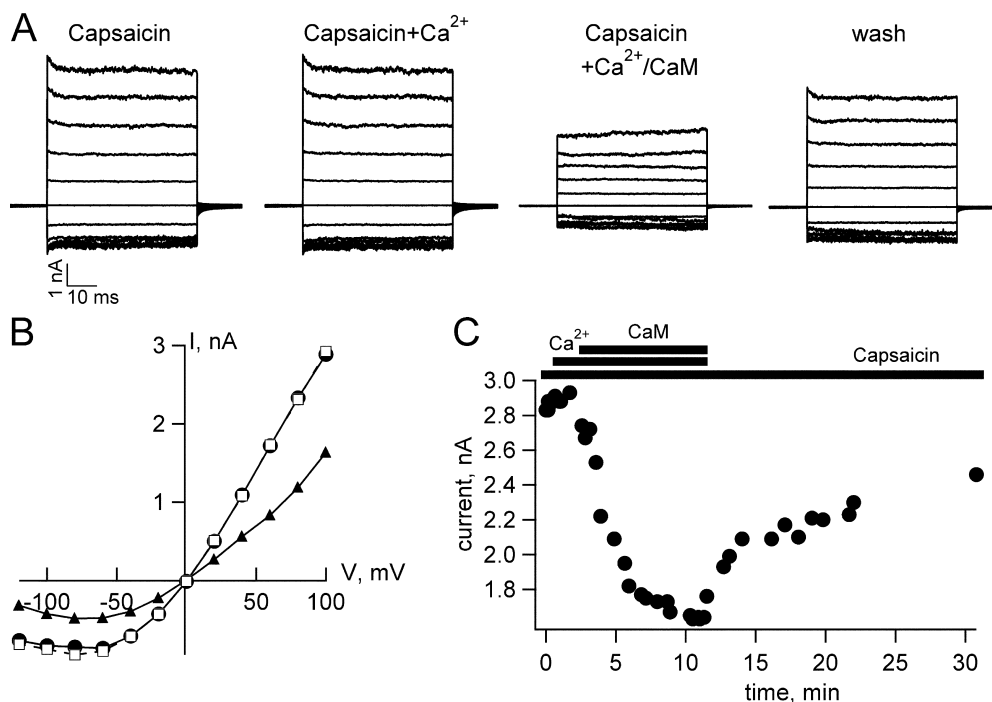
The application of 50 μM free Ca^{2+} without CaM to the intracellular surface of patches produced a small decrease in the capsaicin-activated current (Figs. 1 and 3 A). The mean decrease with 50 μM free Ca^{2+} was 7.4% ($\pm 1.7\%$, SEM, $n = 8$). Could this small decrease in current have been due to endogenous CaM? For this to be the case, CaM must remain associated with the patches even after excision of the membrane from the cell, so that the addition of Ca^{2+} would produce a $\text{Ca}^{2+}/\text{CaM}$ complex that could regulate channel function. This might prove to be a very inefficient process, giving a relatively low CaM concentration in our patches and producing only the small reduction of current observed.

It has recently been reported that, in whole-cell experiments, $\text{Ca}^{2+}/\text{CaM}$ did not appear to modulate TRPV1 channels expressed in HEK 293 cells (Numazaki et al., 2003). It was found that including CaM inhibitors in the pipette and coexpression of mutant CaM did not alter desensitization. We wondered if $\text{Ca}^{2+}/\text{CaM}$ regulation of TRPV1 might have some cell specificity that would indicate the requirement for the presence of an ancillary factor. If this were the case, then the $\text{Ca}^{2+}/\text{CaM}$ effect we observe might be an arti-

fact of the oocyte expression system. To examine this question, we expressed TRPV1 in HEK 293 cells and tested whether $\text{Ca}^{2+}/\text{CaM}$ would inhibit capsaicin-activated currents. As shown in Figs. 2 and 3 B, we found that $\text{Ca}^{2+}/\text{CaM}$ produced inhibition of capsaicin-activated currents that was comparable in magnitude (Fig. 3 B) and kinetics (Fig. 2 C) to that observed with channels expressed in *Xenopus* oocytes. The current decrease produced by Ca^{2+} and $\text{Ca}^{2+}/\text{CaM}$, respectively, were 5% ($\pm 2\%$, $n = 3$) and 43% ($\pm 3\%$, $n = 3$). Thus, it does not seem likely that a difference in the complement of cellular proteins/factors underlies the difference between our data and that previously reported (Numazaki et al., 2003).

The ability of purified CaM to modulate TRPV1 function does not directly address whether the small reduction in current observed with Ca^{2+} alone was mediated by CaM. As discussed above, for this to be the case, CaM would have to remain associated with the patches after their excision from the cell. To determine whether we could manipulate the response to Ca^{2+} in patches by manipulating expression levels of CaM, we coexpressed CaM_{wt} with TRPV1 in both *Xenopus* oocytes and HEK 293 cells. If CaM can be retained in cell-free excised patches, then increasing CaM levels through overexpression should lead to an increased response to Ca^{2+} . We found that in cells overexpressing CaM_{wt} and TRPV1 application of Ca^{2+} alone produced a large reduction in current in both oocytes (Fig. 3 A; $43.5\% \pm 11.8\%$, $n = 4$) and HEK cells (Fig. 3, B and C; $61.9\% \pm 6.6\%$, $n = 6$). This current reduction was statistically significant compared with application of Ca^{2+} onto

FIGURE 2. $\text{Ca}^{2+}/\text{CaM}$ inhibits TRPV1 channels expressed in HEK 293 cells. (A) Current families activated by 20 μM capsaicin, capsaicin + 50 μM free Ca^{2+} , or capsaicin + Ca^{2+} + 500 nM CaM, as indicated. The current family labeled “wash” was recorded in capsaicin after $\text{Ca}^{2+}/\text{CaM}$ had been extensively washed from the patch. (B) IV relations from the current families shown in A. Circles, capsaicin; squares, capsaicin + Ca^{2+} ; and triangles, capsaicin + $\text{Ca}^{2+}/\text{CaM}$. (C) Kinetics of onset and recovery from $\text{Ca}^{2+}/\text{CaM}$ regulation. The presence of capsaicin, Ca^{2+} , and CaM were as indicated by the labeled bars.



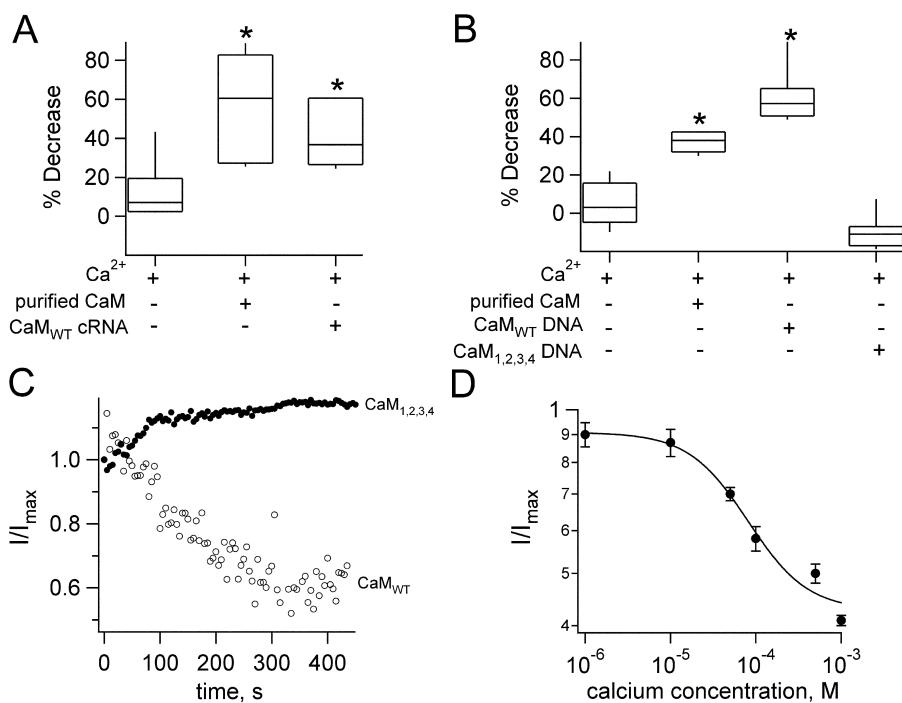


FIGURE 3. Coexpression of wild-type and mutant CaM with TRPV1 alters the effects of Ca²⁺ on channel function in oocytes and HEK cells. (A) Box-plot of the percent decrease of capsaicin-activated (20 μ M) current in *Xenopus* oocytes produced by 50 μ M free Ca²⁺ and no added CaM (left, $n = 8$), 500 nM added CaM (center, $n = 8$), or CaM_{WT} coexpressed with TRPV1 and no added CaM (right, $n = 4$). For the boxes, the line indicates the median of the data, the box surrounds the 25th through 75th percentile of the data, and the whiskers reach the 10th and 90th percentiles. As indicated by the asterisks, both the center and right groups had statistically significant differences compared with Ca²⁺ and no added CaM. (B) Box-plot of the percent decrease of capsaicin-activated (20 μ M) current in HEK cells produced by 50 μ M free Ca²⁺ and either no added CaM (first box, $n = 7$), 500 nM added CaM (second box, $n = 3$), CaM_{WT} coexpressed with TRPV1 and no added CaM (third box, $n = 6$), or CaM_{1,2,3,4} coexpressed with

TRPV1 and no added CaM (fourth box, $n = 6$). As indicated by the asterisks, both the center groups had statistically significant differences compared with Ca²⁺ and no added CaM. For the fourth condition, p exactly equalled 0.05, and therefore did not meet our criterion for significance. (C) Effects of CaM_{WT} and CaM_{1,2,3,4} overexpression in HEK cells on the response to Ca²⁺ (50 μ M). Open circles represent the data from coexpression of TRPV1 and CaM_{WT} and filled circles represent data from coexpression of TRPV1 and CaM_{1,2,3,4}. Data were obtained from pulses to +60 mV every 3 s. Ca²⁺ was introduced at time $t = 0$. (D) Dose-response to different Ca²⁺ concentrations in the presence of exogenous CaM. Data were obtained at a voltage of +60 mV and are normalized to the current obtained in the presence of each Ca²⁺ concentration with CaM. Data are the mean values from three patches. The smooth curve is a fit with the Hill equation, giving an IC₅₀ for inhibition by Ca²⁺ in the presence of CaM of 60 μ M and a Hill slope of 1.25.

patches from cells expressing only TRPV1. Thus, increasing the expression level of CaM facilitated the Ca²⁺-induced inhibition of TRPV1 channels. The simplest explanation of this phenomenon is that CaM remains associated with the patches even after excision, and that it can then modulate TRPV1 function when Ca²⁺ is added.

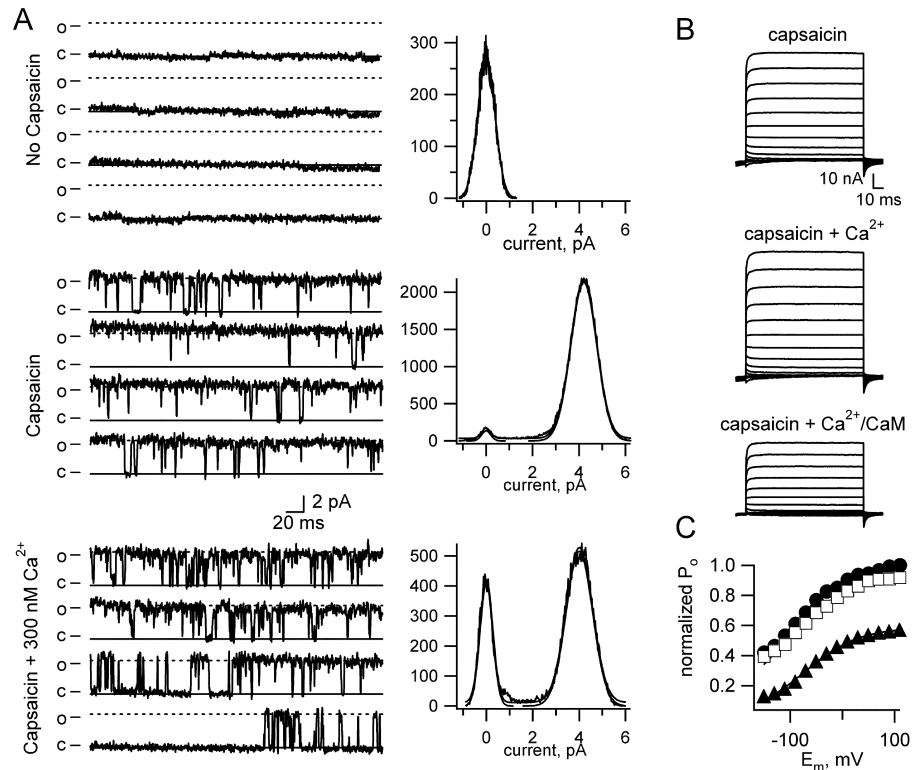
From these experiments we can conclude that CaM remains associated with the patches upon excision, but not whether CaM is associated with the channels themselves. If we could potentiate the effect of Ca²⁺ by overexpressing wild-type CaM, then we should be able to attenuate or eliminate the effect of Ca²⁺ by overexpressing a nonfunctional CaM mutant. The idea here is that the mutant CaM ought to compete with wild-type CaM for association with the channels, but that it would not be able to transduce the changes in Ca²⁺ concentration into changes in channel function. To test this prediction, we cotransfected HEK 293 cells with TRPV1 and CaM_{1,2,3,4}, a mutant in which all four Ca²⁺-binding sites have been crippled. When Ca²⁺ was applied to patches from these cells, the current was not reduced (Fig. 3, B and C). In fact, we observed a slight increase in current upon application of Ca²⁺ to these channels (Fig. 3, B

and C). The simplest interpretation of these results is that CaM_{1,2,3,4} competes with endogenous CaM for association with the channels. These data, together with the biochemical data discussed below, suggest that CaM can remain associated with the channels in the absence of Ca²⁺.

The intracellular concentration of Ca²⁺ varies not only with the activation state of the cell, but also among compartments within the same cell. Furthermore, the apparent affinity of CaM for Ca²⁺ depends on the target protein with which Ca²⁺/CaM interacts. To determine the Ca²⁺ sensitivity of CaM inhibition of the TRPV1, we measured a dose-response relation for inhibition of the channels by different Ca²⁺ concentrations, in the presence of a fixed CaM concentration. As shown in Fig. 3 D, we found that the IC₅₀ for inhibition by Ca²⁺ in the presence of 500 nM CaM was 60 μ M. This is well within the range of Ca²⁺ concentrations expected near these Ca²⁺-permeable channels.

The outward rectification of TRPV1 channels arises from voltage dependence of both gating and unitary conductance (Caterina et al., 1997; but see also Hui et al., 2003). To determine which of these properties is affected by Ca²⁺/CaM, we examined the effect of Ca²⁺

FIGURE 4. $\text{Ca}^{2+}/\text{CaM}$ inhibition of TRPV1 reflects a decrease in channel open probability. (A) Currents from a patch containing a single TRPV1 channel in the absence of both capsaicin and Ca^{2+} (top), in the presence of 4 μM capsaicin but no Ca^{2+} (middle), and in the presence of 4 μM capsaicin and 300 nM free Ca^{2+} (bottom). Consecutive traces at +60 mV are shown. All-points histograms were made from all data collected under each condition (2 s without capsaicin, 12 s with capsaicin in the absence of Ca^{2+} , and 6.1 s for capsaicin plus Ca^{2+}). Open probabilities were taken from the area under the Gaussian representing open channels relative to the sum of the area under the Gaussians representing the closed and open channels, and were as follows: capsaicin alone = 0.99, capsaicin + Ca^{2+} = 0.72. For this experiment, the channels were expressed in *Xenopus* oocytes. (B) Currents in response to steps from a holding potential of -120 mV to between -150 mV and +110 mV, in steps of 20 mV. After the voltage steps the potential was returned to -120 mV. Currents activated by 20 μM capsaicin (top), capsaicin + 50 μM free Ca^{2+} (middle), or capsaicin + 50 μM free Ca^{2+} and 500 nM CaM (bottom). (C) Instantaneous currents measured upon the return of the potential to -120 mV plotted versus the step potential preceding this repolarization. Currents activated by 20 μM capsaicin (circles), capsaicin + 50 μM free Ca^{2+} (squares), or capsaicin + 50 μM free Ca^{2+} and 500 nM CaM (triangles). The currents were normalized to the maximum current activated by capsaicin (without Ca^{2+} or CaM). Solid curves are fits with the Boltzmann equation with the following parameters: capsaicin: $z = 0.41e_0$ (elemental charge), $V_{1/2} = -122$ mV; Ca^{2+} : $z = 0.31e_0$, $V_{1/2} = -109$ mV; $\text{Ca}^{2+}/\text{CaM}$: $z = 0.2e_0$, $V_{1/2} = +40$ mV.



on patches containing only a single TRPV1 channel. As shown in Fig. 4 A, Ca^{2+} decreased the channel open probability, but produced little or no change in unitary conductance. These data indicate that Ca^{2+} altered TRPV1 function by biasing gating toward the closed state. We next examined this question in a second way, looking at tail currents following steps to a range of voltage from -150 to +110 in steps of 20 mV. By examining instantaneous tail currents we could thus get a measure of the relative open probability elicited by the voltage immediately preceding the tail. Fig. 4 B shows an example of a typical current family measured using our tail current protocol, along with normalized tail currents (Fig. 4 C) under the following conditions: capsaicin only (circles), capsaicin + Ca^{2+} (squares), and capsaicin + $\text{Ca}^{2+}/\text{CaM}$ (triangles). The solid curves shown are fits to the data with the Boltzmann equation. Together with the absence of an effect of $\text{Ca}^{2+}/\text{CaM}$ on unitary conductance and channel number in single-channel experiments (Fig. 4 A), these data indicate that the primary effect of $\text{Ca}^{2+}/\text{CaM}$ on channel function is to decrease open probability.

Fast (but not slow) Ca^{2+} chelators eliminate desensitization in whole-cell experiments (Piper et al., 1999),

suggesting that the Ca^{2+} sensor is either part of, or is tightly associated with, TRPV1. To further investigate whether CaM fits this criterion, we examined whether CaM immobilized on agarose beads could bind TRPV1. Cell lysates from TRPV1-expressing oocytes were incubated with CaM-agarose beads and then extensively washed. As shown in Fig. 5 A, the channels remained on the CaM beads and could be subsequently eluted with denaturing sample buffer. The channels did not interact with either naked sepharose beads or with GSH immobilized on agarose beads (Fig. 5 B). We next asked whether the interaction between TRPV1 and CaM in the pull-down assay was Ca^{2+} dependent. We found that the fraction of TRPV1 that bound CaM was the same in the presence of 2 mM Ca^{2+} and 2 mM EGTA with no added Ca^{2+} (Fig. 5 C). The Ca^{2+} -independent association between CaM and TRPV1 is consistent with the association between CaM and TRPV1 we observed in cell-free excised patches, and also consistent with the ability of $\text{CaM}_{1,2,3,4}$ to compete with endogenous wild-type CaM for association with the channels.

TRPV1 channels span the membrane six times and have extensive intracellular NH_2 - and COOH -terminal regions. We hypothesized that (a) the interaction be-

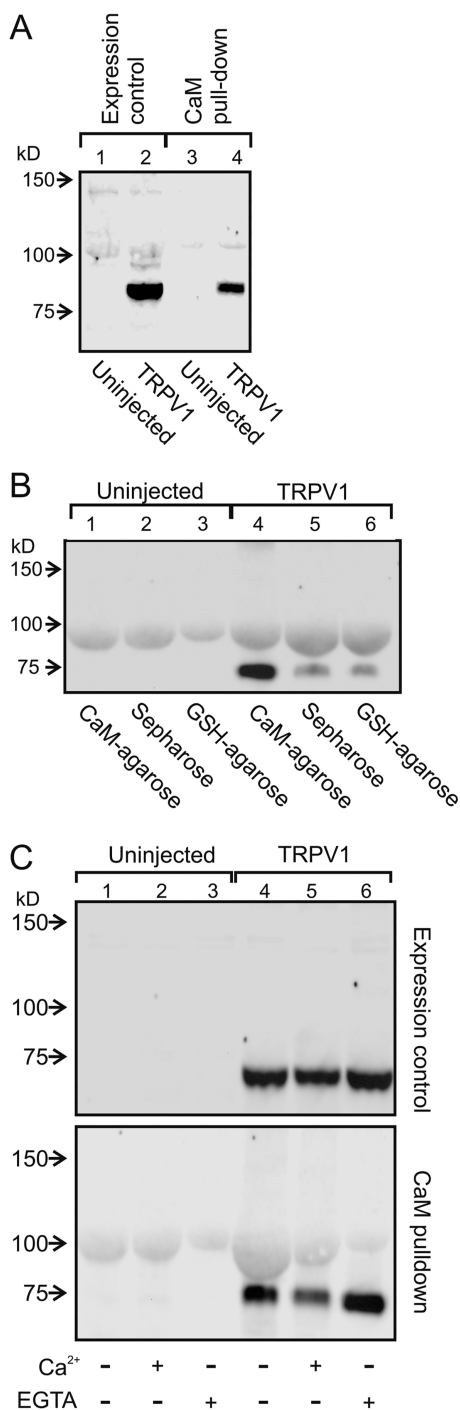


FIGURE 5. TRPV1 channels interact directly with CaM. (A) TRPV1 binds to CaM immobilized on agarose beads. Lanes 1 and 2 represent the input and lanes 3 and 4 represent the protein eluted from the CaM-agarose beads. Lanes 1 and 3 were from uninjected oocytes and lanes 2 and 4 were from oocytes injected with TRPV1 cRNA. (B) TRPV1 does not interact with beads alone. This control demonstrates that the interaction of TRPV1 and CaM is specific. Lanes 1–3 are from uninjected oocytes and lanes 4–6 are from oocytes injected with TRPV1 cRNA. The beads used in each case were as indicated in the figure. (C) The top panel represents inputs and the lower panel represents protein eluted from the CaM beads. Lanes 1–3 are from uninjected oocytes and lanes 4–6 are from oocytes injected with TRPV1 cRNA. Added Ca²⁺ and EGTA were as indicated.

tween CaM and TRPV1 was direct, i.e., not mediated by another protein, and (b) the NH₂-terminal region might be involved in CaM binding. We therefore made seven constructs corresponding to overlapping segments of the TRPV1 NH₂-terminal domain (Fig. 6 A) and expressed them in bacteria as GST-fusion proteins. We purified the GST-fusion proteins, and examined whether (a) they bound to CaM, and (b) binding to CaM was Ca²⁺ dependent. The input (total protein added to each experiment), fusion protein bound in the presence of Ca²⁺, and fusion protein bound in the presence of EGTA are shown for each of these constructs in Fig. 6 B. For two of these fusion proteins, encoding amino acids 149–222 and 189–250, more than half the input was retained by binding CaM. A third protein, consisting of amino acids 247–334, bound more weakly. For all three of these constructs, binding was strongly Ca²⁺ dependent, with 50–100 times as much protein binding in the presence of 2 mM Ca²⁺ compared with 2 mM EGTA and no added Ca²⁺.

The two GST fusion proteins that interacted most strongly with Ca²⁺/CaM overlapped in the region corresponding to amino acids 189–222. These amino acids partly overlap with the first of three Ankyrin repeats in the NH₂-terminal region. Although there is no precedent for Ankyrin repeats binding CaM, these data suggest that Ca²⁺/CaM may modulate channel function by binding directly to this region. We next produced a GST-fusion protein corresponding to amino acids 189–222 to determine if this region is sufficient to allow CaM binding. As shown in Fig. 6 B, this region was indeed sufficient to allow Ca²⁺-dependent CaM binding. To examine whether CaM binding to amino acids 189–222 accounted for the inhibition of functional TRPV1 channels by Ca²⁺/CaM, we made three mutant channels containing deletions in the region of the CaM-binding site: TRPV1Δ189–222, TRPV1Δ200–232, and TRPV1Δ205–222. When expressed in *Xenopus* oocytes, none of these deletion mutants formed capsaicin-activated channels in the plasma membrane.

The lack of expression of functional channels in which the CaM-binding domain has been removed is not unprecedented. For example, KCNQ potassium channels in which the CaM-binding domain is deleted also do not produce detectable currents (Wen and Levitan, 2002). We therefore examined whether lack of protein might account for the lack of capsaicin-activated current we observed. When oocyte lysates were examined with SDS/PAGE and Western blot analysis for expression of these mutants, TRPV1Δ189–222 produced no detectable protein. Expression of TRPV1Δ200–232 and TRPV1Δ205–222 protein was detectable, but reduced compared with wild-type channels (Fig. 7).

Although the TRPV1Δ200–232 and TRPV1Δ205–222 proteins did not produce capsaicin-activated currents, we asked whether they would bind to CaM in the pull-

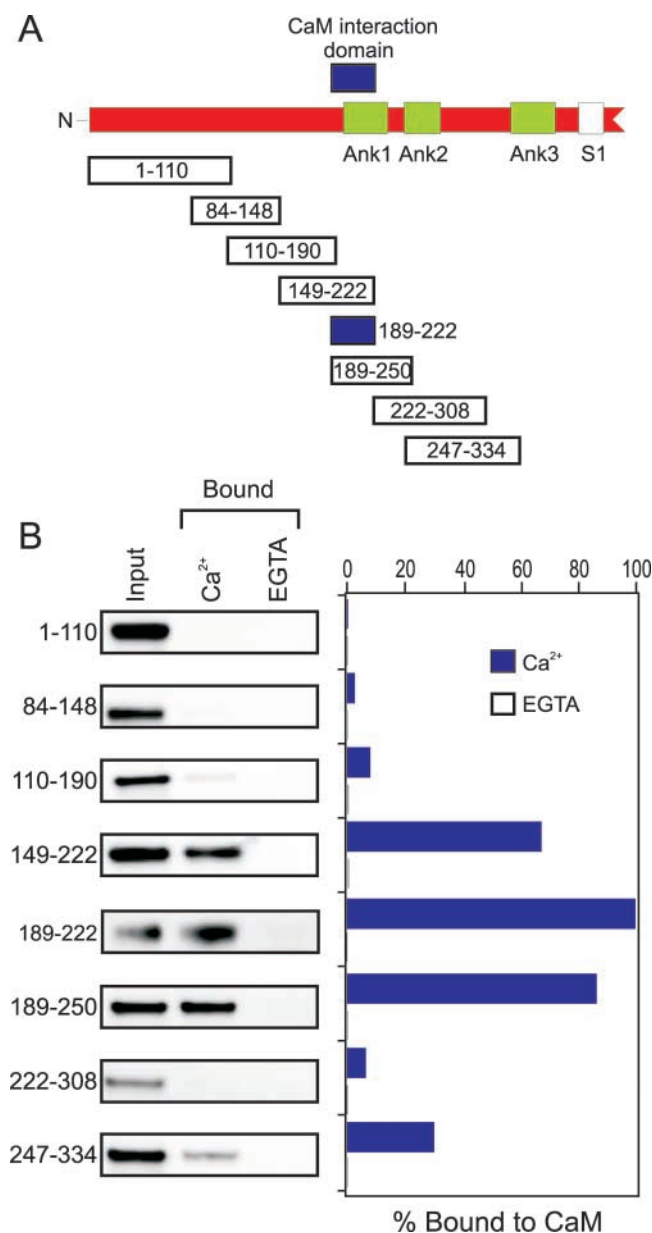


FIGURE 6. The TRPV1 NH₂-terminal region interacts directly with CaM in a Ca²⁺-dependent manner. (A) Cartoon diagram of TRPV1 NH₂-terminal region (not to scale). The three Ankyrin repeats are shown in green (Ank1–3), the region required for interaction with CaM is shown in blue, and the first transmembrane domain (S1) is shown in white. GST-TRPV1 fusion proteins designated below, aligned in-scale to their corresponding sequence in the NH₂ terminus. The numbers within each box refer to the amino acid numbers from TRPV1 encoded in each GST-fusion protein. (B) In vitro CaM binding assay shows that two overlapping GST-fusion proteins can bind CaM in a Ca²⁺-dependent manner. (Left) Western blot of proteins shown in A under three conditions: (1) Input. This represents the amount of each protein that was loaded on the CaM-agarose beads. For the subsequent two lanes, this amount of protein represents that maximum possible amount that can bind. (2) Proteins that bound to CaM agarose beads in the presence of 2 mM Ca²⁺. (3) Proteins that bound to CaM agarose beads in the presence of 2 mM EGTA with no added Ca²⁺. (Right) Bar graph showing the

down assay. We found that the binding of TRPV1Δ200–232 and TRPV1Δ205–222 mutant channels to CaM-agarose beads was decreased by approximately a factor of 10 compared with wild-type channels (Fig. 7). The residual binding of the deletion mutants could have been due to binding of CaM to amino acids 247–334 in the NH₂-terminal region. Alternatively, it could have been due to another binding site within the channel. Although wild-type TRPV1 bound CaM in a Ca²⁺-independent manner in the assay shown in Fig. 5 C, the protein examined in Fig. 7 was not functional, and we have no evidence it was properly folded. These data, then, hint at a second CaM binding region, but their CaM binding cannot be quantitatively compared with functional channels. Nevertheless, the CaM binding ability of the deletion mutants together with the Ca²⁺-independent binding of full-length TRPV1 suggests that multiple regions of channel sequence may interact with CaM in a synergistic manner.

DISCUSSION

Our data show that Ca²⁺/CaM binds to the NH₂-terminal region of TRPV1 channels. The interaction of Ca²⁺/CaM with the channels inhibits gating to reduce open probability. In the peripheral sensory neurons that typically express TRPV1, this would have the effect of interfering with depolarization and decreasing Ca²⁺ influx into the cells. In this way the entry of Ca²⁺ through open channels could feed back on the channels, via their interaction with CaM, to cause channel closure and, perhaps, desensitization to noxious sensory stimuli.

Ion channels in the TRPV and TRPC families contain ankyrin repeats in their intracellular NH₂-terminal regions (Montell et al., 2002). Ankyrin repeats consist of an ~33-residue repeating motif named after the cytoskeletal protein ankyrin, which contains 24 copies of these repeats (Sedgwick and Smerdon, 1999). Present in over 400 proteins, they appear to have diverse functions, many of which involve protein–protein interactions. The function of the ankyrin repeats in TRP channels is not known; speculation has included roles in mediating subunit interactions, interactions with cytoskeletal elements, and interactions with elements of signal transduction cascades. Ankyrin repeats have not been previously shown to interact with calmodulin or to regulate dynamic response properties in ion channels.

It has previously been shown that phosphorylation of an amino acid in the NH₂-terminal region of TRPV1

percent of each GST-fusion protein that bound CaM in the presence of Ca²⁺ (blue) or EGTA (white), as determined by densitometry of the experiment on the left. For each experimental condition (Ca²⁺ and EGTA), the values were normalized to that of the input lane.

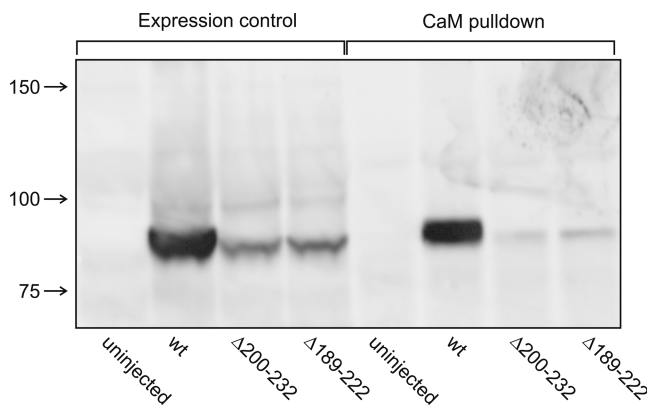


FIGURE 7. NH₂-terminal deletions reduce proteins levels as well as the fraction of the protein that binds to CaM. Lanes 1–4 represent the input and lanes 5–8 represent the protein eluted from the CaM beads. Each channel type is as indicated. The experiment was performed in the presence of 2 mM Ca²⁺.

(S116) by PKA interferes with desensitization to capsaicin in whole-cell experiments (Bhave et al., 2002). Furthermore, S116 and three other residues in the TRPV1 NH₂-terminal region were phosphorylated by PKA in an in vitro PKA phosphorylation assay. None of these four residues fall within the overlapping region of the NH₂-terminal GST fusion proteins that bound CaM in our experiments. However, the localization within the NH₂-terminal region of two apparently disparate mechanisms involved in desensitization raises the question of whether, and to what extent, phosphorylation by PKA and binding of Ca²⁺/CaM interact to regulate TRPV1 open probability.

Several features of the TRPV1 interaction with Ca²⁺/CaM resemble the physiological adaptation to odorants by olfactory receptors (Trudeau and Zagotta, 2003). In that case, Ca²⁺ entering through CNG channels complexes with CaM to inhibit CNG channel activation. CNG channels and TRPV1 channels are both members of the six-membrane spanning domain super-family of ion channels that includes the voltage gated Na⁺, K⁺, and Ca²⁺ channels (Jan and Jan, 1990, 1992), but show no appreciable sequence similarity outside the core transmembrane regions. Interestingly, in olfactory CNG channels CaM also binds to the NH₂-terminal region.

Recently, a CaM-binding site in the COOH-terminal domain of TRPV1 has been identified (Numazaki et al., 2003). This 35-amino acid region bound CaM in GST pull-down assays in a Ca²⁺-dependent manner. Desensitization persisted in functional channels lacking these 35 amino acids, indicating that CaM binding to this region is not required for desensitization. Nevertheless, these data, in combination with the Ca²⁺-independent binding of CaM and full-length TRPV1, indicate that multiple regions of the channels may bind CaM.

Our experiments localized a Ca²⁺-dependent CaM binding site in the TRPV1 NH₂-terminal region. The Ca²⁺ independence of the binding of full-length TRPV1 to CaM suggests that the channels may contain a second, Ca²⁺-independent CaM binding site. There is precedent for the coexistence of Ca²⁺-dependent and Ca²⁺-independent binding sites for CaM within a given protein (Saimi and Kung, 2002). In SK channels for example, the C-lobe of CaM appears to anchor it to the channels in a Ca²⁺-independent manner, whereas the N-lobe influences gating in a Ca²⁺-dependent manner (Keen et al., 1999; Peterson et al., 1999). In L-type Ca²⁺ channels, it is also the N-lobe that is involved in gating and the C-lobe that is involved in anchoring CaM to the channels (Peterson et al., 1999; Zuhlke et al., 1999; Pitt et al., 2001). Thus, the Ca²⁺-independent interaction of full-length TRPV1 with CaM provides a tantalizing suggestion that TRPV1 may indeed participate in such an intricately regulated CaM system.

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