Distinct Modulations of Human Capsaicin Receptor by Protons and Magnesium through Different Domains*

Received for publication, August 25, 2009, and in revised form, January 13, 2010 Published, JBC Papers in Press, February 9, 2010, DOI 10.1074/jbc.M109.058727

Shu Wang‡, Kinning Poon⁴, Robert E. Oswald⁵, and Huai-hu Chuang‡†

From the ‡Departments of Biomedical Sciences and §Molecular Medicine, Cornell University, Ithaca, New York 14853

The capsaicin receptor (TRPV1) is a nonselective cation channel that integrates multiple painful stimuli, including capsaicin, protons, and heat. Protons facilitate the capsaicin- and heat-induced currents by decreasing thermal threshold or increasing agonist potency for TRPV1 activation (Tominaga, M., Caterina, M. J., Malmberg, A. B., Rosen, T. A., Gilbert, H., Skinner, K., Raumann, B. E., Basbaum, A. I., and Julius, D. (1998) Neuron 21, 531–543). In the presence of saturating capsaicin, rat TRPV1 (rTRPV1) reaches full activation, with no further stimulation by protons. Human TRPV1 (hTRPV1), a species ortholog with high homology to rTRPV1, is potentiated by extracellular protons and magnesium, even at saturating capsaicin. We investigated the structural basis for protons and magnesium modulation of fully capsaicin-bound human receptors. By analysis of chimeric channels between hTRPV1 and rTRPV1, we found that transmembrane domain 1–4 (TM1–4) of TRPV1 determines whether protons can further open the fully capsaicin-bound receptors. Mutational analysis identified a titratable glutamate residue (Glu-536) in the linker between TM3 and TM4 critical for further stimulation of fully liganded hTRPV1. In contrast, hTRPV1 TM5–6 is required for magnesium augmentation of capsaicin efficacy. Our results demonstrate that capsaicin efficacy of hTRPV1 correlates with the extracellular ion milieu and unravel the relevant structural basis of modulation by protons and magnesium.

The capsaicin receptor (transient receptor potential vanilloid 1 (TRPV1)), is a transduction channel gated by multiple noxious stimuli, plays a central role in signal integration in pain-sensing neurons (2, 3). Noxious heat (>42 °C), acid, and capsaicin, a pungent natural product from chili peppers, all activate TRPV1 to elicit burning pain (1, 4). Functional synergism among modalities allows TRPV1 to summate different types of subthreshold stimuli and produce a robust response (1, 5, 6). The extent of TRPV1 activation by one or a combination of stimuli determines the intensity of evoked pain (7–12).

Many mammalian TRPV1 agonists exhibit weak receptor activation, even if they may bind the receptor comparably to capsaicin (13, 14). Acidic pH potentiates responses evoked by a low concentration of capsaicin in human and rat TRPV1 alike (1, 15). In contrast, extracellular acidification can enhance only the human receptor currents when capsaicin is applied at a concentration higher than 10 μM (15). Thus, capsaicin functions as a partial agonist for hTRPV1 but a full agonist for the rat receptor. An elevation of temperature or local acidity can in principle augment the efficacies of partial agonists, transforming them from weakly or non-pain-producing ligands into noxious chemicals (16, 17). Thus, pain sensation arising from TRPV1 activation by chemical agonists in humans could conceivably be different from that in rodents, the model species frequently used in biomedical research.

Besides its involvement in pain sensation, TRPV1 displays a low level of activity at normal body temperature (18). Constitutive activity of TRPV1 is essential for regulation of body temperature, evidenced by high fever as a perilous side effect of many TRPV1 blockers during clinical trials for their efficacy in management or prophylaxis of pain (19). Modulators of TRPV1 basal activity and their sites of action are largely unknown; far less known is the variation of basal activities among species in which TRPV1 has been characterized. TRPV1 is a homotetrameric ion channel (20, 21). Hydrophy analysis predicts each subunit to be a six-pass transmembrane polypeptide (4). Earlier work suggests that capsaicin binds to the region between the second and the third transmembrane helices to gate TRPV1 (22). The region between the fifth and the sixth transmembrane segments, including a putative pore loop, forms the ion permeation pathway (23). Several negatively charged amino acid residues in the pore have been shown to be important for sensitization or activation of TRPV1 by cations, particularly protons and magnesium (11, 24). Because of its therapeutic implication, a better understanding of how hTRPV1 opens in response to the extracellular ionic milieu is important. Notably, both protons and magnesium enhance the efficacy of capsaicin as an agonist for hTRPV1. Given its overall similar topological structure and a high percentage of identity (86%) at the amino acid sequence level to rTRPV1, we identified the structural determinants that allow these two ions to specifically sensitize the human receptor. We found that the stimulatory effects of protons and magnesium on fully liganded hTRPV1 require different modular domains. The first four transmembrane segments (TM1–4) of TRPV1 dictate whether the activity of a fully capsaicin-bound receptor can be further enhanced by protons, and a glutamate residue (Glu-536) in the linker between TM3 and TM4 of hTRPV1 is critical in the modulation by protons. TM5–6 of TRPV1 determines the species difference in magnesium modulation of both saturating capsaicin-induced currents and ligand-independent basal currents. A concerted action of the capsaicin-binding domain and the permeation pathway converges at the final stage of hTRPV1 opening.

---

* This work was supported by Cornell University Startup Funds and a scientist development grant from the American Heart Association (to H.-h. C.).
† To whom correspondence should be addressed. Tel.: 607-253-4303; E-mail: huai-hu.chuang@cornell.edu.
‡ The abbreviations used are: TRPV1, transient receptor potential vanilloid 1; TM, transmembrane domain; r, rat; h, human.

The Journal of Biological Chemistry 285 (2010) 11547–11556
Distinct Modulations of Human Capsaicin Receptor

MATERIALS AND METHODS

Molecular Biology—Chimeric channels between human and rat TRPV1 were constructed by overlapped extension PCR of the sequence fragments to be transferred with Pfu polymerase (Stratagene, CA), followed by restriction digestion and ligations into the expression vectors containing wild type cDNAs. Thanks to a high level of sequence homology between the hTRPV1 and rTRPV1, we could select common oligonucleotide primer sets of identical sequences for PCR. We first amplified TM1–4 and TM5–6 fragments using wild type TRPV1 as templates. For TM1–4, we used a pair of oligonucleotides with the following sequences: 5’-CAGGACAAAGTGGGACA-GATT-3’ and 5’- TAGATGCCCATCTGCTGGAA-3’. For TM5–6, the oligonucleotides have the following sequences: 5’-TTCCAGCAGATGGGCATCTA-3’ and 5’-CAGGAT-GATGAAAGACAGCCCTG-3’. The first PCR fragments were extended using external primers outside the domains to be swapped and the wild type recipient receptor plasmid as the templates. The common primer for the N-terminal region has extended using external primers outside the domains to be swapped and the wild type recipient receptor plasmid as the templates. The common primer for the N-terminal region has the following sequence: 5’-AGGCTCTATGATCCGAGGAG-3’ and the C-terminal common primer has the sequence 5’-GCAACTAGAAGGCCACAGTCG-3’ that anneals to the sequence within the vector plasmid. The extended PCR products were digested with BstEII and AflIII, gel-purified, and subcloned into the gapped plasmid vectors containing the cDNA of the recipient receptor. Single point mutations were created by QuikChange mutagenesis following the manufacturer’s protocol (Stratagene, CA). The sequences of mutagenic primers for the sense strand are as follows: H533Q, 5’-CTGTACTTCAGCCTTG-3’ and 5’-TTCCAGCAGATGGGCATCTA-3’. Because channel numbers, single channel currents, and statistical significance was evaluated using unpaired Student’s t test. A probability level of less than 0.05 was considered significant.

Cell Culture and Transfection—HEK293T cells were grown in minimal essential medium plus 10% newborn calf serum with penicillin/streptomycin, incubated at 37 °C in 5% CO2, and transfected using Lipofectamine according to the manufacturer’s protocols (Invitrogen). Cells were transiently transfected with 300 ng of plasmid DNA encoding TRPV1 receptor and 100 ng of reporter plasmid encoding enhanced green fluorescent protein. Electrophysiological recordings were performed 2 days after transfection.

Electrophysiology—Outside-out patch clamp and conventional whole-cell recording methods were used. Pipettes were fabricated from borosilicate glass (World Precision Instruments) and fire-polished to a resistance of ~2 meqohms. For the outside-out patch clamp experiment, the intracellular pipette solution and standard extracellular bath solution were symmetrical and contained 150 mM NaCl, 10 mM HEPES, 1 mM EGTA, pH adjusted to 7.4 or 6.4 with NaOH. For the magnesium potentiation experiments, a 2 mM MgCl2 stock solution was used as the source of Mg2+. It was added into the external solution to achieve a final concentration of 5 mM Mg2+. For whole-cell recordings, we used a cesium solution containing 150 mM CsCl, 10 mM HEPES, 1 mM EGTA. Capsaicin was dissolved in DMSO to make a 0.1 mM stock solution, and diluted into the recording solution at 100 μM concentration before the experiment (0.1% final DMSO). Exchange of external solutions was performed using a gravity-driven perfusion system with manually controlled solenoid valves (ValvebankTM, Automate Scientific Inc.). Voltage clamp experiments were performed at −60-mV holding potential with 320-ms voltage ramps from −120 to 80 mV at 1 Hz. Macroscopic currents were recorded using an Axopatch 200B amplifier (Molecular Devices) and filtered at 2 kHz and digitized at 1 kHz (ITC-18; Instrutech Corp.). Single channel recordings were performed in the outside-out patch clamp configuration. The holding potential was −60 mV. Data were sampled at 5 kHz and low-pass filtered at 2 kHz. Data were acquired and analyzed using Pulse-Pulsefit software (HEKA, Pfalz, Germany), QuB (Program PRE, QUB Suite, Drs. A. Auerbach, F. Qin, and F. Sachs, SUNY, Buffalo, NY), and Origin 8.0 (OriginLab Corp., Northampton, MA). A series of voltage steps for a 150-ms duration was used to induce steady-state currents. Because the tail currents were too small to be measured accurately, the conductance (G) was derived from the steady-state currents at the ends of the voltage steps using the equation: G = I/(V - Vrev), where Vrev is reversal potential (equal to zero in our experiments because we used symmetric solutions). The normalized conductance (G/Gmax) was plotted against the membrane potentials and was fitted with the following equation: G/Gmax = (Gmin/Gmax - 1)/(1 + exp(V - V50)/K) + 1, where V50 is the potential for half-maximal activation, and K is the slope factor. The dose-response curve was fitted with Hill equation: I/Imax = 1/(1 + EC50/C)n. I/Imax is the normalized current at −60 mV; EC50 is the half-maximal effective concentration of capsaicin, and n is the Hill coefficient. Data were presented as means ± S.E., and statistical significance was evaluated using unpaired Student’s t test. A probability level of less than 0.05 was considered significant.

RESULTS

Protons Increase the Efficacy of Capsaicin as a Human TRPV1 Agonist—Extracellular acidosis has been shown to markedly sensitize rTRPV1 by increasing the apparent affinity but not the maximal activation by capsaicin (1). In contrast, the ability of capsaicin to activate hTRPV1 at saturating concentrations depends on extracellular pH. Protons have been shown to per-
Distinct Modulations of Human Capsaicin Receptor

and channel open probability jointly determine the amplitude of macroscopic currents, we asked which factor primarily contributes to the potentiation of hTRPV1 by protons. When we recorded hTRPV1 in the outside-out configuration at −60 mV with 100 μM capsaicin, protons decreased the single channel current of hTRPV1 from 2.62 ± 0.10 (pH 7.4, n = 3) to 1.35 ± 0.15 pA (pH 6.4, n = 3) (Fig. 2). Assuming that the number of channels did not change in the membrane patch subject to acute application of protons, enhancement of hTRPV1 macroscopic current must reflect an increase of channel open probability.

Proton potentiation of hTRPV1 indicates that the saturating concentration of capsaicin alone does not activate the human capsaicin receptor maximally. In principle, any positive channel modulators, in our case protons, will not further potentiate a TRPV1 channel that is maximally activated by capsaicin. We thus examined the biophysical parameters relevant for capsaicin-induced channel activation. Our dose-response analysis of capsaicin-induced currents showed that, in our recording configuration, hTRPV1 and rTRPV1 have similar EC_{50} values for capsaicin (Fig. 3 and Table 2). Therefore, unlike the absence of the capsaicin-binding site in chicken TRPV1 (22), the lower activation of human TRPV1 by capsaicin is not a consequence of a lack of ligand binding but rather is an outcome of the inefficient gating process. Capsaicin activation of TRPV1 is more efficient at more positive membrane potentials, which is clearly observed in the voltage-dependent activation curve (the conductance-voltage plot, or G-V curve) of hTRPV1 currents evoked by 100 μM capsaicin. At −60 mV, the macroscopic channel conductance of hTRPV1 is less than 10% of the conductance at +190 mV in the presence of 100 μM capsaicin (Fig. 4). In comparison, the G-V curve of rTRPV1 under the same recording condition is dramatically left-shifted. At −60 mV, rTRPV1 reaches higher channel opening when all the ligand-binding sites are occupied by capsaicin (~50% of the maximal channel conductance, Fig. 4). This results in almost no significant change of open probability of rTRPV1 from −60 to +80 mV (after correction for the intrinsic rectification of TRPV1 single channel conductance) in response to an increase of membrane voltage, consistent with the idea of nearly maximal activation of rTRPV1 by a high concentration of capsaicin and a consequent lack of proton potentiation. Moreover, proton modulation of hTRPV1 is reduced at more positive membrane potentials, in agreement with the model that a channel with higher open probability is less likely to be further potentiated.

First Four Transmembrane Segments (TM1–4) in Human TRPV1 Are Required for Modulation of Capsaicin Efficacy by Extracellular pH—The stimulatory effect of extracellular protons on TRPV1 arises most likely through interaction with the extracellular domain of the protein or the ion permeation pathway, which is highly water accessible. We decided to subdivide the transmembrane region of TRPV1 into ligand-binding and ion-permeation domains to locate the protons modulation site with respect to the efficacy of capsaicin. Based on analogy to the topological structure of voltage-gated potassium channels (26), we replaced the TM5–6 or TM1–4 of human receptor with the corresponding regions of rTRPV1 to generate chimeric channels between hTRPV1 and rTRPV1, expecting that the former will have a pore region similar to the rat receptor, whereas the
latter will show capsaicin binding and gating mimicking rat TRPV1 (Fig. 5A). These two chimeric channels were named hTRPV1-rTM5–6 and hTRPV1-rTM1–4. We also constructed the reverse chimeric channels, rTRPV1-hTM5–6 and rTRPV1-hTM1–4 (Fig. 5A). Because the wild type and chimeric receptors have similar EC50 values for capsaicin (Fig. 3 and Table 2), we used the standard recording protocol (100 μM capsaicin, −120 to +80 mV voltage ramp) to assess proton modulation. We found that only chimeras that contain the human TM1–4 are further stimulated by protons (Fig. 5, B and C). Protons stimulated the 100 μM capsaicin-induced currents of hTRPV1-rTM5–6 or rTRPV1-hTM1–4 but blocked those of hTRPV1-rTM1–4 or rTRPV1-hTM5–6 (Fig. 5C and Table 1). Apparently, TM1–4 of hTRPV1 or rTRPV1 determines if the receptor is potentiated or blocked by protons in the presence of saturating capsaicin. This is in contrast to the proton sensitization of the TRPV1 current evoked by low doses of capsaicin, which arises from pH titration of a glutamate residue (Glu-600 in rTRPV1 and Glu-601 in hTRPV1) near the pore (24).

Titration of Glu-536 of Human TRPV1 by Protons Mediates Its Enhancement of Capsaicin Efficacy—Protons most likely exert their effect by titrating acidic residues within the TM1–4 segments of hTRPV1. To localize the precise site of proton action, we made single amino acid substitutions with nontitratable glutamine for residues with side chain pKₐ values in the acidic range and examined the effect of protons on the mutant channels. Within the extracellular linker between TM3 and TM4, which has also been shown to be critical for direct activation of rTRPV1 by strong acidic pH (27), a histidine residue is present that is unique to the human receptor (Fig. 6A). Reasoning that titration of this histidine residue (His-533) could lead to enhancement of capsaicin efficacy, we replaced it with the corresponding residue (glutamine) in the rat receptor. Surprisingly, hTRPV1 H533Q (hH533Q) remained substantially potentiated by acidic extracellular pH (Fig. 6, B and D). We then mutated the conserved Glu-536 residue in the linker between TM3 and TM4 into glutamine. The mutation E536Q in the human receptor yields a capsaicin-gated ion channel completely resistant to proton potentiation at 100 μM capsaicin (Fig. 6, C and D). Therefore, the protonation of Glu-536 is necessary for the acid modulation of capsaicin efficacy of hTRPV1. Upon closer inspection of capsaicin activation of hTRPV1 E536Q, we found that its EC₅₀ value for capsaicin is reduced when compared with wild type hTRPV1 (2.34 ± 0.35 versus 0.79 ± 0.03 μM for wild type and mutant receptors, respectively; Fig. 3, and Table 2). More importantly, the voltage for half-maximal activation (V½) of this mutant shifts drastically from V½ of 73 ± 4 mV for wild type hTRPV1 to −28 ± 2 mV for the E536Q mutant (Table 2). Thus, E536Q apparently exhibits a sensitized phenotype regarding capsaicin activation.

Magnesium Stimulation of Human TRPV1 Requires the TM5–6 Region of the Receptor—At the functional level, protons and magnesium share multiple mechanisms of modulation on rat TRPV1. Magnesium can sensitize rTRPV1 current evoked by low doses of capsaicin (11). At a concentration as high as 70 mM, magnesium also directly activates rTRPV1 (11). A previous
study suggests that the two glutamate residues (Glu-600 and Glu-648) critical for proton modulation of rTRPV1 are the ones also involved in magnesium sensitization or activation of the rat receptor (11). Similar to protons, Mg$^{2+}$ only increases the capsaicin efficacy of hTRPV1. As shown in Fig. 7, at a saturating concentration of capsaicin, Mg$^{2+}$ enhances the hTRPV1 current while inhibiting the rTRPV1 current.

Given the highly similar nature of magnesium and proton modulation of hTRPV1, we wondered whether the opposite modulatory effects of Mg$^{2+}$ on human and rat TRPV1 require the same domain as that of protons. We studied the magnesium effects on the same set of four chimeras: hTRPV1-rTM5–6, hTRPV1-rTM1–4, rTRPV1-hTM5–6, and rTRPV1-hTM1–4.

At the holding potential of −60 mV, magnesium inhibits all four chimeric channels (data not shown). A closer inspection of the capsaicin-evoked currents at different membrane voltages, however, reveals that magnesium has more than one action on these chimeric channels. Like calcium and other divalent cations, magnesium is a permeant blocker of TRPV1. The blocking effect of magnesium appeared to be voltage-dependent in the wild type rat receptor, as the inhibition is more pronounced in the negative than in the positive membrane potential range (Fig. 7B). The slight inhibition of the rat receptor by magnesium at positive membrane voltages has the characteristics of a surface potential effect from a high concentration of divalent ions, which displays a right-shifted current-voltage ($I$-$V$) curve with the same slope conductance (Fig. 7B). We reasoned that it may be necessary to analyze the magnesium effect at more extreme membrane potentials so that any possible stimulatory effect will not be obscured by magnesium block, for example at a voltage that magnesium block either does not occur or completely plateaus. So we studied the magnesium effect at −110 mV. We found that at a more negative membrane potential of −110 mV, magnesium actually has a small stimulatory effect on the 100 μM capsaicin-evoked currents of the chimeras with human TM5–6 (Fig. 8, A and C, and Table 1). In contrast, the channels with the rat TM5–6 are still blocked by magnesium at this voltage (Fig. 8, B and C, and Table 1). This result suggests that the TM5–6 region from human TRPV1 is important for mag-

TABLE 2
Activation parameters of TRPV1 wild type and mutant receptors

| Voltage of half-maximal activation (V_{1/2}) | Half-maximal effective concentration of capsaicin (EC_{50}) |
|---------------------------------------------|----------------------------------------------------------|
| Voltage of half-maximal activation (V_{1/2}) | Half-maximal effective concentration of capsaicin (EC_{50}) |
| hTRPV1                                      | 73 ± 4                                                   |
| rTRPV1                                      | −54 ± 6                                                  |
| hTRPV1-rTM1–4                               | −25 ± 6                                                  |
| hTRPV1-rTM5–6                               | −11 ± 2                                                  |
| rTRPV1-hTM1–4                               | −13 ± 3                                                  |
| rTRPV1-hTM5–6                               | −24 ± 2                                                  |
| hE536Q                                      | −28 ± 2                                                  |

hE536Q 28 ± 2 0.79 ± 0.03

FIGURE 4. A and B, representative traces for steady-state currents of hTRPV1 and rTRPV1 induced by a series of voltage steps in the presence of 100 μM capsaicin. C, conductance-voltage relationships (G-V curves) of wild type, chimeras, and hE536Q in the presence of 100 μM capsaicin (see also Table 2).
Magnesium augmentation of capsaicin efficacy. Compared with the wild type human receptor, hTRPV1-rTM5−6 and rTRPV1-hTM5−6 showed much less potentiation by magnesium at −110 mV (Table 1). The reduction of magnesium stimulation in these two chimeras may be an outcome of their higher open probabilities at −110 mV compared with the wild type human receptor (Fig. 4). If the channel has been strongly activated at a particular voltage, magnesium is expected to have more limited stimulatory effects. To test this possibility, we used a combination of acid (pH 6.4) and 100 μM capsaicin to strongly activate hTRPV1. Under this experimental condition, magnesium stimulation of the wild type human receptor is largely diminished, with some residual stimulation seen only at more negative membrane potentials (e.g. −110 mV) (Fig. 9).

In the absence of agonist, TRPV1 also displays slight basal channel activity with strongly voltage-dependent outward rectification (Fig. 10, A and B). The inward basal current is more pronounced when we used cesium instead of sodium as the charge carriers in the extracellular solution for the purpose of accurate measurement. Protons and magnesium both enhance the basal currents of either human or rat TRPV1 measured at room temperature. Magnesium potentiates hTRPV1 basal currents more effectively than rTRPV1, whereas proton enhancement of basal currents is comparable for both channels (4.84 ± 0.78-fold versus 3.05 ± 0.19-fold, p > 0.05, n = 6). Analysis of four chimeras reveals that the TM5−6 region is responsible for the species difference in magnesium modulation of basal currents (Fig. 10C).

DISCUSSION

TRPV1 is the principal transduction channel modulating the excitation of pain-sensing neurons in vertebrate species. Although TRPV1s from all species sense noxious heat and tissue acidosis, the repertoire of chemical agonists for each species homologues displays substantial variability (22, 28–30). The efficacies of capsaicin as well as other small organic TRPV1 agonists are both species- and micro-environment-dependent (13, 17).

It is therefore relevant to elucidate the molecular basis of differential sensitivity to chemical ligands or integration mechanisms between different modalities for a full appreciation of the protective and incapacitating aspects of pain sensation in physiological or pathological settings.

The similarity between human and rat TRPV1 at the primary sequence level is remarkable. The glutamate residue near the pore loop (Glu-600) responsible for enhancement of agonist potency of capsaicin is functionally conserved among species.
One might expect the existence of some titratable amino acid residues unique to the human receptor accounting for proton modulation of agonist efficacy of capsaicin. However, we identified a conserved Glu-536 as the critical residue for proton stimulation of capsaicin efficacy in hTRPV1. Rather than losing its ability to be properly activated by capsaicin due to nonspecific perturbation of the overall structure of the TM1–4 domain, the hE536Q mutant channel behaves as a fully protonated wild type hTRPV1 channel. In support of this view, hE536Q has higher apparent affinity for capsaicin and shows higher channel open probability at saturating concentrations of capsaicin, mimicking a wild type hTRPV1 channel in an acidic environment (pH 6.4). However, the Glu-536 residue is also present in the rat receptor that shows no change in capsaicin efficacy by acidic pH. One possible explanation is that channel open probability of rTRPV1 by capsaicin has already reached its maximum within the voltage range of our recordings so that it is impossible to further enhance the rat receptor current with any positive modulators. As long as saturating capsaicin is present, for membrane potential higher than −60 mV, rat TRPV1 open probability does not even increase in response to voltage increments, a highly effective gating factor for TRPV1 in general. Given that rTRPV1 activated by anandamide can still be potentiated by acidic pH at saturating concentrations of this agonist (13, 16), titration of the Glu-536 residue of the rat receptor may play a role in facilitating channel opening by much lower efficacy agonists, such as anandamide or oxidative lipids.

Previous studies indicate that at the transition between TM3 and the intracellular loop (TM2–3 linker), amino acid residues, including the Tyr-511 and Ser-512, are critical for capsaicin gating of the channel (22). Our results suggest that an amino acid (hGlu-536) within the extracellular loop (linker between TM3 and TM4) serves as a molecular switch to increase the
open probability of ligand-bound human receptors. This linker, although short, constrains the structure of the TM1–4 domain of TRPV1 to impact its function significantly. Amino acid substitutions within this linker had been shown to obliterate the activation of TRPV1 by extremely acidic pH, even if the residues involved are not directly titratable by protons (27).

Although modulatory effects of magnesium on human TRPV1 phenocopy those of protons in both the polarity and the magnitude, immediate sites of actions of these two cations are different. The stimulatory effect of magnesium is associated with pore domains in chimeras. Primary sequences of TRPV1 pore regions among species are even more conserved than other parts of the receptor, the only variation being a small hypervariable segment connecting the distal end of TM5 helix and the completely conserved “pore” loop. This segment likely contributes to refining the positions of several previously identified amino acid residues implicated in magnesium activation of TRPV1 by affecting its binding. The ligand-independent basal currents of TRPV1 from both species variants are stimulated by magnesium. However, there is a significant quantitative difference between human and rat receptors. In contrast, protons stimulate basal currents of both receptors to a similar level.

Multimodal gating is an essential feature of TRP channels. Here, we demonstrated protons and magnesium can further stimulate fully capsaicin-bound hTRPV1 through actions on distinct structures. Various kinetic paradigms have been proposed to explain the generalized gating of the thermo-sensitive

**FIGURE 8.** Human TM5–6 region is required for the Mg2⁺ potentiation of capsaicin efficacy. A, left, at −110 mV, 5 mM Mg2⁺ potentiates the 100 μM capsaicin (CAP)-induced currents of rTRPV1-based chimera with human TM5–6. Right, current-voltage relationships show that Mg2⁺ increases the current at very negative potential. B, Mg2⁺ blocks the capsaicin currents of chimera with rat TM5–6 within the entire range of the voltage ramp. C, summary of the Mg2⁺ effect on the capsaicin currents of the four chimeras at −110 mV.

**FIGURE 9.** Mg2⁺ effect on hTRPV1 is diminished when the channel is substantially opened by the combination of full-dose capsaicin and protons. A, left, at −60 mV, 100 μM capsaicin (CAP)-induced current of hTRPV1 is potentiated by acid (pH 6.4), 5 mM Mg2⁺ blocks the current; right, at −110 mV, Mg2⁺ shows small enhancing effect on the current. B, current-voltage relationships at time points indicated by numbers in A. C, at −60 mV, Mg2⁺ blocks the current by 0.10 ± 0.03-fold, although at −110 mV, Mg2⁺ increases the current by 0.20 ± 0.02-fold.
Transient receptor potential channels (31–34). These models likely will need refinements when more structural or biochemical data become available to correlate with proposed functional states. These models can be quantitatively adjusted to explain differential capsaicin sensitivity of TRPV1 from different species. For example, the efficacy of capsaicin as a TRPV1 agonist is lower for the human receptor than the rat receptor. This is not because capsaicin fails to bind hTRPV1 as well as rTRPV1, as EC50 values of capsaicin for both channels are similar. Instead, capsaicin-binding processes are comparable between hTRPV1 and rTRPV1, but hTRPV1 channel is harder to open. It can be due to either a higher energetic barrier for hTRPV1 to open or because of less efficient coupling between binding and gating in hTRPV1. When we inspect the G-V curves of wild type and mutant channels in this study, it is obvious that maximal left shifts of these curves by capsaicin are determined by the entirety of all six transmembrane segments but not the further subdivided TM1–4 ligand-binding or TM5–6 ion-permeating domain alone. The chimeric channels, no matter with transfer of the TM1–4 capsaicin-binding domain or the TM5–6 pore domain, have the G-V curves fall between the boundaries defined by the two wild type receptors. However, if we compare the pair with the same TM1–6 segments, e.g. the hTRPV1-rTM5–6 and rTRPV1-hTM1–4, they actually have very close V50 values for G-V curves. The human receptor has a TM1–4 domain harder to open by capsaicin. Only by substitution of the Glu-536 residue with a neutral amino acid in hTRPV1, an equivalent of titration of Glu-536 residues in all four channel subunits, can the mutant channel reach the Vi0 value comparable with the chimeric channels containing rat TM1–4 domain, and it acquires resistance to proton modulation. A reduced model where temperature and chemical agonists are treated as gating modifiers to shift the intrinsic voltage-dependent gate of the TRPV1 channel cannot accommodate all the data we obtained without further assumptions (31). Different positive regulators of TRPV1 gating must operate independently so that when one of them reaches its maximal capacity of modulation, the rest of them can still further increase channel opening (32–34). One apparent example is the hTRPV1 E536Q mutant, although protons essentially have no effect on full dose capsaicin-activated currents, an increase in transmembrane voltage still leads to further channel opening. It is expected that for each TRPV1 receptor, wild type or mutants, there must be an upper limit for channel openings even with all possible activators present simultaneously. This upper limit of probability for channel opening may differ from one construct to another. However, a common principle will hold for all kinds of TRPV1 channels that the relative receptor potentiation by a modulator gets smaller when other positive regulators are already present, illustrated by a reduction of Mg2+ effects on proton-potentiated hTRPV1. At the other extreme, one can also view capsaicin as an exceptionally strong activator for rodent receptors. Pepper plants acquire the ability to synthesize this powerful agonist for survival in their ecological niches during evolution. Being an effective deterrent against rodents, capsaicin does not require any other positive regulators to maximally activate rat TRPV1. It is thus tempting to hypothesize that different sensors for agonistic stimuli (capsaicin, heat, protons, and magnesium) are contained in different structural motifs. Although the physical natures of these sensors are currently unknown, our site mapping work will provide some candidate regions to examine once the high resolution structures of TRPV1 are available.

It is worth noting that protons have more complex effects on the capsaicin activation of human TRPV1 than that of rat TRPV1, a property attributable to relatively weak activation of hTRPV1 by capsaicin, namely partial agonism. A lower efficacy of capsaicin on human TRPV1 allows us to appreciate Nature’s design of a signal integrator. Although most physiological activators, including elevation of extracellular acidity, excitatory cations, and endogenous agonists, are all weak activators of TRPV1, synergism of these stimuli can broaden the sensory dimensions of an injury-detecting receptor. Execution of the stimulatory effects through different structural domains of the following figures.
Distinct Modulations of Human Capsaicin Receptor

sensor molecule increases the coding complexity and dynamic range of different categories of signal inputs and its overall efficiency in final receptor activation, particularly when all the stimuli are sub-threshold. This is exemplified in human TRPV1 by a plethora of interactions of protons and magnesium ions on the capsaicin-activated currents, the former through the Glu-536 residue in the linker between TM3 and TM4, as well as the latter acting within the pore. It will be interesting to determine whether the same principle also applied to other endogenously produced TRPV1 agonists with even less efficacy compared with capsaicin.

Acknowledgments—We thank members of the Chuang laboratory for their valuable contribution to this project.

REFERENCES

1. Tominaga, M., Caterina, M. J., Malmberg, A. B., Rosen, T. A., Levine, J. D., and Julius, D. (1998) Neuron 21, 531–543
2. Davis, J. B., Gray, J., Gunthorpe, M. J., Hatcher, J. P., Davey, P. T., Overend, P., Harries, M. H., Latcham, I., Clapham, C., Atkinson, K., Hughes, S. A., Rance, K., Grau, E., Harper, A. J., Pugh, P. L., Rogers, D. C., Bingham, S., Randall, A., and Sheardown, S. A. (2000) Nature 405, 183–187
3. Caterina, M. J., Leffler, A., Malmberg, A. B., Martin, W. J., Trafton, J., Petersen- Zeitz, K. R., Koltzenburg, M., Basbaum, A. I., and Julius, D. (2000) Science 288, 306–313
4. Caterina, M. J., Schumacher, M. A., Tominaga, M., Rosen, T. A., Levine, J. D., and Julius, D. (1997) Nature 389, 816–824
5. Trevisani, M., Smart, D., Gunthorpe, M. J., Tognotto, M., Barbieri, M., Campi, B., Amadesi, S., Gray, J., Jerman, J. C., Brough, S. J., Owen, D., Smith, G. D., Randall, A. D., Harrison, S., Bianchi, A., Davis, J. B., and Geppetti, P. (2002) Nat. Neurosci. 5, 546–551
6. Cornett, P. M., Matta, J. A., and Ahern, G. P. (2008) Mol. Pharmacol. 74, 1261–1268
7. Zhang, N., Inan, S., Inan, S., Cowan, A., Sun, R., Wang, J., Rogers, J. M., Caterina, M., and Oppenheim, J. J. (2005) Proc. Natl. Acad. Sci. U.S.A. 102, 4536–4541
8. Chuang, H. H., Prescott, E. D., Kong, H., Shields, S., Jordt, S. E., Basbaum, A. I., Chao, M. V., and Julius, D. (2001) Nature 411, 957–962
9. Pareek, T. K., Keller, J., Kesavapani, S., Agarwal, N., Kuner, R., Pant, H. C., Iadarola, M. J., Brady, R. O., and Kulkarni, A. B. (2007) Proc. Natl. Acad. Sci. U.S.A. 104, 660–665
10. Zhang, H., Cang, C. L., Kawasaki, Y., Liang, L. L., Zhang, Y. Q., Li, R. R., and Zhao, Z. Q. (2007) J. Neurosci. 27, 12067–12077
11. Ahern, G. P., Brooks, I. M., Miyares, R. L., and Wang, X. B. (2005) J. Neurosci. 25, 5109–5116
12. Zhu, W., Xu, P., Cuascut, F. X., Hall, A. K., and Oxford, G. S. (2007) J. Neurosci. 27, 13770–13780
13. Ross, R. A. (2003) Br. J. Pharmacol. 140, 790–801
14. Hwang, S. W., Cho, H., Kwak, J., Lee, S. Y., Kang, C. J., Jung, I., Cho, S., Min, K. H., Suh, Y. G., Kim, D., and Oh, U. (2000) Proc. Natl. Acad. Sci. U.S.A. 97, 6155–6160
15. Hayes, P., Meadows, H. J., Gunthorpe, M. J., Harries, M. H., Duckworth, D. M., Cairns, W., Harrison, D. C., Clarke, C. E., Ellington, K., Prinjha, R. K., Barton, A. J., Medhurst, A. D., Smith, G. D., Topp, S., Murdock, P., Sanger, G. J., Terrett, J., Jenkins, O., Benham, C. D., Randall, A. D., Gloger, I. S., and Davis, J. B. (2000) Pain 88, 205–215
16. Olah, Z., Karai, L., and Iadarola, M. J. (2001) J. Biol. Chem. 276, 31163–31170
17. Sprague, J., Harrison, C., Rowbotham, D. J., Smart, D., and Lambert, D. G. (2001) Eur. J. Pharmacol. 423, 121–125
18. Gavva, N. R., Bannon, A. W., Surapaneni, S., Hovland, D. N., Jr., Lehto, S. G., Gore, A., Juan, T., Deng, H., Han, B., Klionsky, L., Kung, R., Le, A., Tamir, R., Wang, J., Youngblood, B., Zhu, D., Norman, M. H., Magal, E., Treanor, J. J., and Louis, J. C. (2007) J. Neurosci. 27, 3366–3374
19. Gavva, N. R., Treanor, J. J., Garami, A., Fang, L., Surapaneni, S., Akrami, A., Alvarez, F., Bak, A., Darling, M., Gore, A., Lang, G. R., Kesslak, J. P., Ni, L., Norman, M. H., Pallucchini, G., Rose, M. J., Saffi, M., Tan, E., Romanovsky, A. A., Banfield, C., and Davar, G. (2008) Pain 136, 202–210
20. Kedei, N., Szabo, T., Lile, J. D., Treanor, J. J., Olah, Z., Iadarola, M. J., and Blumberg, P. M. (2001) J. Biol. Chem. 276, 28613–28619
21. Jahnel, R., Dreger, M., Gillen, C., Bender, O., Kurreck, J., and Huch, F. (2001) Eur. J. Biochem. 268, 5489–5496
22. Jordt, S. E., and Julius, D. (2002) Cell 108, 421–430
23. Owsiianik, G., Talavera, K., Voets, T., and Nilius, B. (2006) Annu. Rev. Physiol. 68, 685–717
24. Jordt, S. E., Tominaga, M., and Julius, D. (2000) Proc. Natl. Acad. Sci. U.S.A. 97, 8134–8139
25. Hellwig, N., Plant, T. D., Janson, W., Schäfer, M., Schultz, G., and Schaef er, M. (2004) J. Biol. Chem. 279, 34553–34561
26. Vannier, B., Zhu, X., Brown, D., and Birnbaumer, L. (1998) J. Biol. Chem. 273, 8675–8679
27. Ryu, S., Liu, B., Yao, J., Fu, Q., and Qin, F. (2007) J. Neurosci. 27, 12797–12807
28. Phillips, E., Reeve, A., Bevan, S., and McIntyre, P. (2004) J. Biol. Chem. 279, 17165–17172
29. Gavva, N. R., Klionsky, L., Qu, Y., Shi, L., Tamir, R., Edenson, S., Zhang, T. J., Viswanadhan, V. N., Toth, A., Pearce, L. V., Vanderah, T. W., Porreca, F., Blumberg, P. M., Lile, J., Sun, Y., Wild, K., Louis, J. C., and Treanor, J. J. (2004) J. Biol. Chem. 279, 20283–20295
30. Johnson, D. M., Garrett, E. M., Rutter, R., Bonnett, T. P., Gao, Y. D., Middleton, R. E., and Sutton, K. G. (2006) Mol. Pharmacol. 70, 1005–1012
31. Voets, T., Droogmans, G., Wissenbach, U., Janssens, A., Flockerzi, V., and Nilius, B. (2004) Nature 430, 748–754
32. Matta, J. A., and Ahern, G. P. (2007) J. Physiol. 585, 469–482
33. Latorre, R., Brauchi, S., Orta, G., Zaelzer, C., and Vargas, G. (2007) Cell Calcium 42, 427–438
34. Brauchi, S., Orio, P., and Latorre, R. (2004) Proc. Natl. Acad. Sci. U.S.A. 101, 15494–15499