TRPV1 shows dynamic ionic selectivity during agonist stimulation

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Transient receptor potential vanilloid 1 (TRPV1) is an ion channel that is gated by noxious heat, capsaicin and other diverse stimuli. It is a nonselective cation channel that prefers Ca²⁺ over Na⁺. These permeability characteristics, as in most channels, are widely presumed to be static. On the contrary, we found that activation of native or recombinant rat TRPV1 leads to time- and agonist concentration-dependent increases in relative permeability to large cations and changes in Ca²⁺ permeability. Using the substituted cysteine accessibility method, we saw that these changes were attributable to alterations in the TRPV1 selectivity filter. TRPV1 agonists showed different capabilities for evoking ionic selectivity changes. Furthermore, protein kinase C-dependent phosphorylation of Ser800 in the TRPV1 C terminus potentiated agonist-evoked ionic selectivity changes. Thus, the qualitative signaling properties of TRPV1 are dynamically modulated during channel activation, a process that probably shapes TRPV1 participation in pain, cytotoxicity and neurotransmitter release.

Ionic selectivity is conventionally considered to be an invariant feature of a given ion channel. However, examples of the malleability of this property have emerged¹. For example, K⁺ channels have been shown to undergo a transition, known as C-type inactivation, during which they have transient Na⁺ permeability². More notably, certain ATP-gated P2X channel subtypes show apparent time-dependent increases in their permeabilities to large cations on ATP exposure^{3–6}.

TRPV1, an ion channel that is enriched in sensory neurons involved in pain perception, is the target of action of capsaicin, the pungent ingredient in chili peppers. However, TRPV1 can alternatively be activated by diverse stimuli, including protons, arachidonic acid metabolites and even painfully hot temperatures (>42 °C). TRPV1 is a nonselective cation channel that permeates monovalent cations, but has a ~tenfold preference for Ca²⁺ (ref. 7). TRPV1 has also been shown to mediate the influx of relatively large cations^{8–12}. However, whether such influx is an invariant consequence of TRPV1 activation and whether it involves the TRPV1 pore itself, as opposed to a downstream accessory pore, remains unclear. Recently, we reported that persistent activation of a related heat-gated channel, TRPV3, results in two current phases, characterized by distinct ionic selectivity profiles¹³. In contrast, there has been little effort to determine whether TRPV1 ionic selectivity is dynamically regulated.

We examined these issues and found that TRPV1 selectivity among both monovalent cations and Ca^{2+} changes as a function of time, agonist concentration, ionic conditions and even which agonist is applied. These changes directly involve the TRPV1 pore, are augmented by protein kinase C (PKC) phosphorylation and include increased permeability to large cations. Thus, the intensity and quality of TRPV1 stimuli can impact not only the probability and timing of channel gating, but also the composition of ions that permeate the channel.

RESULTS

Agonist-evoked ionic selectivity changes in TRPV1

We began our study of ionic selectivity in HEK293 cells that stably expressed rat TRPV1 using whole-cell voltage clamp (**Fig. 1**). With the large monovalent cation *N*-methyl-D-glucamine (NMDG) as the sole external cation and Na⁺ as the major internal cation (150 mM Na⁺ and 1 mM Mg²⁺), capsaicin (10 μ M) evoked a steep inward current at -100 mV that peaked in ~10 s (**Fig. 1a**). Thereafter, inward current amplitude typically decreased slightly, followed by a secondary increase that peaked in 1–2 min. The outward current carried by Na⁺ (measured at +20 mV) did not show the secondary rise. When current was measured at –60 mV during voltage ramps (**Fig. 1a**) or when the cell was continuously clamped at –70 mV (**Fig. 1d**), there was a transient outward current component, followed by a sustained inward component.

During the initial seconds of the capsaic in-evoked current response, the reversal potential ($E_{\rm rev}$) was – 79.2 ± 1.8 mV. Over the next 1–2 min, however, it underwent a gradual positive shift to – 34.5 ± 2.5 mV (n = 5, $P < 10^{-3}$, paired *t*-test). This shift became measurable during the initial, transient inward current component and continued during the sustained component before peaking. The overall change in $E_{\rm rev}$ corresponded to a ~5.5-fold increase in TRPV1 permeability to NMDG, relative to Na⁺, ($P_{\rm NMDG}/P_{\rm Na}$, calculated using equation (1), see Methods) from 0.05 ± 0.003 to 0.28 ± 0.025 (n = 5, $P < 10^{-3}$, paired *t*-test). After capsaic in washout, both current amplitude and $E_{\rm rev}$ reverted to values close to baseline.

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ARTICLES

Figure 1 Capsaicin evokes a time-dependent increase in TRPV1 NMDG permeability. (a,b) Top, representative capsaicin (CAP)-evoked currents evaluated at -100 mV (black), -60 mV (red) and –20 mV (blue) during consecutive voltage ramps (-120 to +30 mV over 150 ms at 0.5 Hz) in TRPV1 HEK293 cells. The external solution contained 150 mM NMDG and the internal solution contained 150 mM Na⁺ (int B/ext C). Current amplitudes were normalized to membrane capacitance. Middle, corresponding reversal potential (Erev). Bottom, current-voltage (I-V) relations at indicated time points. Dashed lines indicate zero current level. (c) Representative capsaicin-evoked current from a cultured rat trigeminal ganglion neuron in external solution containing 150 mM NMDG and internal solution containing 165 mM Cs⁺. Data are presented as in a and b. In a-c, the traces labeled a-e in the bottom panels were obtained at the time points labeled a-e in the top and middle panels. (d) Capsaicin-evoked current in a TRPV1 HEK293 cell continuously clamped at -70 mV using int B/ext C. (e) Left, representative current traces evoked at -100 mV by the indicated capsaicin concentrations (applied at arrow) in TRPV1 HEK293 cells in 150 mM NMDG (int A/ext C). The two capsaicin-evoked current components are labeled I_1 and I_2 . Right, capsaicin concentration dependence of I_1 (open circles) and I_2 (filled circles) amplitudes. (f) Capsaicin concentration dependence of initial (open squares), 1 min after



stimulation (open diamonds) and maximal (filled squares) TRPV1 permeability to NMDG, relative to Na⁺ (P_{NMDG}/P_{Na}), calculated from **e** using equation (1). E_{rev} of the first capsaicin-evoked current trace was used for initial values. Mean \pm s.e.m., n = 6 cells per point. Initial data fitted by linear regression (slope, 0.001; *y* intercept, 0.057). Logistic functions used to fit 1 min (EC₅₀, 73 nM; Hill slope, 4.0; max, 0.20) and maximal values (EC₅₀, 62 nM; Hill slope, 6.1; max, 0.24).

The amplitudes of both the initial and second TRPV1 current components in external NMDG at –100 mV were capsaicin concentration dependent (EC₅₀ = 122 nM and 88 nM, respectively; **Fig. 1e**). Indeed, 10 nM capsaicin evoked only a slow monophasic current (**Fig. 1b**). As was seen at 10 μ M, the initial E_{rev} at 10 nM capsaicin was –75 to –80 mV. However, sustained stimulation at 10 nM resulted in little or no E_{rev} change. Similar to current amplitudes, P_{NMDG}/P_{Na} measured at 1 min of stimulation and maximal P_{NMDG}/P_{Na} measured over the entire capsaicin-evoked response both increased in a concentration-dependent manner. In contrast, the initial P_{NMDG}/P_{Na} remained at ~ 0.05 during the response, regardless of capsaicin concentration (**Fig. 1f**). These results demonstrate that TRPV1 has at least two signaling patterns with distinct NMDG permeabilities and argue that the transition between these patterns is determined by the channel-activation level.

Capsaicin-evoked increases in NMDG permeability were also observed in HEK293 cells that were transiently transfected with either rat or mouse *Trpv1* cDNA (data not shown). Under transient transfection conditions, the extent of $E_{\rm rev}$ change was correlated with the functional channel density, defined as the peak outward current density at +20 mV. In fact, in cells showing very high channel densities, even 10 nM capsaicin could evoke a measurable change in $E_{\rm rev}$, although one that was much smaller than that evoked by higher capsaicin concentrations (data not shown). In HEK293 cells transfected with the 5-HT₃ serotonin-gated ion channel, sustained stimulation with 100 µM serotonin induced an $E_{\rm rev}$ shift of ≤ 6.5 mV, despite a range of current amplitudes similar to those of capsaicin-evoked TRPV1 currents (data not shown), which is consistent with previous reports^{3,6}. Thus, altered $P_{\rm NMDG}/P_{\rm Na}$ resulting from prolonged agonist application is not a universal property among ligand-gated cation channels. When the

orientation of ions was switched (intracellular NMDG and extracellular Na⁺), 1 µM capsaicin evoked a reciprocal change in TRPV1 $E_{\rm rev}$ from positive to negative potentials, that was similar in extent ($\Delta E_{\rm rev} = -38.3 \pm 3.9$ mV, n = 4). Capsaicin-evoked changes in $P_{\rm NMDG}/P_{\rm Na}$ were not inhibited by strong chelation of intracellular Ca²⁺ with 5 mM BAPTA, replacement of EDTA in the extracellular solution with EGTA or inhibition of ERK kinase, a downstream target of TRPV1 (ref. 14 and data not shown). The sustained current was inhibited (from -301 ± 15 pA pF⁻¹, n = 9, to -29.6 ± 13.1 pA pF⁻¹, n = 8, $P < 10^{-4}$) by pretreatment with 1 µM ruthenium red, a noncompetitive TRPV1 antagonist⁷.

We also asked whether native TRPV1 expressed in sensory neurons would show agonist-evoked changes in ionic selectivity (**Fig. 1c**). Capsaicin (1 μ M) evoked measurable inward currents in the presence of external NMDG in 25 out of 34 neurons. The initial $E_{\rm rev}$ was -85.9 \pm 0.5 mV (n = 25), which was shifted to more positive potentials over time by 5.8–38.1 mV, corresponding to an increase in $P_{\rm NMDG}/P_{\rm Na}$ from 0.05 \pm 0.01 to 0.10 \pm 0.03 ($P < 10^{-7}$, paired *t*-test, n = 25). As TRPV1 is the only capsaicin-gated ion channel in rodent sensory neurons⁷, these findings demonstrate that native TRPV1 undergoes time- and activation-dependent changes in NMDG permeability.

Changes in the TRPV1 pore accompany altered ionic selectivity

The changes in TRPV1 $P_{\text{NMDG}}/P_{\text{Na}}$ suggest that the effective size of the channel pore may increase following initial channel gating. To examine this possibility, we measured P_X/P_{Na} , where X represents cations of different sizes (NMDG, trishydroxymethylaminomethane (TRIS) and 2-(methyl-amino)-ethanol (2-MAE)) at the earliest moments of response to capsaicin (1 μ M) in TRPV1 HEK293 cells (**Fig. 2**). The initial P_X/P_{Na} was inversely related to cation size, with 2-MAE having



the highest relative TRPV1 permeability and NMDG having the lowest. Application of the excluded volume theory (equation (5) in Methods)^{15,16} allowed us to estimate the initial TRPV1 pore diameter at 10.1 Å (Fig. 2c). With protracted capsaicin stimulation, however, P_X/P_{Na} increased over time for all three cations (Fig. 2a,b), reflecting an apparent enlargement of pore diameter to 12.3 Å (Fig. 2c). Time constants (τ) for increases in P_X/P_{Na} were inversely proportional to cation size (n = 6, P < 0.0001, one-way ANOVA), as was previously reported for P2X₂ (ref. 6; Fig. 2b). The functional TRPV1 pore diameter following prolonged steady-state activation at low capsaicin concentrations (10–30 nM) was 10.4 Å, which was nearly identical to that measured at the beginning of responses to 1 µM capsaicin. Thus, increases in functional TRPV1 pore diameter depend on both the strength and duration of agonist stimulation. Additional experiments revealed that capsaicin activation also produced changes in TRPV1 discrimination among small inorganic cations, but did not lead to TRPV1 anion permeability (Supplementary Fig. 1 online; a summary of capsaicin-evoked E_{rev} and P_X/P_{Na} changes is provided in Supplementary Tables 1 and 2 online).

To ask whether activation-dependent ionic selectivity modulation stems from alterations in TRPV1 per se versus recruitment of a heterologous nonselective pore, we applied the substituted cysteine accessibility method (SCAM) using methanethiosulfonate (MTS) reagents¹⁷. It has been suggested that the architecture of the TRPV channel pore resembles that of the K⁺ channel KcsA. The loop between transmembrane domains 5 and 6 contains a pore helix and a selectivity filter, with the sequence motif of the selectivity filter being relatively well conserved among TRPV1-4 (ref. 18). We individually mutated residues in the putative TRPV1 selectivity filter, from T641 to D646, to cysteine. Among these mutants, T641C, I642C, G643C and G645C generated currents that were too small to permit the evaluation of ionic selectivity. Although TRPV1 D646C showed measurable currents, it was not altered by MTS reagents (data not shown). TRPV1 M644C mediated robust capsaicin-evoked currents. Initial permeability to large cations was decreased in this mutant, consistent with the M644 residue's placement in the TRPV1 selectivity filter, but the extent of capsaicin-evoked change in ionic selectivity was normal (Supplementary Fig. 2 online). Furthermore, with Na⁺ as the external cation, capsaicin-evoked currents mediated by M644C, but not those mediated by wild-type TRPV1, could be strongly inhibited by two bulky MTS

Figure 2 Capsaicin alters TRPV1 permeability to large cations. (a) Capsaicinevoked (1 μ M) currents in the presence of internal Na⁺ and external NMDG, TRIS, 2-MAE or Na⁺ at initial (trace i) and final (trace f) points during 3 min of capsaicin stimulation in TRPV1 HEK293 cells. (b) Changes in *P_X/P_{Na}* (X = NMDG, TRIS or 2-MAE) during prolonged 1 μ M capsaicin application (*n* = 6 cells per cation). (c) Estimation of initial (circles) and final (squares) TRPV1 pore diameter using the excluded volume theory (equation (5)) and data from **b**. Minimum diameters (10.1 Å initial, 12.3 Å at 3 min) were obtained from intercepts of linear fits on the *x*-axis as indicated (initial: a = 1.83, b = -0.36; 3 min: a = 1.78, b = -0.29).

reagents, 2-aminoethyl methanethiosulfonate (MTSEA, 1 mM) and [2-(trimethylammonium)ethyl] methanethiosulfonate (MTSET, 1 mM), but not by negatively charged (2-sulfonatoethyl) methanethiosulfonate (MTSES, 1 mM) (**Supplementary Fig. 3** online). Together, these features allowed us to use TRPV1 M644C to explore whether changes in ionic selectivity arise from changes in the TRPV1 selectivity filter.

We began by examining the effects of MTS reagents on TRPV1 permeability to large cations once that permeability had been increased by capsaicin (Fig. 3). In TRPV1 M644C, as in wild-type, 1 µM capsaicin activated large currents that were accompanied by a positive Erev shift in the presence of extracellular 2-MAE (Fig. 3a,c and Supplementary Fig. 3). However, the subsequent superimposition of MTSET reversed this Erev shift, thus reducing PMAE/PNa. Typically, PMAE/PNa was reduced by 58.4 \pm 0.05% from its value just before MTSET application (n = 6). Concomitantly, MTSET inhibited inward and outward current amplitudes by 72.7 \pm 0.06 and 68.8 \pm 0.04% at -60 and +50 mV, respectively (n = 6). MTSEA reduced P_{MAE}/P_{Na} to a similar extent, but did so more rapidly than MTSET, whereas MTSES showed no effect (Fig. 3c). Using TRIS as the extracellular cation (Fig. 3b,c), MTSET and MTSEA reduced P_{TRIS}/P_{Na} following the capsaicin-evoked P_{TRIS}/P_{Na} increase by 48.8 \pm 0.05 and 53.9 \pm 0.04%, respectively (n = 6), and MTSES had little effect. The MTS-induced inhibition of TRPV1 M644C in the presence of TRIS showed several differences from the inhibition observed in the presence of 2-MAE (Fig. 3c and Supplementary Figs. 3 and 4 online). First, MTSEA showed a peculiar time course of inhibition of $P_{\text{TRIS}}/P_{\text{Na}}$: a rapid initial inhibition of $P_{\text{TRIS}}/P_{\text{Na}}$, followed by a subsequent increase and secondary decrease. This hump in P_{TRIS}/P_{Na} was accompanied by a decrease in current amplitude. Second, the extent of current amplitude inhibition by MTS was less than that of P_{TRIS}/P_{Na}. Third, the onset and time course of the decrease in current amplitude tended to be later and slower than the inhibition of P_{TRIS}/P_{Na}. When MTSEA was applied after the MTSET effect had plateaued, current amplitude was further inhibited by $66.9 \pm 0.04\%$ (n = 8), which was significantly larger than inhibition by MTSET or MTSEA alone (P = 0.001, one-way ANOVA) (Fig. 3d, upper). P_{TRIS} / P_{Na} did not show a substantial additional inhibition (63.3 ± 0.04%) during the same treatment, but showed a larger hump that was synchronized with the larger current inhibition (Fig. 3d, lower). These results suggest that the pore of TRPV1 M644C has multiple accessible cysteine residues whose modification might be required for maximal alteration of $P_{\text{TRIS}}/P_{\text{Na}}$ and current amplitude.

We next investigated whether the modification of the TRPV1 pore by MTS reagents would result in differential rates and/or consequences under conditions where permeability to large cations had been differentially altered. As with wild-type TRPV1, HEK293 cells that were transiently transfected with TRPV1 M644C showed a wide range of current amplitudes and changes in P_X/P_{Na} . We therefore applied capsaicin at a fixed concentration (1 μ M), followed by MTSET, and compared the effects of MTSET on cells in which the capsaicin-evoked increase in $P_{\text{TRIS}}/P_{\text{Na}}$ fell into the bottom 35% of the range recorded in our experiments (low, 0.05 ± 0.01; n = 10) versus those in which the



reagent (black) in the presence of external 2-MAE (top) or TRIS (bottom) (n = 5-6). Data in panels **c**-**f** are presented as mean ± s.e.m. and are normalized to the values measured just before MTS addition. (**d**) Changes in capsaicin-evoked current amplitude (top) and $P_{\text{TRIS}}/P_{\text{Na}}$ (bottom) following consecutive application of MTSET and MTSEA in M644C. Red and blue dashed lines represent averaged values following 2-min application of MTSET or MTSEA alone, respectively (n = 8). (**e**) Comparison of the effects of MTSET on M644C $P_{\text{TRIS}}/P_{\text{Na}}$ (left), outward current amplitudes at +30 mV (middle) and inward current amplitudes at -80 mV (right) in the presence of extracellular TRIS in two groups of cells showing either low (black, n = 10) or high (red, n = 5) $P_{\text{TRIS}}/P_{\text{Na}}$ change in response to 1 μ M capsaicin. The blue trace (left) represents $P_{\text{TRIS}}/P_{\text{Na}}$ change following MTSET application when 50 nM capsaicin was used (n = 6). (**f**) Comparison between low (black) and high (white) $P_{\text{TRIS}}/P_{\text{Na}}$ change groups from **e** with respect to 20–80% decay time (left; P, $P_{\text{TRIS}}/P_{\text{Na}}$; *I*, currents at +30 mV ($I_{+30 \text{ mV}}$) and -80 mV ($I_{-80 \text{ mV}}$) (right). Gray bars represent 50 nM capsaicin group from **e**. *, P < 0.05; ***, P < 0.005; ***, P < 0.001.

increase was in the top 35% (high, 0.24 ± 0.02 ; n = 5). The patterns of inhibition of PTRIS/PNa by MTSET were significantly different between the low and high groups (P < 0.001, two-way ANOVA; Fig. 3e). The low group showed ~ fourfold faster inhibition of $P_{\text{TRIS}}/P_{\text{Na}}$ compared with the high group (Fig. 3f). As a consequence of this, $P_{\text{TRIS}}/P_{\text{Na}}$ was inhibited by 52% in the low group after 30 s of MTSET exposure, but only by 35% in the high group. By 2 min, the extent of inhibition was comparable between groups. Cells treated at a low capsaicin concentration (50 nM), which evoked only modest $P_{\text{TRIS}}/P_{\text{Na}}$ changes $(0.06 \pm 0.01, n = 6)$, showed inhibition by MTSET with an extent and time course similar to those of the low group at 1 µM capsaicin. The magnitude of P_{TRIS}/P_{Na} before MTSET exposure was also correlated with the effects of MTSET on current amplitudes (Fig. 3f). Similar results were obtained using external 2-MAE (Supplementary Fig. 5 online). These data demonstrate that the consequences of TRPV1 M644C exposure to MTS reagents depend on the prior extent of P_X/P_{Na} change and thus argue that changes in permeability to large cations are associated with alterations in the TRPV1 selectivity filter.

TRPV1 agonists change ionic selectivity differentially

We tested whether different agonists would have different capabilities for altering TRPV1 ionic selectivity. In the presence of external NMDG,

capsaicin evoked a biphasic inward current and a slow increase in P_{NMDG}/P_{Na} (Fig. 4a and Supplementary Fig. 6 online). N-arachidonoyl dopamine (NADA)¹⁹ evoked a slower biphasic current (P <0.005) that had less amplitude. Correspondingly, NADA produced a smaller P_{NMDG}/P_{Na} increase than did capsaicin. Piperine²⁰ evoked a large current response that peaked sooner than that evoked by capsaicin. The corresponding $P_{\rm NMDG}/P_{\rm Na}$ rise evoked by piperine was also more rapid ($P < 10^{-3}$), but reached a maximum that was similar to the P_{NMDG}/P_{Na} evoked by capsaicin. Resiniferatoxin (RTX) evoked a slowly developing current without an initial, transient component. The $P_{\rm NMDG}/P_{\rm Na}$ increase evoked by RTX was similar in amplitude to that evoked by capsaicin, but developed more slowly. Heat evoked a transient current without a robust second phase. Moreover, P_{NMDG}/ P_{Na} increased only modestly during heating. Similarly, camphor²¹ evoked only a transient current and a modest transient shift in E_{rev} (by ~ 10 mV). This limited NMDG permeability change is probably not the result of limited efficacy, as maximal camphor-evoked current density was comparable to that evoked by RTX or NADA (Supplementary Fig. 6). Thus, both the rate and extent of TRPV1 ionic selectivity changes vary among different agonists, creating the potential for qualitatively distinct signaling outcomes.

As an independent assay of large cation permeability, we evaluated the uptake of the propidium dye YO-PRO1 (630 kDa, 1 µM). Capsaicin

Figure 4 Differential ionic selectivity changes evoked by TRPV1 agonists. (a) Top, averaged TRPV1 currents evoked by capsaicin (10 µM), NADA (10 µM), piperine (PIP, 100 µM), RTX (10 nM), heat (HT, 25 to 45 °C, $\tau = 4.5$ s) or camphor (CMP, 10 mM) using solutions int A/ext C. Mean baseline-subtracted evoked current amplitudes at -100 mV were normalized to maximum for each agonist and plotted from response onset. Bottom, changes in mean P_{NMDG}/ P_{Na} during agonist exposure (n = 5-6). Error bars omitted for clarity. (b) Top, 10 μM capsaicin– evoked YO-PRO1 (YP) uptake into TRPV1 HEK293 or vector control (pcDNA3) cells. Mean ± s.e.m. from five (pcDNA3) or ten (TRPV1) coverslips. TRPV1 was significantly different from pcDNA3 at 2 min (P = 0.0007). Bottom, rates of increase in cellular YO-PRO1 fluorescence during stimulation of TRPV1 HEK293 cells with different agonists. For cyan trace, ruthenium red (RR, 10 µM) was superimposed on capsaicin at the



indicated time (n = 16-101 cells from 2–8 coverslips per agonist that met criteria for responsiveness). (c) Fluorescence intensity (top) and rate of fluorescence increase (bottom) following addition of 1 μ M capsaicin (at 0 s) and 1 mM MTSET (at 90 s) in the presence of YO-PRO1 in HEK293 cells transfected with GFP and wild-type TRPV1 (black), M644C (red) or pcDNA3 (blue). Intensity was normalized to that just before MTSET application. Mean \pm s.e.m., n = 9-12 cells. Only physically isolated GFP-positive cells were quantified.

evoked a substantial increase in YO-PRO1 uptake in TRPV1 HEK293 cells, but not in pcDNA3 control cells (Fig. 4b, top). In TRPV1expressing cells, the rate of fluorescence change increased approximately fourfold during the first 30 s of capsaicin exposure and reached a plateau thereafter (Fig. 4b, bottom), consistent with a timedependent increase in large cation permeability. Uptake was dependent on continued TRPV1 activity, as it was quickly ablated by ruthenium red. As with NMDG permeability, evoked YO-PRO1 uptake varied in rate and extent among TRPV1 agonists (Fig. 4b, bottom). Carbenoxolone (20 µM), an inhibitor of pannexin-1 that has been reported to block dye uptake mediated by P2X7 (ref. 22), failed to inhibit capsaicinevoked YO-PRO1 uptake (data not shown). In contrast, MTSET suppressed capsaicin-evoked YO-PRO1 uptake by TRPV1 M644C (Fig. 4c). These data provide further evidence that TRPV1 agonists show differential abilities for evoking the influx of large cations and that this influx occurs directly through TRPV1.

Activation-dependent changes in TRPV1 Ca²⁺ selectivity

We next asked whether TRPV1 Ca²⁺ permeability could also be regulated by channel activation. Under bi-ionic conditions (external 112 mM Ca²⁺, internal 150 mM Na⁺), capsaicin-evoked currents were transient, with pronounced desensitization (**Fig. 5a–c**). Capsaicin (100 nM) evoked a current with an initial P_{Ca}/P_{Na} of 17.5 ± 1.8 (n = 8), calculated using equation (2). Over the ensuing 4–7 s, however, E_{rev} decreased, which corresponded to a reduction in P_{Ca}/P_{Na} to 5.9 ± 0.4 ($P < 10^{-3}$, paired *t*-test). P_{Ca}/P_{Na} declined during both the activation and desensitization phases of the current response (**Fig. 5a**) and was 10.2 ± 0.9 at the time of peak current amplitude (n = 8). A lower concentration of capsaicin (3 nM) also reduced TRPV1 P_{Ca}/P_{Na} , albeit more slowly than 100 nM capsaicin (**Fig. 5c**).

In an external solution containing 150 mM NMDG and 1 mM Ca²⁺, capsaicin (1 μ M) again evoked a current that showed rapid activation and desensitization (**Fig. 5d,e**). Initially, this current reversed at -50.9 ± 1.0 mV (n = 6). However, over ~ 20 s, the E_{rev} shifted to -30.9 ± 0.8 mV. When external Ca²⁺ was suddenly quenched with EGTA, E_{rev} rapidly reverted to -75.3 ± 1.3 mV (n = 6). Because NMDG was the only remaining external cation at this point, this value corresponds to a P_{NMDG}/P_{Na}

of 0.05 ± 0.002 (n = 6), which is similar to the initial TRPV1 $P_{\text{NMDG}}/P_{\text{Na}}$ that we measured in the absence of Ca²⁺ (**Fig. 1e** and **Supplementary Fig. 6**). Although Ca²⁺ limited changes in TRPV1 $P_{\text{NMDG}}/P_{\text{Na}}$, further experiments showed that $P_{\text{NMDG}}/P_{\text{Na}}$ can increase even at physiologically relevant Ca²⁺ concentrations (**Supplementary Fig. 7** online).

The modest capsaicin-evoked change in $P_{\rm NMDG}/P_{\rm Na}$ at 1 mM Ca²⁺ implied that the $E_{\rm rev}$ changes measured during the capsaicin-evoked response in the presence of 1 mM Ca²⁺ were predominantly attributable to changes in $P_{\rm Ca}/P_{\rm Na}$. $P_{\rm Ca}/P_{\rm Na}$ could therefore be calculated using equation (3), with a correction for the underlying $P_{\rm NMDG}/P_{\rm Na}$ at the time of Ca²⁺ quenching. In contrast with the capsaicin-evoked decrease in TRPV1 $P_{\rm Ca}/P_{\rm Na}$ that we observed in 112 mM external Ca²⁺, $P_{\rm Ca}/P_{\rm Na}$ at 1 mM Ca²⁺ increased from 7.6 ± 0.6 (n = 6) to a maximum of 24.9 ± 1.0 (n = 6, $P < 10^{-7}$, paired *t*-test) during prolonged capsaicin activation. The extent of this temporal rise in $P_{\rm Ca}/P_{\rm Na}$ increased with capsaicin concentration (EC₅₀ = 136 nM; Fig. 5f). Thus, TRPV1 $P_{\rm Ca}/P_{\rm Na}$ can be modulated over a fivefold range, with the starting and ending points being dependent on both capsaicin and Ca²⁺ concentration (a more extensive analysis of this relationship is presented in **Supplementary Fig. 8** online).

We next examined agonist-evoked ionic selectivity changes in a TRPV1 mutant in which aspartate 646, located at the external boundary of the putative selectivity filter²³, was substituted with asparagine (D646N). Mutation of this same residue alters divalent cation permeability, decreasing $P_{Mg}/P_K^{23,24}$. In 150 mM NMDG in the absence of Ca²⁺, the initial P_{NMDG}/P_{Na} was greater in D646N than in wild-type TRPV1 in response to 1 μ M capsaicin (D646N, 0.07 \pm 0.02, n = 11; wild type, 0.05 ± 0.002 , n = 11; $P < 10^{-6}$), whereas the maximal P_{NMDG}/P_{Na} over the course of the response was statistically indistinguishable from wild type (D646N, 0.22 ± 0.03 , n = 11; wild type, $0.23 \pm$ 0.03, n = 11). In 150 mM NMDG and 1 mM Ca²⁺, the initial P_{Ca}/P_{Na} during capsaicin stimulation was 71% lower in D646N than in wildtype TRPV1, but increased over time by a similar absolute amount to reach a value that was 38% lower than that of wild type (Fig. 5g,h). At high external Ca²⁺ (112 mM), the initial and maximal P_{Ca}/P_{Na} during stimulation with 3 nM capsaicin were 49% and 44% lower in the D646N mutant than in the wild type, respectively (Fig. 5i,j). As

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Figure 5 Capsaicin- and $[Ca^{2+}]_o$ -dependent changes in TRPV1 P_{Ca}/P_{Na} . (a) Representative capsaicin-evoked current at -20 mV (top) and corresponding E_{rev} (bottom) in the presence of 112 mM $[Ca^{2+}]_o$ (int A/ext G) in TRPV1 HEK293 cells. (b) *I-V* relations at the time points indicated in panel **a**. (c) P_{Ca}/P_{Na} changes during application of 3 nM (filled) and 100 nM (open circles) capsaicin in 112 mM $[Ca^{2+}]_o$ (int A/ext G) (n = 4-8 cells per point). (d) Current density at -100 mV (top) and corresponding E_{rev} (bottom) during stimulation with 1 μ M capsaicin in 1 mM $Ca^{2+}/150$ mM NMDG (int A/ext D), followed by quenching of external Ca^{2+} (ext E, open bar). (e) *I-V* relations at the time points indicated in **d**. (f) Initial (open) and maximum (filled) P_{Ca}/P_{Na} values across capsaicin concentrations under the conditions described in **d** (n = 6 cells per point). (g) Representative capsaicin-evoked currents at -100 mV (top), E_{rev} (middle) and P_{Ca}/P_{Na} (bottom) under the conditions described in **d** in cells expressing wild-type (black) or D646N (gray) TRPV1. (h) Left, initial (a) and peak (b) P_{Ca}/P_{Na} calculated from the data in **g**. Right, P_{NMDG}/P_{Na} measured immediately after quenching external Ca^{2+} (point c in **g**). *, P = 0.009; **, P = 0.0002; ***, $P < 10^{-5}$; n = 6. (i) Representative 3 nM capsaicin-evoked P_{Ca}/P_{Na} changes at 112 mM external Ca^{2+} (int A/ext G) in TRPV1 wild-type (black) or D646N (gray). (j) Corresponding initial, maximal and minimum-following maximal P_{Ca}/P_{Na} for TRPV1 wild-type (black) and D646N (gray) (*, $P < 10^{-5}$; n = 8 for WT, 9 for D646N).

capsaicin activation continued, however, P_{Ca}/P_{Na} decreased to approximately the same level as was seen in wild-type TRPV1. Thus, neutralization of D646 compresses the dynamic range of agonist-evoked changes in both P_{NMDG}/P_{Na} and P_{Ca}/P_{Na} .

Capsaicin also evoked concentration- and time-dependent changes in TRPV1 P_{Ca}/P_{Na} in the absence of NMDG at 150 mM Na⁺ and 10 mM Ca²⁺ (Supplementary Fig. 9 online). RTX produced robust time-dependent changes in P_{Ca}/P_{Na} that fell into two patterns, similar to capsaicin (Fig. 6a). Four of eight cells began at or near their maximal P_{Ca}/P_{Na} , whereas the others showed a lower initial P_{Ca}/P_{Na} that increased over 30 s to reach a comparable maximal value. Across all eight cells, however, there was an 87% decline from that maximum by 3 min of stimulation. The RTX-evoked inward current amplitude typically showed an inflection at the time of the most abrupt drop in $P_{\rm Ca}/P_{\rm Na}$ (Fig. 6a). RTX-evoked changes in $P_{\rm Ca}/P_{\rm Na}$ were also observed in rat trigeminal ganglion neurons in 150 mM Na⁺ and 10 mM Ca²⁺ (Fig. 6b). Neuronal TRPV1 currents showed a 59% decrease in P_{Ca}/P_{Na} over 5 min of stimulation, with an initial small increase occurring in a few cells. In neurons, as in TRPV1 HEK293 cells, an inflection in current amplitude was observed at the point of sharpest P_{Ca}/P_{Na} decline (Fig. 6b). Thus, both native and recombinant TRPV1 show agonistand time-dependent changes in P_{Ca}/P_{Na} .

TRPV1 phosphorylation modulates ionic selectivity changes

Because PKC-mediated TRPV1 phosphorylation at cytosolic serine and threonine residues has been shown to enhance agonist activation of TRPV1 (refs. 25–27), we sought to determine whether PKC might also modulate capsaicin-evoked ionic selectivity changes (**Fig. 7**). We pretreated TRPV1 HEK293 cells with the PKC activator phorbol myristoyl acetate (PMA, 50 nM) or vehicle before application of 10 nM capsaicin, and compared the resulting current amplitude and $E_{\rm rev}$ changes in 150 mM NMDG. Vehicle-treated cells showed only modest capsaicin (10 nM)-evoked changes in $E_{\rm rev}$ and $P_{\rm NMDG}/P_{\rm Na}$ (**Fig. 7a**). In contrast, we observed a 3.5-fold capsaicin-evoked $P_{\rm NMDG}/P_{\rm Na}$ increase in PMA-pretreated cells (**Fig. 7b**). Such sensitization by PMA was accompanied by a ~ 13-fold enhancement in peak inward current, whereas the $P_{\rm NMDG}/P_{\rm Na}$ increase evoked by 1 μ M capsaicin was not significantly different between vehicle and PMA groups (P = 0.58, data not shown). Pretreatment with 1 μ M bisindolemaleimide, a PKC inhibitor, prevented the effect of PMA on $P_{\rm NMDG}/P_{\rm Na}$ increase (data not shown).

To determine whether direct TRPV1 phosphorylation might account for the PMA augmentation of ionic selectivity changes, we analyzed TRPV1 S800A, a mutant channel shown to be impaired in phosphorylation and sensitization by PKC^{25,26}. As in stable TRPV1 HEK293 cells, PMA (100 nM) pretreatment of cells that were transiently transfected with wild-type TRPV1 resulted in an increase in the amplitude of currents evoked by 10 nM capsaicin (at –100 mV) (**Fig. 7e**), as well as enhancement of the capsaicin-evoked change in $P_{\text{NMDG}}/P_{\text{Na}}$ (**Fig. 7f**). TRPV1 S800A showed a slightly greater capsaicin-evoked $P_{\text{NMDG}}/P_{\text{Na}}$ change than wild-type TRPV1 after vehicle pretreatment, but we observed no sensitization of either inward current amplitude or



Figure 6 Agonist-evoked changes in TRPV1 P_{Ca}/P_{Na} in the presence of 10 mM Ca²⁺ and 150 mM Na⁺. (a) Top left, representative currents (at -80 mV) (top) during the application of 1 nM RTX to TRPV1 HEK293 cells in external solution containing 10 mM Ca²⁺/150 mM Na⁺ (int A/ext F). Bottom left, corresponding P_{Ca}/P_{Na} (calculated using equation (5)) measured during the responses. Right, mean \pm s.e.m. initial (open bar), maximum (black bar) and final (at 3 min, gray bar) P_{Ca}/P_{Na} (n = 8). Arrows in **a** and **b** highlight abrupt changes in current and P_{Ca}/P_{Na} . (**b**) Representative currents (at -80 mV) (top left) and corresponding P_{Ca}/P_{Na} (bottom left) measured during the application of 1 nM RTX to rat trigeminal ganglion neurons in the same external solution used in **a**. Right, mean + s.e.m. initial (open bar), maximum (black bar) and final (at 5 min, gray bar) P_{Ca}/P_{Na} (n = 5). For all panels, * indicates P < 0.01 and ** indicates P < 0.001, paired Student's *t*-test.

capsaicin-evoked $P_{\text{NMDG}}/P_{\text{Na}}$ change following PMA pretreatment. Despite these differences, currents evoked by 10 nM capsaicin measured at +20 mV were not significantly different (vehicle, P = 0.17; PMA, P = 0.72) between wild-type and S800A channels.

PKC activation also increased the change in TRPV1 P_{Ca}/P_{Na} that was evoked by 10 nM capsaicin. When we assayed stable TRPV1 HEK293 cells in 150 mM NMDG and 1 mM Ca²⁺, P_{Ca}/P_{Na} increased modestly from 10 to 14 during 10 nM capsaicin exposure (**Fig. 7c,d**). Pretreatment with PMA significantly enhanced the peak P_{Ca}/P_{Na} to 21 (P = 0.002) without a significant change in initial P_{Ca}/P_{Na} (P = 0.11). This change was accompanied by a ~ twofold increase in peak inward

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Figure 7 Protein kinase C sensitizes capsaicinevoked TRPV1 ionic selectivity changes. (a,b) Left, representative currents at -100 mV in external NMDG (ext C) during consecutive stimulation of TRPV1 HEK293 cells with 10 nM and 1 µM capsaicin, following pretreatment with either vehicle (ethanol, a) or 50 nM phorbol myristoyl acetate (PMA, b). Right, I-V relations at the times indicated (left). (c,d) TRPV1 currents, in the presence of 1 mM Ca²⁺ at -100 mV, evoked by 10 nM capsaicin (left) and corresponding I-V relations (right) after pretreatment with vehicle (c) or 0.1 µM PMA (d). The switch between 1 mM Ca²⁺/150 mM NMDG and EGTA/150 mM NMDG (ext D and ext E) is indicated by the topmost horizontal bars. (e) Currents evoked in external NMDG (ext C) by 10 nM capsaicin in HEK293 cells that were transiently transfected with wild-type TRPV1 (WT) or S800A, and recorded at +20 mV (left) or -100 mV (right) following pretreatment with vehicle (open bars) or 0.1 µM PMA (filled bars). **, P < 0.001, n = 10-12. (f) Initial and maximal $P_{\rm NMDG}/P_{\rm Na}$ measured during the experiment in **e**. **, P < 0.001, n = 10-12. (g) Initial and maximal P_{Ca}/P_{Na} (at 1 mM Ca²⁺)

a Vehicle **b** PMA 10 nM CAP 10 nM <u>CAP</u> 1 μM CAP 0.3 Current Current 0.0 0.0 (nA) (nA) 0.5 nA С d 0.4 nA d -0.3 1 min 1 min -100 -0.3 -60 -20-100 -60 -20 Ч Voltage (mV) Voltage (mV) 1 Ca²⁺+ EGTA-С d 150 NMDG 150 NMDG 1 Ca²⁺+ EGTA+ 80 80 150 NMDG 150 NMDG РМА 10 nM CAP Current Current 40 40 Vehicle 10 nM CAP 0 0 è -40 PA a -40 P h ~ ٠d , d, -80 0.5 nA -80 0.3 nA 30 s -60 -20 -100 -60 -100-2020 s Voltage (mV) Voltage (mV) e t g 30 0.20 100 <u>م</u> 20.16 مر Ь Current density Ч 0.3 rent density -80 ط² 20 P U Aq) -60 0.12 0.2 P_{NMDG}/ ž 2 -40 °° 0.08 +20 0. 8 0.04 at 0.00 S800A Max Ini Max Ini Ini TRPV1 WT S800A WT WT S800A WT S800A □ Vehicle PMA D Vehicle PMA D Vehicle PMA

during 10 nM capsaicin stimulation in vehicle- (open bars) or 0.1 μ M PMA- (filled bars) pretreated cells that transiently expressed WT or S800A. *, P < 0.05; n = 9-12. Internal solution int B was used in all panels.

current. As with $P_{\rm NMDG}/P_{\rm Na}$, PMA failed to sensitize the capsaicin-evoked $P_{\rm Ca}/P_{\rm Na}$ change in TRPV1 S800A (**Fig. 7g**). Thus, agonistevoked changes in both $P_{\rm NMDG}/P_{\rm Na}$ and $P_{\rm Ca}/P_{\rm Na}$ are sensitized by PKC-mediated TRPV1 phosphorylation.

DISCUSSION

Our data demonstrate that, following initial gating by agonist, TRPV1 shows timedependent changes in its discrimination among monvalent and divalent cations that occur over a time span of seconds to several minutes. Although considerable attention has previously been focused on the quantitative regulation of TRPV1 activity (that is, whether the channel is open), these findings identify ionic selectivity as a qualitative signaling property that is influenced by such parameters as TRPV1 phosphorylation, the extent of TRPV1 activation and even which agonist is applied.

One consideration when interpreting changes in $E_{\rm rev}$ is whether they result from

the accumulation of ions inside the cell or in an adjacent extracellular compartment. Several observations argue that this does not explain our findings. First, when TRPV1-expressing cells were held at -70 mV, the transient outward flow of Na⁺ ions was small compared with the subsequent sustained inward flow of NMDG ions. In fact, temporarily halting bath perfusion during capsaicin stimulation, which should augment extracellular Na⁺ accumulation, had no effect on E_{rev} (M.-K.C. and M.J.C., unpublished observation). Second, time-dependent agonist-evoked increases in the TRPV1 YO-PRO1 uptake rate cannot be readily explained by ion accumulation. Third, capsaicin- and RTX-evoked changes in P_{Ca}/P_{Na} are bidirectional, with both increasing and decreasing

phases, depending on the extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$). Finally, two TRPV1 mutants, D646N and S800A, showed smaller changes in $P_{\rm NMDG}/P_{\rm Na}$ than did wild-type TRPV1, despite similar outward current amplitudes measured at +20 mV. These findings support our interpretation that ionic selectivity changes, rather than ion accumulation, explain the observed $E_{\rm rev}$ changes.

Other studies have reported TRPV1-dependent influx of large cations such as FM1-43, H₃O⁺ ions, tetraethylammonium and NMDG in response to agonist stimulation^{10,11}. Endogenous polyamines and cationic aminoglycoside antibiotics have also been reported to permeate TRPV1 (refs. 8,12). Recently, another study⁹ ingeniously exploited this ability of TRPV1 to mediate the flux of large cations by demonstrating that coadministration with capsaicin facilitated the access of the charged local anesthetic molecule QX-314 to its intracellular site of action in voltage-gated Na⁺ channels. However, in all of these studies, neither the potentially dynamic nature of TRPV1 permeability to large cations nor the direct involvement of the TRPV1 pore was addressed. It has also previously been reported that capsaicin can evoke a current response in cultured trigeminal ganglion neurons consisting of two phases with distinct E_{rev}^{28} . However, that study left it unclear whether this represented activation of one or multiple capsaicin receptor subtypes or what the ionic basis of these changes might be. Overall, our findings concur with previously published data and, more importantly, provide the first demonstration to our knowledge of dynamic TRPV1 ionic selectivity.

Our SCAM findings strongly support the argument that changes in the TRPV1 selectivity filter itself account for the observed changes in ionic discrimination that occur on protracted agonist exposure. Together with the absence of carbenoxolone effects on TRPV1-mediated YO-PRO1 uptake, they also argue against the recruitment of a secondary, nonselective pore, such as pannexin 1, as the basis for large cation permeability under these conditions. Our results also suggest that the TRPV1 pore can simultaneously accommodate MTSET, MTSEA and TRIS molecules, substantiating the permeation of TRPV1 by larger cations such as NMDG or YO-PRO1. Furthermore, the fact that covalent modification of a residue in the middle of the selectivity filter reduces permeability to both small and large cations argues against a multi-barreled pore in the TRPV1 tetrameric complex. However, we cannot exclude the possibility that alterations in TRPV1 ionic selectivity require allosteric input from other protein or nonprotein molecules.

Although a functional increase in pore diameter is indicated by our excluded volume calculations, this does not necessitate a rigid concentric pore enlargement, but could alternatively be explained on the basis of increased conformational flexibility that more readily permits flux by large cations. Regardless, structural rearrangements in and around the pore loop domain leading to altered TRPV1 ionic selectivity are likely to occur. Several mutations in the pore loop domain have been shown to enhance the sensitivity of TRPV1 to capsaicin²³, whereas neutralization of glutamic acid 648 to alanine reduces TRPV1 activation by protons or other extracellular cations^{7,8,29}. In TRPV5, alkaline conditions result in a dilation of the pore diameter and changes in Ca²⁺ permeability, which accompany the rotation of the pore helix domain³⁰. Among other nonselective cation channels, agonist-evoked conformational changes of the pore helix domain were also reported in cyclic nucleotide-gated channels³¹. Therefore, conformational changes in the TRPV1 pore loop region might represent integral features of channel activation, as well as contributors to dynamic ionic selectivity.

Although the differences between low and high P_X/P_{Na} groups in our SCAM analysis indicate that the pore has changed, they do not define the nature of that change. The extent of inhibition is not necessarily a function solely of MTSET accessibility, as we are directly modifying an

ionic permeation path. The pore conformation could also determine the extent to which MTS reagents hinder the flow of ions; a narrower pore would be expected to be more greatly affected by a given modification. In addition, strong activation of TRPV1 might result in multiple modifications of cysteine residues in the tetrameric pore, further complicating the picture. More advanced structural analyses will be required to better understand these processes.

Strong selectivity for Ca²⁺ in L-type Ca²⁺ channels or TRPV6 is achieved not by a sieving mechanism, as the size of Ca²⁺ is similar to that of Na⁺ and smaller than that of the pore, but rather by high-affinity binding of Ca²⁺ to the carboxyl side chains of single acidic residues in the selectivity filter¹⁸. Although P_{Ca}/P_{Na} is much lower in TRPV1 than in L-type channels or TRPV6, the TRPV1 pore still favors Ca²⁺ over Na⁺. The TRPV1 pore size appears to be larger than that of the other two channels, again arguing against a sieving mechanism. High-affinity binding at D646 evidently contributes to TRPV1 Ca²⁺ selectivity. Our results, however, suggest that the TRPV1 pore has at least one more Ca²⁺ binding site, deeper in the pore, which may be resistant to external EGTA quenching and responsible for the D646-independent changes in P_{Ca}/P_{Na} . The crystal structure of a bacterial nonselective cationic channel (NaK) has shown that its pore is architecturally similar to the K⁺ channel pore, but has two Ca²⁺ binding sites³². One is just outside of the selectivity filter. The second, one of two cationic binding sites in the selectivity filter, is equivalent to position 3 of the K⁺ channel filter. Although the outer Ca²⁺ binding site of NaK requires an acidic residue, backbone carbonyl oxygen atoms form both sites, in contrast with the side chain-based Ca²⁺ binding in highly Ca²⁺-selective TRPV6 and L-type channels. Given the failure of MTS reagents to modify TRPV1 D646C, it is tempting to envision that TRPV1 has a pore structure that is similar to that of NaK.

By monitoring P_{Ca}/P_{Na} over time, we have uncovered substantial changes in this parameter that depend on both capsaicin and Ca²⁺ concentration during the course of TRPV1 stimulation. At higher $[Ca^{2+}]_{0}$, when Ca^{2+} is able to outcompete other ions from the outset of the response, the P_{Ca}/P_{Na} reduction is the most evident consequence of activation-evoked changes in the TRPV1 pore, particularly at high agonist concentrations. This effect is reminiscent of the elimination of Ca²⁺ block that we previously reported following persistent stimulation of TRPV3 (ref. 13). In both cases, a homologous aspartic acid (D646 in TRPV1, D641 in TRPV3) accounts for only a portion of the Ca²⁺ selectivity of the channel, suggesting that Ca²⁺ selectivity is contributed to by other residues. When [Ca²⁺]_o is relatively low, TRPV1 shows a time-dependent increase in P_{Ca}/P_{Na}. This might reflect an activationdependent increase in the rate of Ca²⁺ binding to sites deeper in the pore than D646 that could increase the competitive advantage of Ca²⁺ over other external ions for permeation through the channel. At intermediate [Ca²⁺]_o, P_{Ca}/P_{Na} first rises and then falls, consistent with a continuum of events. Therefore, Ca2+ and capsaicin concentration-dependent dynamic changes in PCa/PNa may be the results of altered rates of Ca²⁺ interactions with the Ca²⁺ binding sites that are induced by activation-dependent conformational changes, rather than being a simple consequence of changes in TRPV1 pore size.

TRPV1 has been reported to undergo both Ca^{2+} -dependent and -independent desensitization^{7,33,34}. Consistent with previous reports^{21,34}, we observed that TRPV1 currents evoked by either heat or camphor desensitized by 90% in 30 s when placed in external Ca^{2+} free buffer containing 150 mM Na⁺ (M.-K.C. and M.J.C., unpublished observation). This finding is intriguing, given the apparently weak ability of heat or camphor to alter P_{NMDG}/P_{Na} . Ca^{2+} -independent TRPV1 desensitization by heat or camphor may preclude subsequent, slower events that change the selectivity filter. Alternatively, 2008 Nature Publishing Group http://www.nature.com/natureneuroscience

conformational changes that are evoked by heat or camphor might mask ongoing parallel changes that would otherwise result in increased P_{NMDG}/P_{Na}. A reciprocal situation exists for RTX, which evokes a substantial change in TRPV1 NMDG permeability, but does not evoke a transient inward current component under $[Ca^{2+}]_0$ -free conditions. Furthermore, the sustained component of RTX-evoked currents shows little desensitization, even in the presence of $[Ca^{2+}]_{0}$. This apparent relationship between dynamic ionic selectivity and TRPV1 desensitization warrants further exploration. Analogous inverse relationships between current desensitization kinetics and activitydependent changes in ionic selectivity have been reported for P2X₂ and $P2X_4$ (ref. 35), suggesting that the mechanisms underlying dynamic ionic selectivity might overlap among structurally diverse channel families. Another parallel observation is that PKC phosphorylation of a cytosolic domain is either required or facilitatory for ionic selectivity changes in both TRPV1 and P2X₂ (ref. 36).

In vivo, changes in TRPV1 permeability to large cations, such as channel blockers9, polyamines8 or aminoglycosides12, might facilitate the therapeutic, physiological or toxic effects, respectively, of these compounds. A potentially greater physiological result of dynamic TRPV1 ionic selectivity, however, is Ca2+ influx, which has been proposed to regulate not only TRPV1 desensitization, but also heterologous desensitization of voltage-gated channels³⁷, activation of MAP kinase and PI3 kinase¹⁴, peripheral release of inflammatory peptides³⁸, central release of fast neurotransmitters³⁹ and degeneration of sensory nerve terminals⁴⁰. The latter is a major contributor to the ability of high-dose topical capsaicin to ameliorate burning sensory neuropathies and urinary bladder hyperactivity⁴¹. Modulation of TRPV1 P_{Ca}/P_{Na} over a three- to fivefold range could certainly impact these processes. By analogy, the relative calcium permeability of hippocampal NMDA receptors can be augmented twofold by neuronal activity or PKA-mediated channel phosphorylation, with consequences on long-term potentiation^{42,43}. The differential abilities of distinct agonists to alter TRPV1 ionic selectivity might account, at least in part, for their varying abilities to trigger TRPV1-mediated processes^{44,45}. Moreover, the enhancement of agonist-evoked changes in TRPV1 Ca2+ selectivity by PKC provides a previously unknown mechanism by which inflammatory mediators such as bradykinin and prostaglandins might shape the qualitative, as well as quantitative, features of TRPV1dependent nociceptive signaling during tissue injury.

METHODS

Patch-clamp electrophysiology. We applied the whole-cell voltage-clamp technique to HEK293 cells that stably expressed TRPV1 or were transiently transfected with wild-type or mutant TRPV1 cDNAs, using several solutions: (A) 150 mM NaOH and 10 EDTA, (B) 150 mM NaOH, 1 mM MgCl₂ and 5 mM EGTA, (C) 150 mM NMDG-OH and 10 mM EDTA, (D) 150 mM NMDG-OH and 1 mM CaCl₂, (E) 150 mM NMDG-OH and 10 mM EGTA, (F) 150 mM NaOH and 10 mM CaCl₂, and (G) 110 mM CaCl₂ and 2.3 mM Ca(OH)₂. Every solution contained 10 mM HEPES, sufficient HCl to adjust the pH to 7.4 and mannitol to adjust the osmolarity to 290 ± 10 mOsm. The Cl⁻ concentration was calculated when necessary. When a given solution (X) was used as an internal or external solution, it is indicated as int X or ext X, respectively. Unless otherwise indicated, we excluded divalent cations from the bath and included EDTA to minimize the potential effects of residual divalent cations.

Trigeminal ganglion neurons were cultured from Sprague-Dawley rats (postnatal days 1–14, Charles River) and were assayed 16 to 24 h later. All experiments were conducted in accordance with protocols approved by the Johns Hopkins Animal Care and Use Committee. Neurons were superfused with bath solution containing 140 mM NaCl, 3 mM KCl, 2 mM CaCl₂, 0.6 mM MgCl₂, 10 mM HEPES and 10 mM glucose (pH 7.4 with NaOH). Pipettes contained internal solution consisting of 135 mM CsCl, 30 mM CsOH, 10 mM HEPES, 5 mM BAPTA and 2 mM magnesium ATP (pH 7.4, 300 mOsm with

mannitol). After establishing the whole-cell mode, we exchanged the external solution with one containing 150 mM NMDG, 10 mM HEPES and 10 mM glucose (pH 7.4 with HCl, 310 mOsm with mannitol) to measure $P_{\text{NMDG}}/P_{\text{Na}}$. To evaluate $P_{\text{Ca}}/P_{\text{Na}}$, the bath solution was switched with one containing 150 mM NaOH, 10 mM CaCl₂, 10 mM HEPES and 10 mM glucose (pH 7.4 with HCl). To minimize contamination from voltage-gated Na⁺ and Ca²⁺ channel currents, we gradually depolarized the holding potential from -70 mV to 0 mV over ~1 min in the NMDG solution and held it at 0 mV for 5 to 10 min before the recording, until the obvious inward current component disappeared.

For SCAM, int A was used in every experiment. The external solution contained 150 mM X-OH (X = Na⁺, 2-MAE⁺ or TRIS⁺), 10 mM HEPES and 10 mM EDTA (pH 7.4 adjusted with HCl, ~290 mOsm adjusted with mannitol). MTSET bromide, MTSEA bromide and sodium MTSES were obtained from Toronto Research Chemicals and stored at -20 °C as powder. Each day the reagents were dissolved in external solution at 100 mM, stored on ice and diluted to 1 mM immediately before use.

Cells were visualized using an inverted microscope (Nikon) and recorded using an Axopatch 200B amplifier with pClamp 9 software (Axon Instruments). Borosilicate glass electrodes had tip resistances of 3–5 M Ω and series resistance was compensated >75%. A 3 M KCl agar salt bridge was used throughout the experiments. To evaluate the current amplitude and the E_{rev} at the same time, we applied repetitive 100–200-ms voltage ramp pulses (1 mV ms⁻¹ at 0.5 or 1 Hz). The cells were clamped at 0 mV unless otherwise indicated.

Data analysis. In every case, we calculated E_{rev} and the current amplitude after subtracting the endogenous baseline currents resembling Mg²⁺-inhibited current⁴⁶ that was recorded before agonist application. E_{rev} was corrected for liquid junction potential (range –0.2 to 7.2 mV), which was calculated using pClamp software before the calculation of P_X/P_{Na} , using the following equation: $V_M =$ $V_P - (V_{L1} + V_{L2})$, where V_M is the corrected membrane potential, V_P is the pipette potential, V_{L1} is the potential of the solution with respect to the pipette and V_{L2} is the potential of the second solution with respect to the first solution when the bath solution was changed into new solution during the recording. The relative mobilities that we used for 2-MAE and EDTA were 0.49 and 0.27, respectively. To calculate P_X/P_{Na} , we used variations of the Goldman-Hodgkin-Katz equation⁴⁷. For monovalent cations, we used:

$$\frac{P_{\rm X}}{P_{\rm Na}} = \frac{\left[{\rm X}^+\right]_{\rm o}}{\left[{\rm Na}^+\right]_{\rm o}} \times e^{\frac{\Delta E_{\rm rev}F}{RT}} \tag{1}$$

where *F* is the Faraday constant, *R* is the gas constant, $\Delta E_{\rm rev}$ is the difference in $E_{\rm rev}$ between the specific cation X⁺ and the $E_{\rm rev}$ measured in parallel cells in the presence of extracellular Na⁺, *T* is absolute temperature (room temperature, 297.15K; heat, 318.15K), and [X⁺] is the activity of ion X⁺. The subscripts i and o represent intra- and extracellular, respectively. The activity coefficients (γ) for each ion used for calculating [X⁺] are as follows: $\gamma = 0.75$ for Na⁺, Li⁺, K⁺ and Cs⁺, $\gamma_{2-\rm MAE} = 0.78$, $\gamma_{\rm TRIS} = 0.79$, $\gamma_{\rm NMDG} = 0.81$, $\gamma_{\rm Ca} = 0.36$ for 1 to 5 mM Ca²⁺, 0.34 for 10 mM Ca²⁺ and 0.28 for ext G. $E_{\rm rev}$ in the presence of external Na⁺ was ~0 mV when internal solutions containing Na⁺ were used and ~9.5 mV when internal solutions containing Cs⁺ were used in trigeminal ganglion neuron recordings. To calculate $P_{\rm Ca}/P_{\rm Na}$ using int A/ext G, when the Ca²⁺ was the only permeant cation in the bath, we used:

$$\frac{P_{Ca}}{P_{Na}} = \frac{[Na^+]_i}{4[Ca^{2+}]_o} \times e^{\frac{E_{rev}F}{RT}} \times (e^{\frac{E_{rev}F}{RT}} + 1)$$
(2)

assuming $E_{Na}=0\,$ mV. To calculate P_{Ca}/P_{Na} using int A or B/ext D and E, we used:

$$\frac{P_{Ca}}{P_{Na}} = \alpha \frac{[\text{NMDG}]_o}{4[\text{Ca}^{2+}]_o} \times \left(\frac{\Delta E_{\text{Rev}}F}{RT} - 1 \right) \times \left(e^{\frac{E_{Ca}F}{RT}} + 1 \right)$$
(3)

where α is $P_{\text{NMDG}}/P_{\text{Na}}$, calculated after quenching the external Ca²⁺ with ext E, E_{Ca} is the E_{rev} in ext D, and ΔE_{rev} is the shift in E_{rev} when ext D was replaced with ext E.

In the experiment using int A/ext F, we evaluated P_{Ca}/P_{Na} in int A/ext F and ext A using:

$$\frac{P_{\text{Ca}}}{P_{\text{Na}}} = \frac{[\text{Na}^+]_o}{4[\text{Ca}^{2+}]_o} \times (e^{\frac{\Delta F_{\text{rev}}F}{RT}} - 1) \times (e^{\frac{E_{\text{Ca}}F}{RT}} + 1)$$
(4)

ARTICLES

The size of the narrowest region of the pore was estimated using excluded volume theory 15,16 according to:

$$\frac{P_{\rm X}}{P_{\rm Na}} = \left(\frac{R_{\rm C} - R_{\rm X}}{R_{\rm C} - R_{\rm Na}}\right)^2 \tag{5}$$

where $R_{\rm C}$ is the radius of the channel pore, and $R_{\rm X}$ and $R_{\rm Na}$ are the radii of ions

X⁺ or Na⁺, respectively. A square root transformation gives $\frac{P_X}{P_{Na}} = a - bR_X$, where $a = R_C/R_C - R_{Na}$ and $b = 1/R_C - R_{Na}$. The values of *a* and *b* can be obtained from the linear regressions of plots of $\frac{P_X}{P_{Na}}$ versus R_X . The diameter of the narrowest region of the pore is -2a/b. Friction was not taken into account. The radii of organic cations used for the calculation were 3.1 Å for 2-MAE, 3.7 Å for TRIS and 4.5 Å for NMDG¹⁶.

The E_{rev} and current-voltage plot in all figures are raw data without the correction of junction potential or the subtraction of baseline unless otherwise indicated. Data are presented as mean \pm s.e.m. and statistical comparisons were made using an unpaired two-tailed Student's *t*-test unless otherwise indicated.

YO-PRO1 uptake. HEK293 cells were perfused in ext A or imaging buffer containing 130 mM NaCl, 3 mM KCl, 10 mM EGTA, 0.6 mM MgCl₂, 10 mM HEPES, 1.2 mM NaHCO₃ and 10 mM glucose, adjusted to pH 7.45 with NaOH. Fluorescence intensity (470-nm excitation, 525-nm emission) was monitored in isolated cells.

Supplementary Methods and Supplementary Table 3 are available online.

Note: Supplementary information is available on the Nature Neuroscience website.

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AUTHOR CONTRIBUTIONS

All authors designed and interpreted the experiments. M.-K.C. and A.D.G. carried out the experiments, M.J.C. and M.-K.C. wrote the manuscript and M.J.C. supervised the project.

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