Supporting Information

Li et al. 10.1073/pnas.1018550108

SI Materials and Methods

Synthesis of Capsaicinoids. Boc-aminoethyl bromide (262 mg, 1.17 mmol, prepared from ethanolamine) was dissolved in 3 mL of dry THF and added to capsaicin (305 mg, 1.0 mmol), potassium *tert*-butoxide (112 mg, 1.0 mmol), and 18-crown-6 (264 mg, 1.0 mmol) dissolved in 7 mL of dry THF. The reaction was stirred overnight, concentrated, and partitioned between EtOAc and saturated NH_4Cl solution. The EtOAc was washed well with water, then dried and concentrated to 439 mg of white solid. Of this, 150 mg (0.33 mmol) was dissolved in 1 mL of CH_2Cl_2, 35 μL (0.35 mmol) of thiophenol, and 0.5 mL of TFA. After 30 min, the solvent was evaporated and the residue purified by reverse phase HPLC on a semipreparative C_{18} column, eluting with a linear gradient of 20–70% CH_3CN in 50 mM NH_4OAc, pH 4.5, over 50 min. Cap-ethylamine (cap-EA) eluted at 50% CH_3CN and unreacted capsaicin at 68%. Fractions were lyophilized twice to a white powder (81 mg, 54%). ^1H NMR (DMSO-d_6, 400 MHz) showed additional peaks relative to capsaicin at δ 4.66 (*t*, 2H) and 3.39 (*t*, 2H). MS calcd for $C_{20}H_{33}N_2O_3$ (MH)+: 349.2. Found: 349.2.

The acetate salt of cap-EA (46 mg, 0.11 mmol) was further dissolved in 2 mL of dry DMF. Diisopropylethylamine (65 μL, 0.37 mmol) and iodomethane (22 μL, 0.35 mmol) were added and the reaction stirred overnight. HPLC using the conditions described above gave a cap-ET peak eluting at 46% CH_3CN. Fractions were lyophilized twice to a white powder (39 mg, 91%). ^1H NMR (DMSO-d_6, 400 MHz) showed additional peaks relative to capsaicin at δ 4.52 (*t*, 2H), 3.30 (*t*, 2H), and 3.22 (*s*, 9H). MS calcd for $C_{23}H_{39}N_2O_3^+$ (M)+: 391.3. Found: 391.3.

Cap-BT was similarly prepared by full methylation of cap-butylamine and was cap-ETEA prepared by full ethylation of cap-ethylamine using ethyl iodide.

Water/Octanol Partition. A 10-μL aliquot of 10 mM cap-ET in water or 50 mM capsaicin in octanol was added to 0.5 mL of 10 mM NaH_2PO_4 (pH 7.4) and 0.5 mL of octanol, which formed two layers. The tubes were vortexed well and centrifuged briefly to separate the layers. UV absorbance (at 280 nm) of the top (octanol) and bottom (water) layers was measured in a spectrophotometer.

Cell Culture. ***rTRPV1 stable line.*** HEK (human embryonic kidney) 293 cells stably expressing rat TRPV1 were grown in DMEM/F12 with 10% newborn calf serum and antibiotics and cultured in a 5% CO_2 aerated incubator at 37 °C. Cells were trypsinized and plated on 96-well plates or matrix-coated coverslips for Ca^{2+} imaging, YO-PRO-1 imaging, plate-reader assays, or electrophysiological recording. Calcium imaging, fluorescent dye uptake assays, and electrophysiological experiments were all performed 24–72 h after plating.

Culture of primary sensory neurons. Newborn rats (postnatal day 0–2, *Rattus norvegicus*) were killed by acute decapitation following guidelines of Cornell University Institutional Animal Care and Use Committee. Dorsal root ganglia were dissected out, rinsed with Hanks' buffer, and digested in the same solution containing 1 mg/mL type 1 collagenease (Worthington Biochemical Corporation) for 15–30 min at 37 °C. Partially digested tissues were triturated with plastic pipette tips to release neurons into the suspension. The debris was discarded. Neurons in the suspension were centrifuged at 1,000 rpm for 3–5 min. Pellets containing neurons and fibroblasts were resuspended in 0.25% trypsin and digested for an additional 5 min at 37 °C. Cells were spun down, aliquotted onto coverslips coated with poly-D-lysine (0.1 mg/mL) and cultured in MEM supplemented with 10% newborn calf serum and glutamine in an incubator (5% CO_2, 37 °C).

Electrophysiology. ***HEK293 cells.*** Inside-out or cell-attached membrane patches were formed in cells expressing rat TRPV1, bathed in a standard solution containing 10 mM Hepes (Na), 140 mM NaCl, 1 mM $MgCl_2$, and 0.1 mM EGTA, pH to 7.4. The extracellular (pipette) solution had a similar composition to the standard bath solution except 0.1 mM EGTA was replaced with 1 mM $CaCl_2$. For the experiments to determine extracellular block of 100 μM cap-ET, standard bath solution was used as the pipette solution. The solution for agonist perfusion further replaced all chloride ions with gluconate. The patch pipettes were pulled from borosilicate capillaries and fire polished until final tip diameters of 8–12 μm with access resistance 0.2–0.3 MΩ. Data were filtered at 2 kHz and sampled at 1 kHz using the Pulse-Pulsefit (HEKA) software. Patch membranes were held either at constant membrane potential (−60mV) or given a 320-ms ramp pulse from −120 to +80 mV every second for continuous recordings. For derivation of voltage–current (I–V) relationships, 200-msec voltage steps of 10-mV decrements (ranging from +100 mV to −110 mV) were applied to membrane patches. Amplitudes of steady-state currents were used to plot against potentials of test pulses for construction of all I–V plots.

Neuronal Na^+ Currents. ***Pretreatment for cap-ET and/or QX-314 loading.*** Neurons were incubated in artificial cerebrospinal fluid (ACSF) with the following composition (in mM): 10 Hepes, 10 glucose, 150 NaCl, 4.4 KCl, 1.2 $MgCl_2$, 1 Na_2HPO_4, pH 7.4. The agonist or blocker was loaded in two stages. In the first phase, neurons were incubated in ACSF containing either PAO (10 μM) or cap-ET (10 μM), or a combination of both (10 μM PAO + 10 or 50 μM cap-ET) for 15 min. The coverslips were then rinsed with ACSF extensively. In the second phase, cells were incubated in 5 mM QX-314 (in ACSF) for another 15 min.

Whole-cell recording. After finishing loading chemicals into neurons, the coverslips were rinse in ACSF several times and transferred to the recording chamber containing the following extracellular solution composed of (in mM): 10 Hepes, 10 glucose, 60 NaCl, 60 NMGCl, 4.4. KCl, 1 $MgCl_2$, 0.1 $CdCl_2$, 15 TEA, 5 4-aminopyridine, pH 7.4. Neurons were recorded using the conventional tight seal whole-cell voltage clamp method. The intracellular solution for recording contained (in mM): 110 CsCl, 2 $MgCl_2$, 1 $CaCl_2$, 11 EGTA, 10 Hepes, pH adjusted to 7.4 with about 25 mM CsOH. Neuronal Na^+ currents were recorded after breaking into the whole cell mode, immediately following capacitance cancellation, and using a protocol with 200-ms voltage steps from -70 to +30mV. Patch pipettes resistances were in the range of 2–3 MΩ. Maximal peak amplitudes of Na^+ currents were measured and divided by cellular capacitance to obtain current density. All neuronal data were plotted in Fig. 6*C*. Each circle represented one neuron.

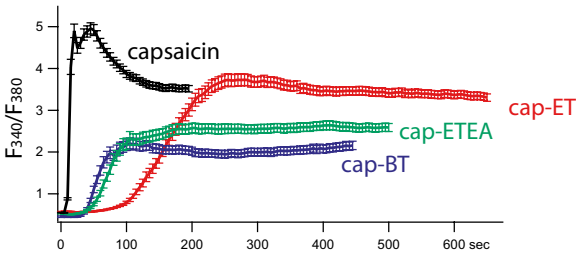


Fig. S1. Raw data of ratiometric Ca^{2+} images without normalization.

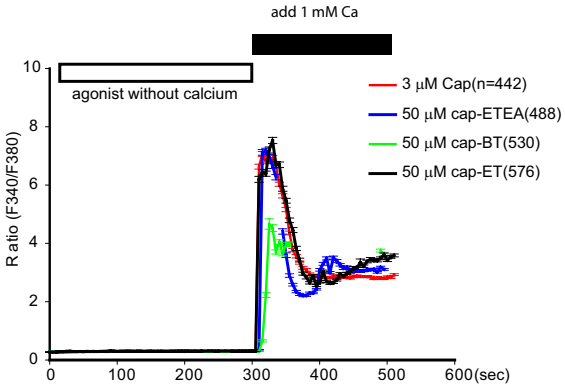


Fig. S2. Capsaicin and capsaicinoid-evoked Ca^{2+} signals were mediated by entry. Cells were initially incubated in a nominally Ca^{2+}-free solution with agonists of specified concentrations for 5 min. At the end of a 5-min incubation, extracellular Ca^{2+} concentration was raised to 1 mM and robust signals were observed. No latency was observed when agonists had been preincubated.

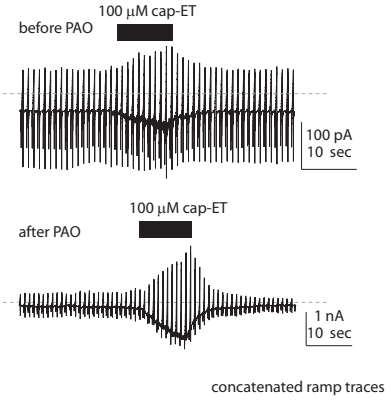


Fig. S3. Blow-up traces of concatenated traces evoked by repetitive voltage ramps were displayed at high magnification. Direct application of cap-ET to the cytoplasmic face of the excised patches elicited currents without latency.

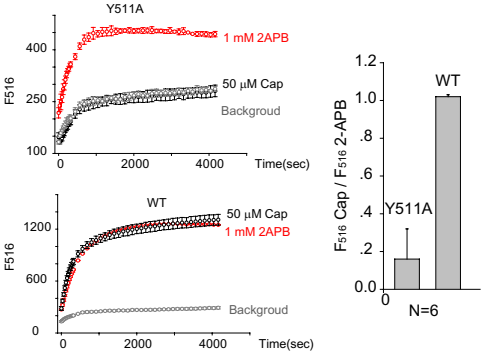


Fig. S4. The rTRPV1 Y511A mutant channel can facilitate YO-PRO-1 entry, provided that the nonvanilloid ligand 2-aminoethoxydiphenyl borate (2-APB) was used to activate the receptor. For the wild-type receptor, 2-APB and capsaicin elicited comparable YO-PRO-1 fluorescence. The bar graph shows the statistics of these experiments.

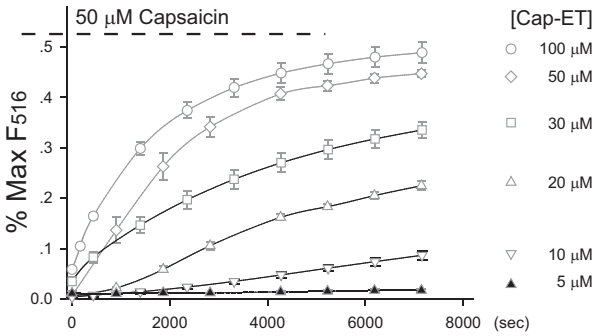


Fig. S5. Kinetics of cap-ET–evoked YO-PRO-1 uptake is slower than that of capsaicin.

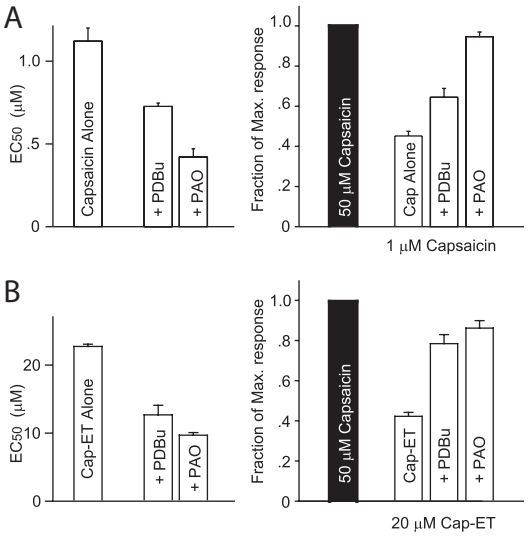
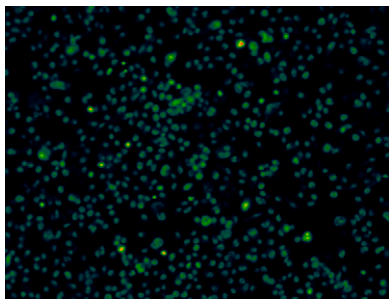


Fig. S6. Summarized statistics for PKC activation and receptor oxidation enhancement of cap-ET–evoked YO-PRO-1 entry.

Table S1. The water/octanol partition coefficient was calculated as the ratio of the two measurements of UV absorbance

	Water/octanol partition coefficient
Capsaicin	0.0054 ± 0.0008
Cap-ET	1.78 ± 0.10

The ratio (capET/capsaicin) = 331 ± 68.



Movie S1. Time-dependent increase of YO-PRO-1 fluorescence in HEK cells stably expressing rat TRPV1 and stimulated by 1 μM capsaicin.

Movie S1