Supplementary Information

Unless otherwise noted, all reagents and chemicals were purchased from Sigma Aldrich.

Protein Expression and Purification

Rat TRPM8 cDNA was a generous gift from Dr. Eleonora Zakharian and Dr. Tibor Rohacs. A TRPM8 construct with 6x histidine tag at the amino terminus was generated by subcloning TRPM8 into pET21b vector (EMD Biosciences) and transforming that into BL21(DE3) *E. coli* (Invitrogen) for protein expression. The protocol for protein expression and purification was adapted from Zakharian *et al.*,[1] with slight modifications described below. Cells were harvested and resuspended in lysis buffer (50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 5 mM EDTA, 0.1% NaN_3, 0.5% Triton X-100, 0.1 mM PMSF, and 1 mM DTT). The resuspended pellet was sonicated and 10 mM $MgSO_4$ was added to chelate the EDTA. DNase (0.01 mg/mL) and lysozyme (0.1 mg/mL) were added to the lysate and left to incubate at room temperature for 20 min. The lysate was centrifuged three times (28,000 x g, 15 min, 4°C) to collect inclusion bodies, with the supernatant being discarded each time. The final pellet was resuspended in equilibration buffer (20 mM HEPES (pH 7.5), 400 mM LiCl, 1 mM $MgCl_2$, and 15% glycerol). The inclusion body pellet was then solubilized with the addition of 0.5% n-dodecyl-β-D-maltopyranoside (DDM) (Anatrace) and left to incubate overnight at 4°C. Nonsolubilized debris was removed by centrifugation (30,000 x g, 1 hour, 4°C). Solubilized inclusion bodies were applied to a Ni-NTA metal affinity column (Qiagen), washed with equilibration buffer containing 20 mM imidazole, and eluted with equilibration buffer containing 350 mM imidazole and 2 mM DDM. Eluted protein was then concentrated and further purified on a Superdex-200 gel filtration column. TRPM8 was eluted with equilibration buffer in the presence of 2 mM DDM. Protein expression and purification was verified with SDS-PAGE gels and Western blots (Fig. S1).

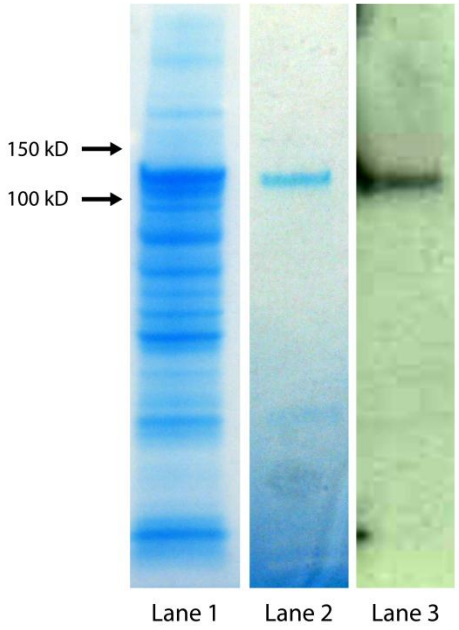

Fig. S1 Proteins were electrophoretically separated at a constant voltage of 125 V with 4–20% Mini-PROTEAN® TGX™ Precast Gel (Bio-Rad) using Tris-glycine SDS buffer (Bio-Rad). Protein bands were visualized by staining with Bio-Safe™ Coomassie G-250 Stain (Bio-Rad). For Western blot analysis, protein was transferred onto nitrocellulose membranes (Bio-Rad) in 10 mM CAPS, 0.07% SDS buffer at 30 V overnight. The TRPM8 protein was detected with anti-CMR IgG antibodies (Santa Cruz Biotechnology, Inc.). Lane 1—inclusion body fraction of BL21(DE3) *E. coli* expressing TRPM8; Lane 2—Coomassie blue staining of TRPM8 protein purified on Ni-NTA metal affinity column (Qiagen); Lane 3—Western blot of purified TRPM8 protein detected with anti-CMR IgG. Protein bands observed are at an apparent molecular weight of ~129 kDa, which corresponds to a monomer of TRPM8.

TRPM8 Proteoliposome Reconstitution

Following purification, TRPM8 was reconstituted into liposomes, adapted from Long *et al.*[2] and Tao *et al.*[3] In brief, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine

(POPE) (Avanti Polar Lipids) at a ratio of 3:1 (w:w) were dried under an argon stream, resuspended at 5 mg/mL in reconstitution buffer (RB, 20 mM HEPES (pH 7.2), 150 mM KCl, 0.2 mM $MgCl_2$), and extruded through 200 nm filters (Avanti Polar Lipids) to form unilamellar liposomes. n-octyl-β-D-maltopyranoside (OM) (Anatrace) was added to a final concentration of 10 mM and incubated at room temperature for 30 min. Purified TRPM8 was added to the liposome solution at the desired protein:lipid (w:w) ratio; three different protein:lipid ratios (1:100, 1:1,000, and 1:10,000) were used. The concentration of OM was then increased to 17.5 mM and the protein/lipid mixture was incubated for 45 min. Detergent was removed with the addition of Bio-Beads SM-2 Adsorbents (Bio-Rad) every hour for six hours at 4°C. The resulting proteoliposomes were aliquoted and stored at –80°C.

PI(4,5)P_2-Dependent Ensemble Measurements of TRPM8

We studied the effect of varying PI(4,5)P_2 concentration on TRPM8 ensemble currents for comparison to previous studies at the ensemble[4] and single-channel level.[5, 6] Proteoliposome solutions of 1:1,000 (w:w) protein to lipid ratio with 250 μM menthol and no PI(4,5)P_2 were measured with no resultant channel activity, as expected (Fig. S2). During this measurement, the PI(4,5)P_2 concentration was increased to 0.8 μM with the addition of 2.4 μL PI(4,5)P_2 stock solution (100 μM) to the 300 μL lower aqueous phase. At a PI(4,5)P_2 concentration of 0.8 μM, channel activity increased, but very infrequent. Increasing the PI(4,5)P_2 concentration to 1.6 μM resulted in almost complete activation of the reconstituted TRPM8. A close look at the sudden rise in current revealed discrete single- and multi- channel openings that closely matched the conductance value determined in single-channel studies (Fig. S2—Inset). As the concentration of PI(4,5)P_2 was increased from 1.6 μM to 2.4 μM, the measured maximum current remained the same, but the frequency of single-channel closing events decreased, indicating further increase in P_{open}.

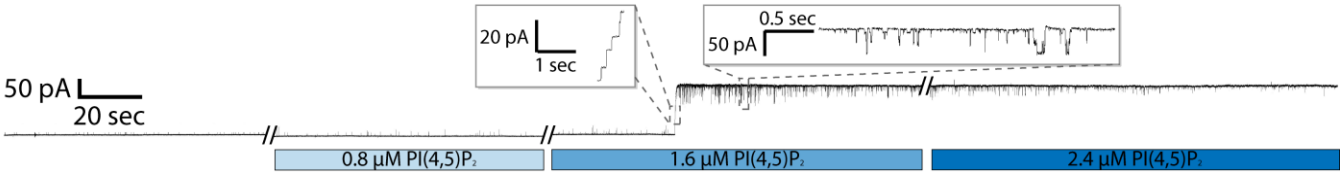

Fig. S2. Ensemble recordings of TRPM8 currents while varying PI(4,5)P_2 concentration in the presence of 250 μM menthol and a clamping potential of +100 mV. Data were filtered at 200 Hz. Measurements began in the absence of PI(4,5)P_2. After ten minutes at each concentration, the concentration of PI(4,5)P_2 was then increased by 0.8 μM with the addition of 2.4 μL PI(4,5)P_2 stock solution (100 μM) to the 200 μL lower aqueous solution. With no PI(4,5)P_2 in the measurement solution, there was no observed channel activity. As the concentration of PI(4,5)P_2 increased, frequency of channel opening increased. At 2.4 μM PI(4,5)P_2, the maximum current did not increase further. Throughout experiments, temperature was kept constant at 20°C.

TRPM8 Single-Channel Studies

Previous work has shown that the signaling phospholipid PI(4,5)P_2 is necessary for TRPM8 function.[7, 4] We measured no channel currents in the absence of PI(4,5)P_2. Hence, in all experiments PI(4,5)P_2 was present in the reconstitution buffer at 2.5 μM.

Following bilayer formation, a potential of 0 mV was applied followed by voltage steps from -100 mV to +100 mV, in 20 mV increments for 10 seconds each, and the resulting current measured. We first explored the effect of temperature on open probability (P_{open}) by measuring TRPM8 single-channel currents while decreasing the experimental temperature from 30°C to 20°C. At 30°C, the channel was predominantly closed, with a P_{open} of 0.035 ± 0.012 (n = 7), which increased to 0.410 ± 0.035 (n = 7) at 20°C (Fig. S3a), matching previous published values.[2] Similar to effect of decreasing temperature, menthol was also seen to activate TRPM8 (at ~20°C) at the single-channel level, with P_{open} increasing from 0.410 ± 0.035 (n = 7) at 0 μM (Fig. S3b) to 0.639 ± 0.029 (n = 9) at 50 μM and 0.967 ± 0.013 (n = 9) at 500 μM (Fig. S3b). In all single-channel measurements, the single-channel conductance was found to be 64 ± 6 pS (n = 21), in agreement with previous work.[2, 3]

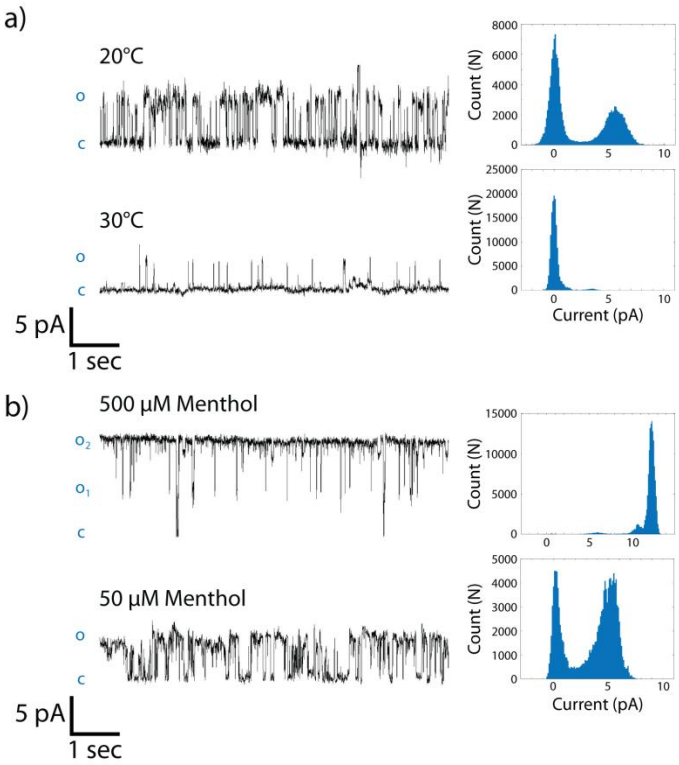

Fig. S3. Cold and menthol activation of TRPM8. Representative single-channel recordings (left panels) and corresponding all points' histograms (right panels) shown with applied potential of +100 mV. Data were filtered at 200 Hz. All single-channel measurements were done using proteoliposomes of a protein:lipid ratio of 1:10,000 (w:w). (a) Temperature-dependent activation of TRPM8. Measurements made in the presence of 2.5 µM $PI(4,5)P_2$ and in the absence of menthol. Single-channel temperature experiments began at 20°C and the temperature then increased to 30°C using an alcohol lamp (see Materials and Methods). P_{open} increased 12-fold from 0.035 ± 0.012 (n = 7) to 0.410 ± 0.035 (n = 7) with a corresponding decrease in temperature from 30°C (bottom panel) to 20°C (top panel). (b) Menthol-dependent activation of TRPM8. Measurements made in the presence of 2.5 µM $PI(4,5)P_2$ while the menthol concentration was varied. Temperature was kept constant at 20°C. Increasing the menthol concentration from 50 µM (bottom panel) to 500 µM (top panel), increased P_{open} from 0.639 ± 0.029 (n = 9) to 0.967 ± 0.013 (n = 9).

Temperature-Dependent Ensemble Measurements of TRPM8

Similarly, reconstituted TRPM8 ensembles were also responsive to temperature changes (Fig. S4). It has previously been shown that TRPM8 responds to temperature without the aid of secondary membrane components.[8, 9] Previous work has also shown that the channel conductance increases at temperatures below ≈28°C and is maximal at temperatures ≈10°C and below.[10] Measurements in the presence of 250 µM Menthol and 2.5 µM $PI(4,5)P_2$ began at 20°C and showed immediate ensemble channel currents of 250 pA magnitude. There was little change in these currents as the temperature was raised to 23°C, but further increases in temperature showed a progressive decrease in measured current, extinguishing completely at 28°C. As a control, bilayers formed from POPC:POPE (3:1) liposomes without reconstituted TRPM8 were measured as a function of temperature and the current did not exceed 10 pA (while capacitance remained constant) through temperatures greater than 38°C.

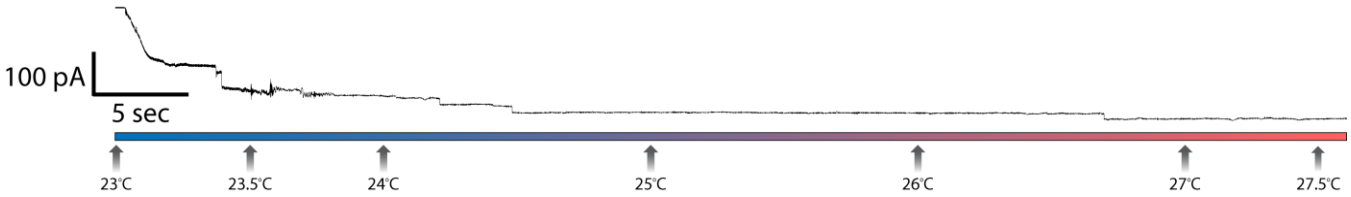

Fig. S4. Ensemble recordings of TRPM8 currents while varying temperature in the presence of 250 μM menthol and a clamping potential of +100 mV. Data were filtered at 200 Hz. Measurements made in the presence of 2.5 μM $PI(4,5)P_2$ and at a starting temperature of 20°C. After recording channel activity at 20°C for five minutes, alcohol lamp was then ignited and chamber temperature was slowly increased to 35°C. In representative trace, maximal current (275 pA) was achieved at 20°C and, upon warming, remained constant until 23°C. For temperatures greater than 23°C, the measured current progressively decreased and was finally extinguished at 28°C.

References

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