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The Super-Cooling Agent Icilin Reveals a Mechanism of Coincidence Detection by a Temperature-Sensitive TRP Channel

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Summary

TRPM8, a member of the transient receptor potential family of ion channels, depolarizes somatosensory neurons in response to cold. TRPM8 is also activated by the cooling agents menthol and icilin. When exposed to menthol or cold, TRPM8 behaves like many ligand-gated channels, exhibiting rapid activation followed by moderate Ca^{2+}-dependent adaptation. In contrast, icilin activates TRPM8 with extremely variable latency followed by extensive desensitization, provided that calcium is present. Here, we show that, to achieve full efficacy, icilin requires simultaneous elevation of cytosolic Ca^{2+}, either via permeation through TRPM8 channels or by release from intracellular stores. Thus, two stimuli must be paired to elicit full channel activation, illustrating the potential for coincidence detection by TRP channels. Determinants of icilin sensitivity map to a region of TRPM8 that corresponds to the capsaicin binding site on the noxious heat receptor TRPV1, suggesting a conserved molecular logic for gating of these thermosensitive channels by chemical agonists.

Introduction

The transient receptor potential (TRP) family encompasses a large number of nonselective Ca^{2+}-permeable cation channels that are widely expressed among eukaryotes, where they are found in a variety of tissues and cell types. Although physiological roles have yet to be determined for most members of this family, pharmacological and genetic evidence has clearly implicated certain TRP channels in the detection or transduction of sensory stimuli (Clapham, 2003; Montell, 2001). One notable example is provided by the mammalian TRP channels TRPV1 and TRPM8, which are believed to function as thermosensors on primary afferent nerve fibers of the somatosensory system (Jordt et al., 2003; Julius and Basbaum, 2001; Patapoutian et al., 2003). In addition to being activated by changes in ambient temperature, these channels are also gated by chemical agonists that elicit psychophysical sensations of heat or cold, respectively (Caterina et al., 1997; McKemy et al., 2002; Peier et al., 2002). Thus, rat TRPM8 is activated by cold ($<$26°C), as well as by natural cooling compounds such as menthol and eucalyptol. TRPM8 is also activated by the synthetic "super-cooling agent" icilin (AG-3-5), which is structurally unrelated to menthol and ~200-fold more potent (Behrendt et al., 2004; McKemy et al., 2002; Wei and Seid, 1983). Because these agonists shift the temperature threshold at which cold-sensitive neurons or the cloned TRPM8 channel can be activated (Hensel and Zotterman, 1951; McKemy et al., 2002; Peier et al., 2002; Reid et al., 2002), it is likely that cooling agents generally serve as positive allosteric modulators that enable the channel to open at higher than normal temperatures. At the same time, there is evidence to suggest that menthol and icilin activate TRPM8 through distinct mechanisms. Most notably, menthol activates TRPM8 in the absence of extracellular calcium, whereas icilin is essentially inactive in the absence of calcium (McKemy et al., 2002). These and other observations raise a number of interesting questions regarding the mechanisms of cooling agent action: whether they are one and the same and how they relate to gating of the channel by cold.

Besides or in addition to functioning as primary detectors of sensory stimuli, many TRP channels are gated or modulated by metabotropic receptors that activate phospholipase C (PLC) signaling systems. While there has been much debate with regard to how TRP channels are regulated downstream of PLC-coupled receptors (Hardie, 2003b), it now seems likely that members of this structurally divergent class of ion channels respond to different consequences of PLC activation, such as the hydrolysis of PIP_2 (Chuang et al., 2001), production of diacylglycerol (Hofmann et al., 1999) or free fatty acid metabolites (Chyb et al., 1999; Hardie, 2003a), or the release of Ca^{2+} from intracellular stores (Launay et al., 2002; Liu and Liman, 2003; Perez et al., 2002; Prawitt et al., 2003). Regardless of the exact mechanism, the ability of some TRP channels to be regulated by PLC-coupled receptors endows them with the capacity to respond to more than one stimulus and to therefore serve as polymodal signal detectors. For example, the sensitivity of TRPV1 to noxious heat can be greatly enhanced by inflammatory agents that activate PLC signaling pathways (Cesare and McNaughton, 1996; Chuang et al., 2001; Premkumar and Ahern, 2000; Tominaga et al., 2001). Such integration of physical and chemical inputs allows TRPV1 to detect subthreshold stimuli, providing one way through which tissue injury produces thermal hypersensitivity (Julius and Basbaum, 2001). Mechanistically, each of these inputs alone can open the channel to a significant extent, and synergistic activation by multiple stimuli—even at low levels—can also drive channel open probability toward the maximum achievable by any single stimulus (Tominaga et al., 1998).

While some TRP channels, such as TRPV1, TRPV4, and TRPM8, show such a capacity for synergistic stimulus integration (Caterina et al., 1997; McKemy et al., 2002; Nilius et al., 2003), we know of no case for TRP channels in which there is codependency of activation, wherein channel gating requires the simultaneous presence of two independent coagonists. Here, we illustrate the potential for this mechanism as it applies to TRP channels by demonstrating that significant or maximal

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activation of TRPM8 by icilin requires simultaneous exposure of the channel to intracellular calcium ions. In contrast, menthol or cold can directly and robustly gate TRPM8 in a membrane-delimited fashion, even when all calcium ions are strongly buffered. This differential requirement for coagonists illustrates the potential for TRPM8 to detect various combinations of environmental cues as a mechanism for expanding its sensory repertoire. While many TRP channels have been identified in the genome, little is known about their gating mechanisms. Our findings suggest that some TRP channels may serve as coincidence detectors whose full activation requires the simultaneous presence of two or more physiological stimuli. Finally, comparative analysis of avian and mammalian TRPM8 channels reveals specific amino acid residues within the putative intracellular loop connecting transmembrane domains 2 and 3 that are essential for icilin activation. Remarkably, a topologically equivalent domain has previously been implicated in the binding of capsaicin and other vanilloid ligands to TRPV1 (Jordt and Julius, 2002; Gavva et al., 2004), suggesting that TRP channels use a conserved structural logic to mediate interactions with chemical agonists.

Results

Voltage-clamp recordings from TRPM8-expressing *Xenopus* oocytes or HEK293 cells have shown that cold or menthol evokes rapidly developing and slowly desensitizing nonselective cationic currents similar to those observed in cultured sensory neurons (McKemy et al., 2002; Reid et al., 2002). In contrast, we found that icilin-evoked currents displayed completely different kinetic profiles compared to menthol- or cold-activated currents, exhibiting highly variable latencies with pronounced desensitization (Figures 1A and 1B). Moreover, icilin displayed very little agonist activity in the absence of extracellular Ca^{2+}, whereas menthol or cold produced robust and immediate responses under these same conditions (Figures 1C and 1D; McKemy et al., 2002). This reduction of icilin activity in Ca^{2+}-free conditions does not appear to reflect a decrease of agonist potency, since increasing icilin concentration from 10 to 100 μM did not produce larger currents under Ca^{2+}-free conditions. A striking recovery of agonist efficacy was observed when Ca^{2+} was replenished in the extracellular recording solution (Figure 1C). Like menthol-activated TRPM8 currents, icilin-evoked responses were suppressed by raising the bath temperature such that at 33°C even prolonged application of icilin in Ca^{2+}-containing solutions produced no TRPM8 currents (data not shown).

To analyze how the extracellular Ca^{2+} ions assist icilin in activating TRPM8, we first asked whether other divalent cations can substitute for Ca^{2+} in this process. Interestingly, none of the divalent ions that were tested, including Mg^{2+}, Sr^{2+}, Ba^{2+}, Mn^{2+}, Co^{2+}, and Zn^{2+}, were able to replace Ca^{2+} in supporting full agonist activity of icilin (Figure 2A). Ca^{2+} might serve as a coagonist of TRPM8 by interacting directly with the channel at an extracellular binding site, in a manner resembling the sensitizing effect of extracellular protons on the capsaicin/heat receptor TRPV1 (Jordt et al., 2000; Tominaga et al., 1998; Welch et al., 2000). Such a mechanism predicts that one would see a rapid effect of Ca^{2+} upon extracellular application, but this is not consistent with the variable latencies that we observed when icilin-evoked channel activation was measured in the whole-cell configuration in the presence of extracellular calcium (Figure 1B). Alternatively, influx of extracellular Ca^{2+} into the cell may be required to enhance icilin's efficacy. Indeed, this appears to be the case, since icilin-evoked currents in TRPM8-expressing oocytes were abolished when the Ca^{2+} chelator BAPTA was injected into the cell, whereas activation by menthol was unaffected (Figure 2B). These results suggest that extracellular Ca^{2+} serves as an icilin cofactor by entering the cell and acting at an intracellular site. How, then, does icilin stimulate Ca^{2+} influx across the plasma membrane or otherwise increase cytoplasmic Ca^{2+} concentrations? First, we asked whether icilin could elicit increases in intracellular Ca^{2+} independent of TRPM8 expression. This possibility was ruled out, since mock-transfected HEK293 cells showed no evidence of icilin-induced cytosolic Ca^{2+} rise, even when the bath solution contained 2 mM Ca^{2+}. Moreover, TRPM8-expressing HEK293 cells showed icilin-evoked increases in intracellular Ca^{2+} only when Ca^{2+} was present in the bath solution (Figure 2C), excluding a mechanism involving release of Ca^{2+} from intracellular stores. Taken together, our results suggest that icilin-evoked increases in intracellular Ca^{2+} are initiated by influx through TRPM8 channels themselves. This model presupposes that icilin can activate TRPM8 to some small but finite degree in the absence of extracellular Ca^{2+}, thereby generating a positive feedback loop to further enhance efficacy of icilin and subsequent channel activation. Indeed, whole-cell recordings from TRPM8-transfected HEK293 cells revealed a low level of icilin-evoked activity, even when EGTA was included in the bath solution (Figure 3A). Because TRPM8 shows slight activation at room temperature (21°C), this small icilin-induced current in EGTA-containing bath solution is likely due to a potentiation of basal thermally evoked channel activity.

Activation of TRPM8 by icilin in EGTA-containing bath solution, even at a concentration of 100 μM, was still dramatically reduced compared to currents evoked by 10 μM icilin in the presence of 1 mM extracellular Ca^{2+} (Figure 3B). Furthermore, we found that icilin readily potentiated cold-evoked currents (Figure 3C) and partially reduced menthol-evoked currents (data not shown) in TRPM8-expressing oocytes bathed in Ca^{2+}-free solution. Taken together, these results indicate that icilin retains the ability to interact with the channel, even in the absence of extracellular calcium, and behaves as a partial agonist under Ca^{2+}-free conditions, as long as the recording is performed at temperatures below (i.e., colder than) the threshold for channel activation. Thus, one factor contributing to the variable onset of icilin action may be the delay in achieving a critical concentration of intracellular free Ca^{2+} needed for full channel activation.

In light of the clear dependence of icilin activity on intracellular calcium, we asked whether inhibitors of major calcium-dependent signaling pathways, including calmodulin (calmidazolin), SERCA pumps (thapsigar-

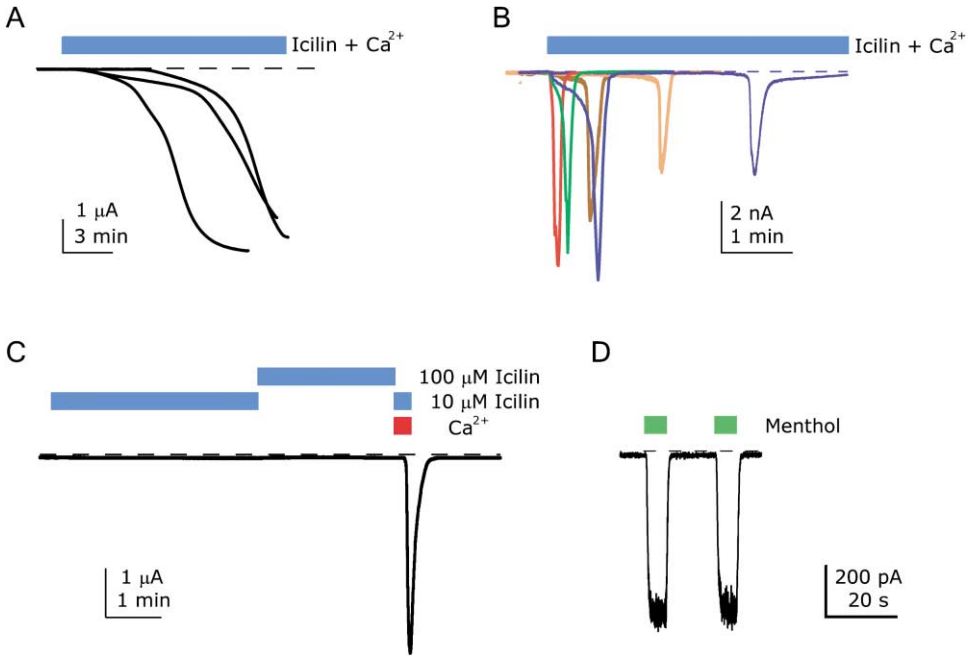

Figure 1. Icilin-Evoked TRPM8 Currents Show Variable Activation Kinetics and Ca^{2+} Dependence

In voltage-clamped TRPM8-expressing oocytes (A) or HEK293 cells (B), icilin (10 μM) activates membrane currents with variable delay of onset. (C) Omission of Ca^{2+} from the extracellular solution greatly reduces agonist efficacy of icilin (10 μM or 100 μM). (D) TRPM8 activation by menthol (200 μM) remains robust and immediate when extracellular calcium is chelated with EGTA (1 mM). Dashed lines in all traces indicate zero current.

gin), and calcium-dependent kinases (K252a), could abrogate icilin-evoked channel activation. The failure of any of these agents to alter icilin-evoked currents (data not shown) prompted us to ask whether icilin, Ca^{2+}, or both could activate TRPM8 when applied directly to inside-out membrane patches excised from oocytes. Application of icilin alone did not produce appreciable currents, even though the channel still responded to menthol (Figure 3D). Unfortunately, the endogenous Ca^{2+}-activated Cl^- conductance in the oocyte membrane precluded our attempts to observe effects of Ca^{2+} on TRPM8 when applied directly to the excised patch. In addition, TRPM8 channel activity exhibited rapid rundown upon patch excision from transfected HEK293 cells, further hindering our efforts to address this issue. To circumvent these technical limitations, we developed an alternative recording mode consisting of a modified half-cell giant membrane patch in the cell-attached configuration. Membrane seals were initially established to a large surface area (30%) of the cell, with EGTA inside

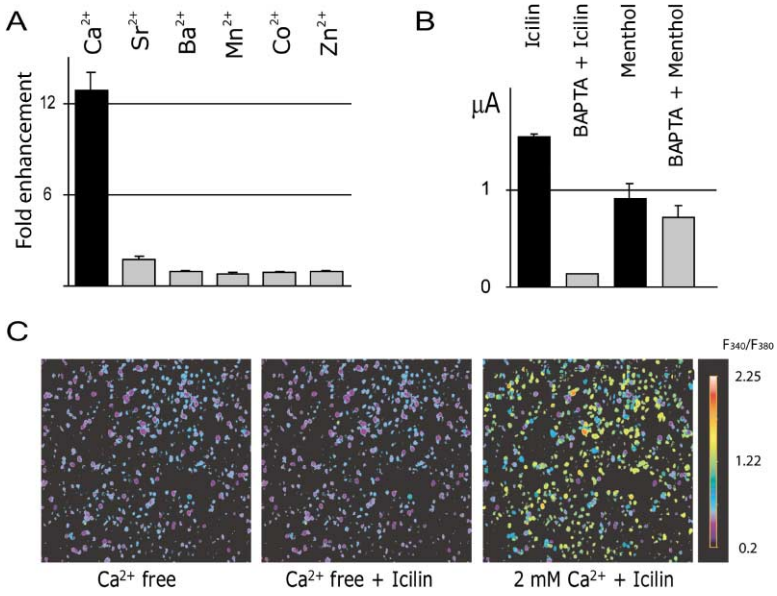

Figure 2. Ca^{2+} Plays a Key Role in Icilin-Mediated Activation of TRPM8

(A) Among several divalent cations tested, Ca^{2+} was the only one that supports full agonist efficacy of icilin in TRPM8-expressing oocytes.

(B) Intracellular injection of BAPTA (100 mM, 23 nl) eliminated agonist efficacy of icilin but not that of menthol (n = 5–9 cells).

(C) Icilin produces an increase in intracellular free Ca^{2+} by promoting Ca^{2+} entry. Intracellular Ca^{2+} levels remained low before (left) or after (middle) addition of icilin (10 μM) to TRPM8-expressing HEK293 cells bathed in nominal Ca^{2+}-free solution. Addition of 2 mM Ca^{2+} to bath solution containing icilin evoked a robust intracellular Ca^{2+} rise (right).

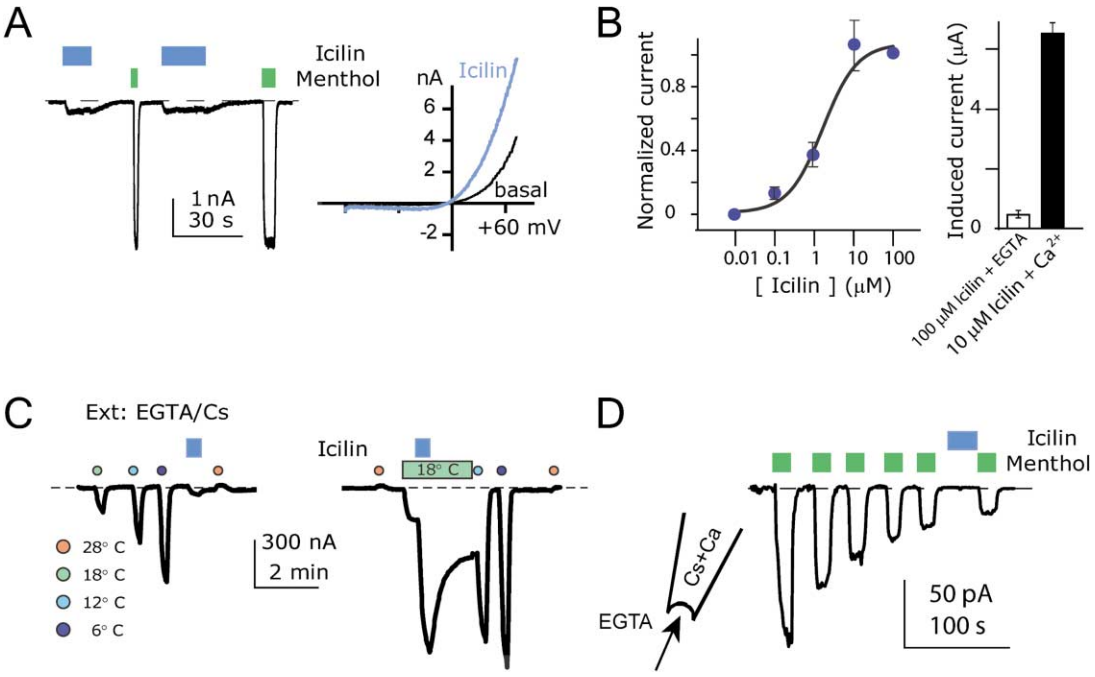

Figure 3. Icilin Has a Low but Measurable Efficacy in the Absence of Extracellular Ca^{2+}

(A) (Left) With all the Ca^{2+} ions chelated by EGTA in the bath solution, icilin produced minimal activation of TRPM8 in HEK293 cells, whereas menthol activation remained robust. (Right) Icilin-evoked current in EGTA displays characteristic strong outward rectification.

(B) Icilin activates TRPM8 in EGTA in a dose-dependent manner (EC_{50} = 1.4 μM; Hill coefficient = 1.59; χ^2 = 0.013; n = 7; and normalized to the 100 μM icilin-induced current). The bar graph shows that icilin behaves like a partial agonist in EGTA.

(C) Icilin potentiates cold-activated TRPM8 currents in a calcium-independent manner. (Left) Cold activates TRPM8 in an intensity-dependent manner. (Right) In the same cell, administration of icilin together with cold induced a much larger current than either stimulus alone, even in the presence of EGTA (1 mM).

(D) Icilin (10 μM) failed to activate TRPM8 after excision of an inside-out membrane patch from an oocyte into "intracellular" solution containing EGTA, whereas menthol (1 mM) still elicited sizeable currents.

the pipette. Since TRPM8 is slightly activated at room temperature (21°C), small basal TRPM8-mediated currents were observed just as the seal was formed (Figure 4A). We then perfused the exposed half of the cell with an icilin (10 μM) solution containing EGTA (1 mM). Icilin was apparently able to diffuse freely across the plasma membrane and cytoplasm to activate channels in the patch, as evidenced by a small stimulation of the basal current. Subsequent addition of icilin plus Ca^{2+} (1 mM) produced biphasic modulation of TRPM8 current, characterized by an initial stimulation followed by desensitization (Figure 4A), similar to that which we observed in standard whole-cell recordings. All currents subsided immediately upon icilin washout, and no responses were observed in vector-transfected control cells exposed to icilin plus Ca^{2+} (data not shown). Based on these observations, we conclude that resting cytoplasmic Ca^{2+} is sufficient to support weak activation of TRPM8 by icilin or that icilin facilitates thermal activation of the channel at room temperature; subsequent influx of Ca^{2+} through open TRPM8 channels on the exposed half of the cell further stimulates icilin bound receptors within the patch pipette. This is supported by the fact that addition of 1 mM La^{3+} (which blocks open TRPM8 channels; data not shown) to the perfusate attenuated the stimulatory effect of Ca^{2+} (Figure 4B). Moreover, icilin was absolutely required for the observed stimulatory effect of Ca^{2+}, since perfusate containing 1 mM Ca^{2+} plus a Ca^{2+} ionophore was not sufficient to activate the channel in the absence of icilin. Apparently, interaction of Ca^{2+} with the extracellular domain of TRPM8 was not required for channel stimulation, since currents were readily observed when Ca^{2+} in the patch electrode was completely buffered by EGTA. We also tested the effects of calcium on other chemical or physical activators of TRPM8, including menthol, isopulegol, WS-3 (N-ethyl-p-methane-3-carboxamide), and cold, and found that stimulation is specific for icilin (Figure 4C). Indeed, coapplication of Ca^{2+} with menthol resulted in only a modest desensitization of TRPM8 currents (Figure 4D). Icilin thus defines a special modality for TRPM8 activation that mandates intracellular Ca^{2+} as a cofactor for achieving full agonist efficacy.

We next asked whether an increase in intracellular free Ca^{2+} from sources other than entry via TRPM8 channels could exert a similar stimulatory effect. We therefore coexpressed the phospholipase C-coupled m1 muscarinic acetylcholine receptor (mAChR) with TRPM8 in HEK293 cells and recorded in cell-attached configuration while buffering extracellular calcium by including EGTA (1 mM) in both the perfusate and pipette solution. Under these conditions, acetylcholine (ACh)-mediated release of Ca^{2+} from IP_3-sensitive stores greatly potentiated icilin-evoked TRPM8 currents (Figure 5A), which were blocked by La^{3+} in the negative voltage range (data not shown). ACh alone did not activate or potentiate

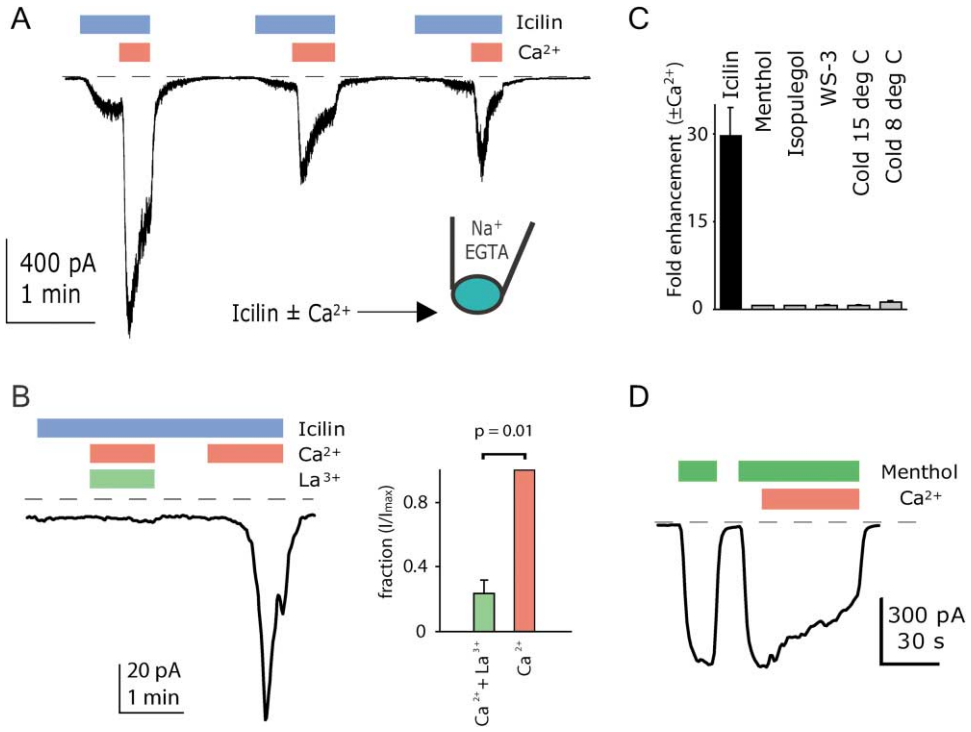

Figure 4. Ca^{2+} Selectively Enhances Icilin-Activated TRPM8 Currents

(A) Application of icilin (10 μM) without Ca^{2+} to the exposed half of a TRPM8-expressing HEK293 cell (in attached half-cell configuration) produced very modest activation. Substantial potentiation was observed when Ca^{2+} (1 mM) was subsequently applied with icilin.

(B) The stimulatory effect of Ca^{2+} on icilin-evoked current was significantly suppressed by the TRPM8 channel blocker La^{3+} (1 mM) ($n = 9$; $p = 0.0098$; paired Student's t test). "I," the size of the evoked current after 60 s application of icilin, Ca^{2+}, and La^{3+}; "I_{max}," the maximal current observed within 1 min application of icilin and Ca^{2+}.

(C) Among known TRPM8 agonists, icilin is uniquely potentiated by Ca^{2+}. Data were derived from two-electrode voltage-clamp recording of TRPM8-expressing oocytes.

(D) In the cell-attached HEK293 cell recording, menthol-evoked TRPM8 current was partially inhibited by coapplication of Ca^{2+}.

TRPM8 responses at room temperature, nor did ACh plus icilin activate any currents in cells expressing mAChR alone (data not shown). Because the cell was bathed in EGTA, robust mAChR stimulation essentially depleted the IP_3-sensitive Ca^{2+} store so that a second application of ACh no longer elevated intracellular Ca^{2+} levels. Consequently, we observed only a transient stimulatory effect of the icilin-induced TRPM8 current, even during continuous perfusion with ACh.

These results demonstrate that an increase in intracellular Ca^{2+} is sufficient to enhance icilin-induced TRPM8 current, but they still do not say whether Ca^{2+} exerts its stimulatory effect by a direct action on the channel or via other Ca^{2+} effectors. To address this issue, we developed a preparation aimed at reducing the extent of TRPM8 rundown while facilitating access to the cytoplasmic side of the receptor. This was achieved by scraping the exposed portion of the patch-clamped HEK293 cell so as to establish a "permeabilized half-cell" preparation. To validate this method, we showed that calcium-dependent activation of human SK potassium channels in transfected HEK293 cells was only observed after scraping (Figure 5B). TRPM8-evoked currents could be recorded for approximately 2 to 5 min in this configuration, albeit with some reduction (5- to 10-fold) in agonist sensitivity. Nonetheless, we readily observed instantaneous and robust stimulation of icilin-induced TRPM8 current when 100 μM Ca^{2+} was coapplied to the permeabilized surface of the cell. This stimulation could be elicited two or three times in the same permeabilized half-cell before the receptors ran down completely, as assessed by exposure to any of the three activating stimuli (menthol, cold, or icilin). Moreover, responses reversed immediately upon chelation of calcium (>90% decrease within 1 s) in the continued presence of icilin (Figure 5B). The stimulatory effect of Ca^{2+} on icilin activation had a steep dependence on Ca^{2+} levels in this permeabilized half-cell mode (Figure 5B) and exhibited agonist specificity in that similar treatment of menthol-activated currents simply displayed moderate inhibition, as observed in whole-cell recordings (Figure 5C). Simultaneous application of icilin and Ca^{2+} to the permeabilized cell induced TRPM8 currents with little or no latency in all cells examined, as compared with the variable latency associated with whole-cell recordings. Furthermore, these responses were of comparable size to those evoked by menthol, demonstrating that the variable latency and efficacy associated with icilin in the whole-cell configuration can be circumvented by providing access of calcium to the cytoplasm.

We also asked whether other IIA group divalent ions could replace Ca^{2+} in this stimulatory action. Ba^{2+}, a

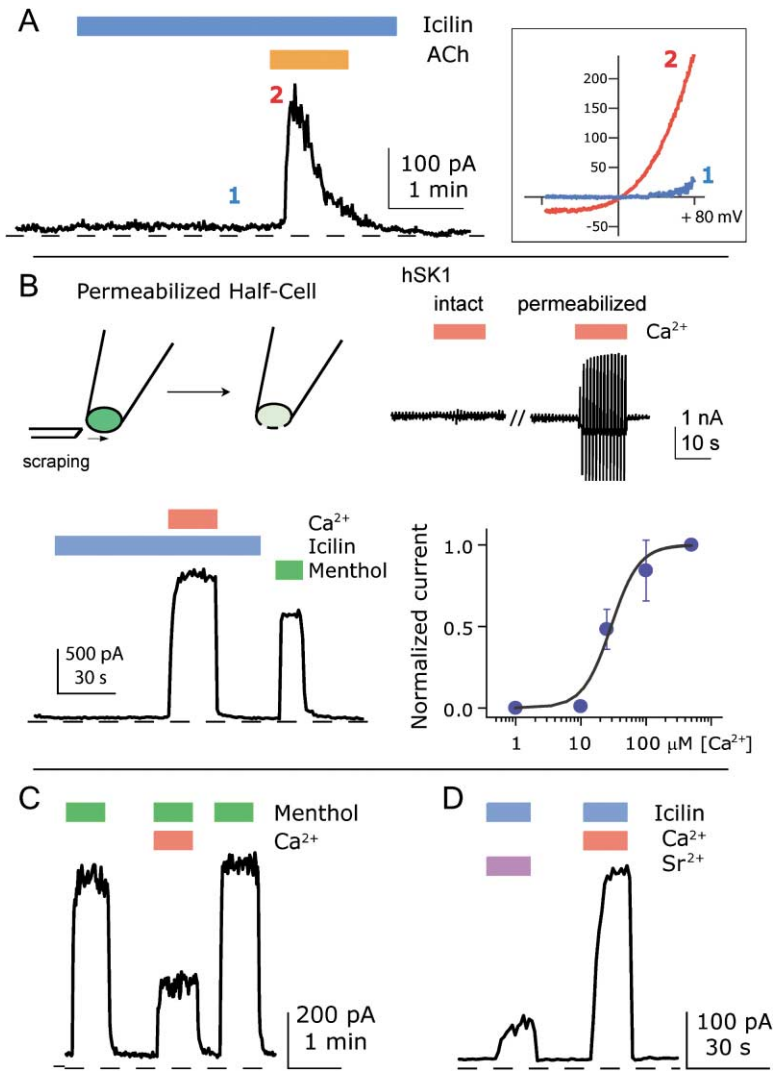

Figure 5. Intracellular Ca^{2+} Rise Stimulates Icilin-Activated TRPM8 Current

(A) Icilin-evoked currents were recorded from HEK293 cells cotransfected with TRPM8 and the m1 muscarinic acetylcholine receptor (mAChR). The experiment was performed in the cell-attached configuration to avoid untoward effects of dialysis on intracellular contents as well as on Ca^{2+} waves due to chelators used in conventional whole-cell recording. Inset shows the current-voltage relationships of icilin-activated TRPM8 current before and during coapplication of ACh (50 μM).

(B) (Upper left) A schematic representation of formation of the permeabilized half-cell. (Upper right) A control experiment with the intact versus permeabilized half-cell recording from a HEK293 cell transiently expressing the human small conductance Ca^{2+}-activated potassium channels (hSK1) demonstrates accessibility of Ca^{2+} to the cytoplasmic side of the SK channels in the permeabilized configuration. Vertical deflections indicate currents evoked by voltage pulses. (Lower left) In the permeabilized half-cell recording configuration, coapplication of icilin and Ca^{2+} to the cytoplasmic surface of the cell evoked robust TRPM8 current with rapid onset/offset and little variability. The relative efficacy of icilin to menthol was also restored when Ca^{2+} was coadministered with icilin to the cytoplasmic side of the membrane. (Lower right) In this configuration, the stimulatory effect of Ca^{2+} is dose dependent (EC_{50} = 29.1 μM; Hill coefficient = 2.02; χ^2 = 0.018; n = 5–9 cells; and normalized to currents evoked by 500 μM Ca^{2+} and 10 μM icilin).

(C) In the permeabilized configuration, menthol activation of TRPM8 was inhibited by intracellular Ca^{2+} rise, as observed with intact whole cells.

(D) Very high concentration of intracellular Sr^{2+} (0.5 mM) can slightly mimic the stimulatory effects of Ca^{2+} on TRPM8 activation by icilin.

divalent ion known to activate calmodulin, was ineffective as a stimulant of icilin, even when applied at high concentrations (5 mM); Sr^{2+} produced only marginal stimulation when applied at a concentration of 0.5 mM (Figure 5D). While it is possible that a Ca^{2+} binding protein closely associated with the receptor serves as the genuine effector for the stimulatory phenomenon, the onset, reversibility, and persistence of this effect in permeabilized cells is suggestive of a direct action of Ca^{2+} or Sr^{2+} on the channel complex, in the absence of freely soluble cofactors.

In light of the very selective effects of Ca^{2+} on icilin action, we were curious to know whether sensitivity of TRPM8 to icilin could be structurally dissociated from that of other agonists. To address this question, we asked whether species orthologs of TRPM8 might exhibit differential sensitivities to chemical and physical stimuli, in which case such functional differences could be linked to minor genetic drift in protein sequence. Indeed, this approach has been used to great effect for identifying critical regions of the capsaicin receptor TRPV1 that specifically mediate sensitivity to vanilloid compounds (Jordt and Julius, 2002; Chou et al., 2004; Gavva et al., 2004; Phillips et al., 2004). For this study, we focused on birds, because they are homeothermic vertebrates that are distantly related to mammals, but whose sensory nerve fibers can also be activated by cold (Schafer et al., 1989). A search of the EST database revealed a sequence from chicken lymphocytes that displays significant sequence identity to rat TRPM8 (rTRPM8). Using an oligonucleotide probe derived from this sequence, we identified a full-length TRPM8 ortholog from a chicken dorsal root ganglion cDNA library whose predicted amino acid sequence shows 79% identity and 88% similarity to rTRPM8. When expressed in oocytes, chicken TRPM8 (cTRPM8) was activated by cold thermal stimuli, albeit with an apparently higher thermal threshold (34°C) compared to rTRPM8 (26°C) (Figure 6A), presumably reflecting the higher core body temperature of birds compared with mammals. Menthol also produced robust responses in cTRPM8-expressing oocytes, with somewhat greater potency compared to

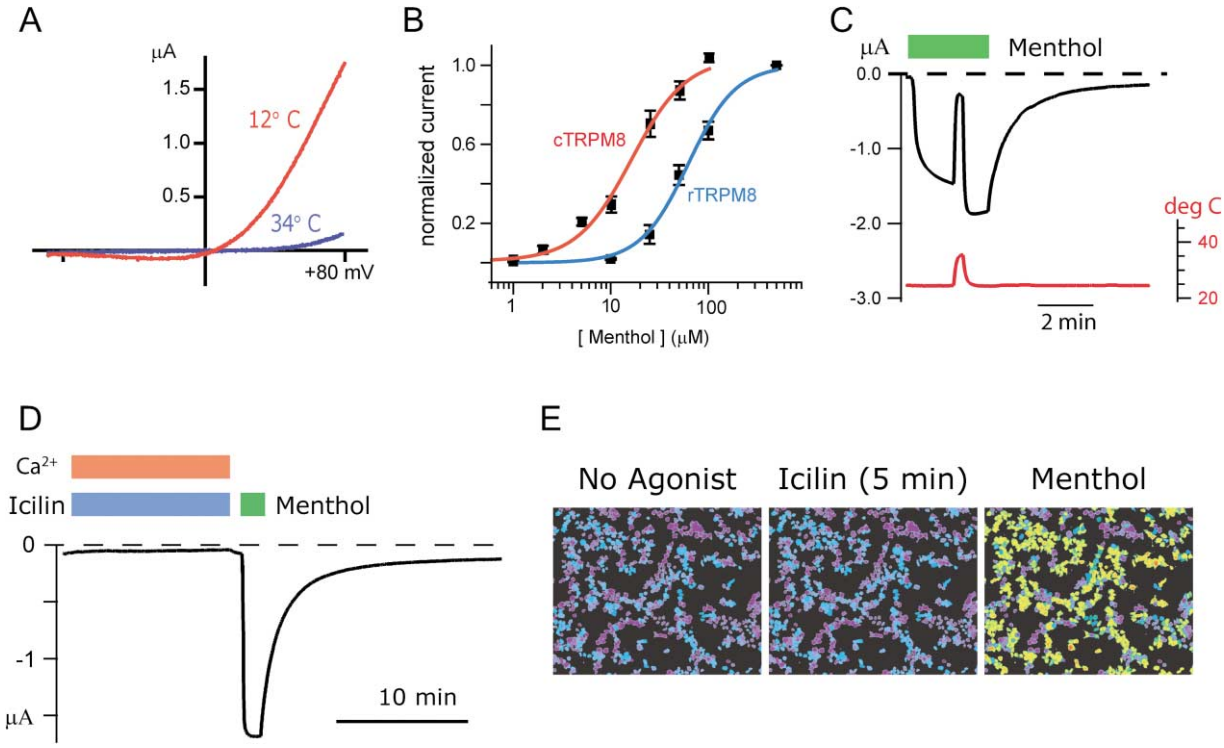

Figure 6. Chick TRPM8 Is Activated by Cold or Menthol but Not by Icilin

(A) cTRPM8 is activated by cold temperature (12°C) and exhibits strong outward rectification. Since small residual current was still observed at 34°C, the apparent temperature threshold for cTRPM8 is higher than reported for rTRPM8.

(B) The menthol dose-response curve of cTRPM8 is left shifted compared to rTRPM8.

(C) Menthol-activated cTRPM8 currents were inhibited by warm temperature.

(D) A 10 min application of icilin to cTRPM8 did not elicit any current, while menthol-evoked response in the same cell was robust.

(E) Insensitivity of cTRPM8 to icilin was also observed in ratiometric fura-2 Ca^{2+} imaging experiments using transfected HEK293 cells.

the rat receptor (EC_{50} = 15.0 ± 1.2 μM for cTRPM8 versus 62.1 ± 3.1 μM for rTRPM8) (Figure 6B). Moreover, as previously observed with rTRPM8, warm temperatures inhibited menthol-evoked responses in cells expressing the chick channel (Figure 6C). In striking contrast to these functional similarities, the avian receptor was completely insensitive to icilin, even when this agonist was applied together with extracellular Ca^{2+} for a prolonged period (>10 min) and at 10-fold greater concentrations (100 μM) than that required to maximally activate rTRPM8 (Figure 6D). A selective loss of icilin sensitivity was also observed by calcium imaging in cTRPM8-expressing HEK293 cells (Figure 6E).

To identify the structural determinants underlying icilin sensitivity, we generated a series of rat-chicken TRPM8 chimeras (Supplemental Figure S1 at http://www.neuron.org/cgi/content/full/43/6/859/DC1). Transfer of the putative TM2 and TM3 regions from the rat to chicken TRPM8 channel was sufficient to confer icilin sensitivity to the resulting chimera (rS23cL). Within these segments, rat and avian receptors differ at only five amino acids (Figure 7A). Mutating rTRPM8 to the corresponding residue in the chick receptor at four of these positions (V743I, A747V, L806I, or F815L) had no effect on cold, menthol, or icilin sensitivity. In contrast, replacement of glycine with alanine at position 805 (G805A) in the rat channel led to a selective loss of icilin sensitivity, thereby phenocopying the chicken channel (Figures 7B and 7D). Moreover, substitution of the corresponding residue in cTRPM8 to that found in the rat receptor (A796G) conferred robust icilin sensitivity to the chicken channel (Figure 7C).

These results demonstrate that a single residue within the third transmembrane domain, at position 805 of rTRPM8, accounts for differential sensitivity to icilin. However, other residues conserved between chicken and rat receptors might also contribute to icilin sensitivity. To identify such sites, we performed alanine scanning mutagenesis within a portion of the channel flanking position 805, covering a segment spanning TM2 through TM3 in rat TRPM8. The resulting mutants were expressed in HEK293 cells and analyzed for sensitivity to cooling compounds by calcium imaging. Mutations at two additional positions, N799 and D802, were found to abrogate icilin sensitivity while retaining sensitivity to menthol (Figure 7D) or cold. Further substitution of the negatively charged aspartate at position 802 with aromatic (Y), polar (S), acidic (E), neutral (N and Q), or basic (H and K) amino acids resulted in icilin-insensitive receptors exhibiting wild-type responses to menthol or cold, indicating a specific requirement for aspartate at this position with regard to icilin sensitivity. In contrast, the asparagine at position 799 could be replaced with aspartate or glutamine without affecting icilin sensitivity, but not by glutamate or tyrosine (data not shown). Alanine replacements at all other positions in the region span-

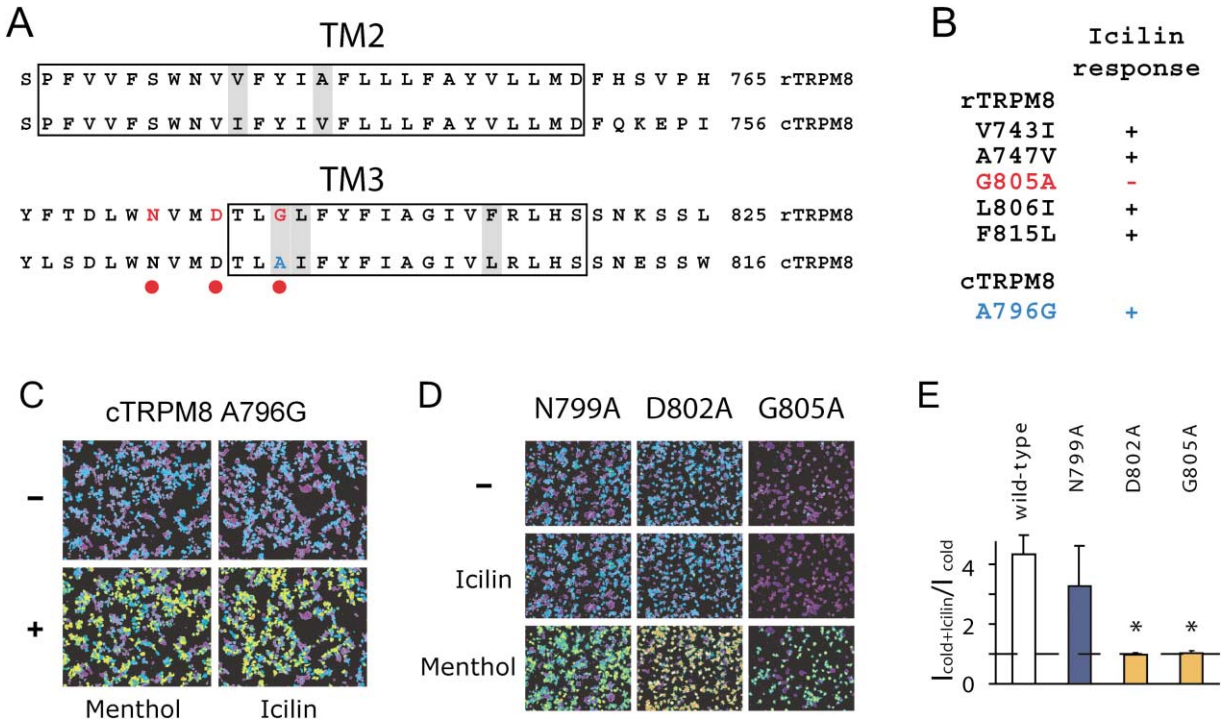

Figure 7. Specific Amino Acid Residues at the Junction between Cytoplasm and Plasma Membrane of the Putative TM3 Domain Are Required for Icilin Sensitivity of Mammalian TRPM8

(A) Sequence comparison of rat and chicken TRPM8 within the region mapped by chimeric analysis highlights variation at five positions within the TM2 and TM3 segments (gray shading). Residues contributing to species-specific icilin sensitivity of TRPM8 are marked with red dots.

(B) A table summarizing amino acid substitution experiments performed within the TM2 and TM3 segments reveals the critical importance of the glycine at position 805 of rat TRPM8 for icilin response.

(C) A single point mutation, A796G, in cTRPM8 is sufficient to convert the chick receptor to an icilin-sensitive ion channel.

(D) Three critical residues in rTRPM8 show specific abrogation of channel response to icilin but not menthol in calcium imaging experiments.

(E) Icilin potentiates cold (18°C)-evoked currents of wild-type rTRPM8 and N799A but not D802A or G805A (n = 8–10; plotted as mean ± SD; asterisks denote $p < 0.01$).

ning TM2 and TM3 had no specific effect on icilin sensitivity or led to a generalized decrease in channel expression or response to all three stimuli (data not shown). Taken together, these results highlight three residues within TM3 or the cytoplasmic loop between TM2 and TM3 as specifically determining sensitivity of TRPM8 to icilin.

Mutations in this short stretch can presumably affect the ability of icilin to serve as a full agonist by disrupting the interaction of the channel with either icilin or Ca^{2+}. To discriminate between these possibilities, we asked whether any of these three mutants preserve the ability of icilin to potentiate cold-evoked TRPM8 currents in the absence of extracellular Ca^{2+}, as this would test whether icilin is still capable of functionally interacting with the channel. We found that icilin no longer potentiated cold-evoked currents in cells expressing the D802A or G805A mutants, consistent with a loss of icilin interaction. Interestingly, icilin remained capable of potentiating cold-induced currents in cells expressing the N799A mutant (Figure 7E). Thus, even within this small region of the channel, we can observe two types of structural changes that differentially affect icilin action: the residue located within the putative cytoplasmic loop (N799) appears to have a greater effect on Ca^{2+}, while those closer to the membrane interface (D802 and G805) may have a greater effect on interaction with icilin. We also asked whether mutations in this region abrogate the Ca^{2+}-dependent desensitization of menthol-activated TRPM8 currents (as shown in Figure 4D) but found that all three mutants remained desensitized upon exposure of the cells to menthol plus Ca^{2+} (data not shown). Thus, Ca^{2+}-dependent channel desensitization and Ca^{2+}-dependent potentiation of icilin action appear to be mediated by different mechanisms.

Discussion

Several TRP channels thus far identified in sensory systems can respond to multiple physical and/or chemical stimuli. TRPV1, the capsaicin receptor, is activated by vanilloid compounds, anandamide, noxious heat, or acid (Caterina et al., 1997; Zygmunt et al., 1999; Smart et al., 2000); TRPV4 responds to hypoosmolar challenge, heat, and metabolites of arachidonic acid or anandamide (Guler et al., 2002; Liedtke et al., 2000; Strotmann et al., 2000; Watanabe et al., 2003); TRPM8 can be activated by cold or chemical cooling agents (McKemy et al., 2002; Peier et al., 2002). In contrast to its immediate activation by menthol (or cold), gating of TRPM8 by icilin exhibits significant temporal delay, comparable to that reported for the activation of TRPV4 by arachidonic acid and

suggestive of a signaling mechanism involving more than a simple ligand-receptor interaction. For TRPV4, the delay in activation by arachidonic acid has been attributed to a requirement for cytochrome P450-mediated conversion of arachidonate to an active metabolite. In the case of TRPM8, we show that the temporal delay in icilin-evoked activation can be explained by a different mechanism, namely, one in which intracellular Ca^{2+} serves as an icilin-selective coagonist. Increases in cytosolic calcium sufficient to support robust icilin-evoked responses can be mediated by calcium release from intracellular stores or by local Ca^{2+} entry via a small number of open TRPM8 channels. Indeed, our measurements of calcium sensitivity for facilitation of icilin action (EC_{50} = 29.1 μM) are well within the range of Ca^{2+} increases achieved within close proximity of calcium-permeable ion channels, as observed in TRP-mediated depolarization of fly photoreceptors (Hardie, 2001). Moreover, this level of sensitivity matches that recently reported for the direct activation of TRPM5 channels by intracellular Ca^{2+} alone (Hofmann et al., 2003; Liu and Liman, 2003; Prawitt et al., 2003).

In contrast to TRPM5, Ca^{2+} by itself has no apparent agonist action on TRPM8 but serves only as a coagonist for icilin. This characteristic, together with the significant permeability of the channel for Ca^{2+} ions, endows TRPM8 with the capacity to generate complex receptor potentials or Ca^{2+} waves. For example, a low concentration of icilin (or an icilin-like endogenous agonist) would induce a cold sensation only when paired with another stimulus that promotes an increase in intracellular calcium, such as an inflammatory agent that activates PLC. This priming effect of Ca^{2+} on icilin-liganded TRPM8 should allow the cell to integrate Ca^{2+} signals from other transmitter pathways and tune sensitivity of the channel to a specific chemical stimulus. Thus, icilin becomes a robust agonist when the cell experiences coincident input from another excitatory stimulus, or after sufficient delay to allow for channel-dependent influx of extracellular calcium. This biophysical design greatly increases the information capacity of one channel, a characteristic that can be particularly useful for receptors involved in sensory transduction. This is in contrast to TRPV1 (Caterina and Julius, 2001) or TRPC6-like channels (Albert and Large, 2003), where simultaneous exposure to two agonists demonstrates synergy but not codependence.

Calcium is also required for TRPM8 desensitization (to cold, menthol, or icilin), demonstrating that a single second messenger can mediate both stimulatory and desensitizing effects. While this may seem paradoxical at first, such an arrangement can effectively broaden the dynamic range of agonist action and sharpen the temporal resolution of signal input. Furthermore, a similar principle is also exploited by *Drosophila* or *Limulus* photoreceptors, where a rise of intracellular Ca^{2+} plays dual roles in both excitation and adaptation to light (Hardie, 2001; Lisman et al., 2002).

Our physiological studies suggest that menthol (and cold) activate TRPM8 in a manner that is mechanistically distinct from that of icilin. Indeed, functional separation of stimulus sensitivities is consistent with recent findings that intracellular pH differentially modulates responses of TRPM8 to menthol versus cold and icilin (Andersson et al., 2004). Our comparative and mutagenesis studies with avian and mammalian TRPM8 channels now provide compelling evidence that these functional differences can be attributed to distinct structural requirements for channel activation, as evidenced by the fact that substitutions at a single amino acid can specifically abrogate or confer calcium-dependent icilin sensitivity without affecting activation by cold or menthol. Taken together, our mutational analysis highlights residues N799, D802, and G805 (of rat TRPM8) as being critical for icilin sensitivity. These amino acids are located in the cytoplasmic loop connecting putative transmembrane domains 2 and 3, where they are positioned at three-residue intervals resembling a helical turn. It is therefore conceivable that side chains from these residues are located on the same side of an α helix facing the cytoplasm, where they may function as an interaction site for icilin, Ca^{2+}, or both. Unfortunately, radioligands or competitive antagonists for TRPM8 have not been developed, and thus direct measurements of agonist binding are not yet possible.

Remarkably, residues that are critical for activation of TRPV1 by capsaicin (Y511 and S512) map to an analogous position within the intracellular loop connecting TM2 and TM3 of this channel (Jordt and Julius, 2002; Gavva et al., 2004). Additionally, mutations within this domain also impair activation of TRPV4 by phorbol ester or heat (Vriens et al., 2004). Thus, while these channels show relatively low amino acid sequence conservation (TRPV1 and TRPM8 share only 15% identity and 27% similarity), residues crucial for activation by chemical agonists map to the same region, implicating a conserved structural and functional topology for ligand binding, activation, and/or gating of these temperature-sensitive TRP channels. These findings also suggest that natural products, such as capsaicin, have evolved to target this region of the protein so as to maximize their effects on channel gating and sensory neuron activation. Mutational studies have also shown that interaction of TRPV1 with larger pharmacophores, such as resiniferatoxin and capsazepine, may require additional residues within the TM3 and TM4 domains. Thus, the TM2-TM4 region appears to constitute a major site through which chemical ligands mediate their effects on channel gating, which is interesting in light of the contribution of this corresponding domain to voltage-dependent gating of topologically related K_v channels (Papazian et al., 2002).

For some TRP channels, a single stimulus may be sufficient to evoke channel activation, as in the case of TRPM8, where cold or menthol robustly gates the channel, or TRPV1, for which heat or capsaicin is an efficacious agonist. However, there is also evidence from genetic studies of *Drosophila* phototransduction to suggest that maximal activation of TRP channels by PLC-coupled rhodopsin requires the concerted integration of downstream cellular events, including PIP_2 metabolism (Hardie and Minke, 1995), Ca^{2+} entry via activated TRP channels (Hardie et al., 2001), or the generation of lipid second messengers (Hardie, 2004). Although icilin is not a naturally occurring or endogenously produced molecule, it has served as a valuable pharmacological tool to support the hypothesis that some TRP channels function as coincidence detectors, measuring

multiple outcomes of a single signal transduction process (i.e., PLC activation) or of different signaling pathways simultaneously. Indeed, the search for relevant physiological stimuli or endogenous agonists for members of the TRP channel family may, in some cases, require that multiple inputs be present simultaneously.

Experimental Procedures

Molecular Biology

A functional cTRPM8 clone was obtained by screening a chicken dorsal root ganglion (DRG) cDNA lambda phage library (kindly provided by Dr. Y. Barde) with a ^{32}P-labeled oligonucleotide probe (5′-GACCATACCCTTCCCTTTTGTCATCTTTGCCTAC-3′) derived from a chicken lymphocyte EST (emb|AJ456804.1). Sequences from positive clones were used to design additional oligonucleotide probes representing regions 5′- to the EST sequence of the insert lacking the 5′ initiation ATG codon (5′-CTTCACCAATGACCGAAACTGGG AGTCTGCTG-3′ and 5′-CAGCACTGGCACCTGAAAACCCCTAACC TTG-3′). These probes were further used to rescreen the DRG library, and a full-length cTRPM8 cDNA, including 2 kb of 3′ UTR sequence, was thus obtained from partial, overlapping clones. cTRPM8 encodes a predicted protein of 1095 amino acids (GenBank accession number AY700573). The insert full-length open reading frame was subcloned into pcDNA3 for functional expression in *Xenopus* oocytes and human embryonic kidney cell (HEK293). Point mutations and chimeras were generated using oligonucleotide-directed mutagenesis and high-fidelity PCR, as previously described (Jordt et al., 2000).

Mammalian Cell Culture, Ca^{2+} Microfluorimetry, and Electrophysiology

HEK293 cells were cultured in DMEM containing 10% fetal bovine serum and transfected (Lipofectamine 2000, Invitrogen) with 1 μg plasmid DNA or additional 100 ng pEGFPN1 plasmid for Ca^{2+} imaging experiments and electrophysiological experiments, respectively. Cells were plated onto polyornithine-coated coverslips the next day and examined 2 days after transfection using ratiometric Ca^{2+} microfluorimetry (10 μM fura-2-AM loaded) in a solution containing 10 mM HEPES, 135 mM NaCl, 4 mM KCl, 2 mM $MgCl_2$, and 2 mM $CaCl_2$ (pH to 7.4) with NaOH. All mammalian cell electrophysiological recordings were performed at room temperature (21°C). The standard extracellular solution contained 10 mM HEPES, 150 mM NaCl or CsCl, 1 mM $MgCl_2$, and 1 mM EGTA for Ca^{2+}-free solution or 1 mM $CaCl_2$ for Ca^{2+}-containing solution (pH 7.4). The intracellular solution contained 10 mM HEPES, 150 mM NaCl, 1 mM $MgCl_2$, and 1 mM EGTA (pH 7.3). Due to fast rundown of TRPM8 in the inside-out patch configuration, we adopted the permeabilized half-cell configuration to prolong channel activity. We also added 1 mM Na_3VO_4 to the intracellular (bath) solution, as this was found to modestly prolong channel lifetime in this configuration (by approximately 2-fold). EGTA was replaced with 100 μM $CaCl_2$ or mixed with variable concentration of $CaCl_2$ to achieve different concentrations of free Ca^{2+} in the perfusate, to facilitate icilin activation of TRPM8. The perfusion was controlled by a pinch valve system (AutoMate, CA) and has an exchange rate ~1 s between different solutions. Patch electrodes were pulled from borosilicate capillaries and fabricated to a final tip size of ~1 μm (2–4 MΩ resistance) for whole-cell recordings, 3–5 μm for cell-attached recording, or 10–12 μm (0.1–0.4 MΩ resistance) for half-cell recordings. Holding currents at −60 mV or currents at +60 mV during a voltage ramp from −120 mV to +80 mV were used for quantitative analysis of channel activation.

Oocyte Electrophysiology

cRNA transcripts were synthesized and injected into *Xenopus laevis* oocytes (2–10 ng per cell) using Ambion mMessage machine kits according to manufacturer's protocol. Two-electrode voltage-clamp recordings were performed 2–7 days postinjection at room temperature unless otherwise specified. The standard bath solution contained 10 mM HEPES, 120 mM CsCl, and 2 mM $MgCl_2$ with either 1 mM EGTA or 1 mM $CaCl_2$ (pH 7.4). Menthol or icilin was diluted into physiological buffer from a DMSO-based stock solution (200 mM or 10 mM, respectively). Icilin (AG-3-5) was generously provided by Dr. Edward Wei, UC Berkeley. Temperature ramps were generated by heating (35°C) or cooling (4°C) the perfusate using a controlled temperature feedback Peltier element (Reid et al., 2002) and monitored with a thermistor placed near the oocyte.

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Accession Numbers

The GenBank accession number for the chicken TRPM8 cDNA described here is AY700573.