Inhibition of TRPM8 by Icilin Distinct from Desensitization Induced by Menthol and Menthol Derivatives*

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TRPM8 is a cation channel activated by cold temperatures and the chemical stimuli menthol and icilin. Both compounds use different mechanisms of current activation; amino acid residues within the S2-S3 linker have been identified critical for current activation by icilin but not by menthol. Current decline in the course of menthol stimulation reflects Ca\(^{2+}\)-dependent desensitization attributed to phosphatidylinositol 4,5-bisphosphate depletion. Carboxyamide derivatives chemically resembling menthol have been described as activators of TRPM8 analogous to icilin. Our aim was a detailed analysis of whether differences exist between all these substances with respect to their activation and inactivation of currents. We studied wild-type TRPM8 as well as an s3-TRPM8 mutant with mutations in the S2-S3 linker region that could not be activated by icilin. Menthol and menthol derivatives behaved indistinguishable in evoking currents through both channels in a Ca\(^{2+}\)-independent manner as well as inducing Ca\(^{2+}\)-dependent desensitization. Icilin, in contrast, activated currents only in wild type TRPM8 and in the presence of Ca\(^{2+}\). Moreover, it completely reversed currents induced by menthol, menthol derivatives, and cold temperatures in wild type TRPM8 and s3-TRPM8; this current inhibition was independent of Ca\(^{2+}\). Finally, icilin suppressed current activation by the other agonists. None of the inhibiting effects of icilin occurred in the cation channel TRPA1 that is also stimulated by both menthol and icilin. Thus, icilin specifically inhibits TRPM8 independently of its interaction site within the S2-S3 linker through a process distinct from desensitization.

The sensitivity to temperature is mediated by ion channels of the transient receptor potential (TRP)\(^2\) superfamily. Until now, several temperature-sensitive TRP channels which belong to the TRPA (ankyrin), TRPM (melastatin), and TRPV (vanilloid) subfamilies have been described (1–10). A growing number of chemical agonists of natural and synthetic origin are also capable of stimulating these thermo-TRPs and, therefore, mediate the sensation of heat or cold (1, 3, 4, 11).

To date TRPM8 is one of the most intensively studied temperature-gated channels. Interestingly, TRPM8 is not only expressed in thermo-sensitive neurons; an enhanced expression has been demonstrated in several malignant tumors from tissues including breast, lung, colon, and prostate (12). These tissues encounter little temperature variations, suggesting that further currently unknown physiological activation mechanisms may exist. Under experimental conditions, TRPM8 is activated by various stimuli including voltage, cold temperatures (<28 °C), and several chemical compounds that open the channel either alone or through a synergistic interaction (13–15). Most intensively studied are the effects of menthol, the natural cooling compound from the mint plant (11, 16), and the synthetic cooling agent icilin (17, 18). Interestingly, both these compounds stimulate human TRPA1 channels as well (8, 19–21).

There is experimental evidence that menthol and icilin activate TRPM8 through distinct mechanisms. For instance, only the activation of TRPM8 by icilin depends on the presence of intracellular Ca\(^{2+}\) (3, 18) and is modulated by intracellular pH in the physiological range (22). Furthermore, although both icilin and menthol induce only transient currents, the current decline is much slower when menthol is the stimulus (18, 23, 24). It has been proposed that the decline after menthol stimulation represents desensitization involving depletion of phosphatidylinositol 4,5-bisphosphate; the desensitization in turn depends on intracellular Ca\(^{2+}\) or on Ca\(^{2+}\) influx through the open channel (13). Remarkably, a TRPM8 mutant that does not pass Ca\(^{2+}\) does not show desensitization (25).

Each of the three amino acid residues, Asn-799, Asp-802, and Gly-805, localized in the cytoplasmic loop connecting the transmembrane segments S2 and S3 is required for icilin sensitivity of mammalian TRPM8 but not for channel activation by menthol or cold (18). This domain may represent a binding site for icilin comparable with a topologically equivalent domain previously described for the binding of capsaicin and other vanilloid ligands (26, 27). On the other hand, several point mutations have been reported to affect activation of TRPM8 by menthol (15, 28), but these mutations are widely scattered throughout the channel structure (within transmembrane segments S2, S4, the S4-S5 linker, and the channel segment downstream of S6) and affect the sensitivity of the channel to icilin, cold, and voltage as well, at least in part. Thus, a distinct channel region that specifically controls menthol sensitivity has not yet been identified. Recently, some carboxyamide derivatives were characterized as potent TRPM8 agonists and proposed as potential candidates for the development of anti-cancer drugs (23, 24). Although the chemical structures of the carboxyamides more closely resemble that of menthol than of icilin, it has been suggested that at least one of these compounds,
WS-12, acts like icilin on TRPM8 (23). However, this proposition has not been verified in the icilin-insensitive variant.

In the present study we aimed at a more rigorous test of whether these agents act like icilin or like menthol. In addition to our comparison of the processes of current induction, we were particularly interested in the current decline after stimulation and whether differences might exist in between the various stimuli. We report that icilin exerts specific inhibitory effects on human TRPM8 that are distinct from desensitization, do not require the interaction site in the S2-S3 loop, and affect current activation by menthol, menthol derivatives and cold temperatures.

**EXPERIMENTAL PROCEDURES**

Molecular Cloning—The cDNAs of human TRPM8 and human TRPA1 were subcloned in pIRES-hrGFP-2a vector (Stratagene). For the generation of a TRPM8 channel variant insensitive to icilin, the amino acid sequence $^{803}$TLGLFY$^{808}$ of the human TRPM2 channel using the QuikChange site-directed mutagenesis kit (Stratagene). The mutations were verified by DNA sequencing with the Big-DYE-Terminator Kit (PerkinElmer Life Sciences, Roche Applied Science). The presence of inadvertent mutations in other regions of the channel could be excluded because two independent clones were tested yielding the same results. All procedures were performed in accordance to the respective manufacturers’ instructions if not indicated otherwise.

Cell Culture and Transfection—Stable expression of wild-type and chimerical TRPM8 channels as well as wild-type TRPA1 channel was achieved as follows. Each of the core expression constructs pIRES-wtTRPM8, pIRES-s3TRPM8, and pIRES-wtTRPA1 was recombined with a neomycin resistance module (EC-Neo, Stratagene) according to the manufacturers’ instructions. This recombination construct was transiently transfected into human embryonic kidney (HEK-293) cells using the Trans-Fast transfection reagent (Promega). The transfected cells were selected by growth in Dulbecco’s modified Eagle’s medium supplemented with 4 mM l-glutamine, 1 mM sodium pyruvate, 10% (v/v) fetal calf serum, and containing Geneticin (G418 sulfate, 1 mg/ml) (Invitrogen). Surviving clones that were visibly positive for the expression of enhanced green fluorescent protein were isolated and frozen at early passage numbers. These stocks were propagated in the presence of Geneticin (1 mg/ml) for further studies.

Electrophysiology—Patch clamp recordings were performed in the conventional whole-cell mode. The standard bath solution contained 140 mM NaCl, 1.2 mM MgCl$_2$, 1.2 mM CaCl$_2$, 5 mM KCl, 10 mM HEPES, pH 7.4 (NaOH). For Na$^+$ free solutions, Na$^+$ was replaced by 150 mM N-methyl-D-glucamine (NMDG), and the titration was performed with HCl. The divalent free bath solution (DFV) contained 150 mM NaCl, 10 mM EGTA, 10 mM HEPES, pH 7.4 (NaOH). The pipette solution contained 145 mM cesium glutamate, 8 mM NaCl, 2 mM MgCl$_2$, 10 mM HEPES, pH 7.2 (CsOH), and the Ca$^{2+}$ concentration was adjusted to either <10 mM (10 mM Cs-EGTA) or 1 mM (0.886 mM CaCl$_2$ and 1 mM Cs-EGTA).

The Ca$^{2+}$ concentration of the solutions were calculated using the MAXC program. In all experiments Cs$^+$ and glutamate were the main intracellular ions to minimize potential contaminating currents through K$^+$ and Cl$^-$ channels. For the stimulation of TRPM8 currents, each of the following agonists was added directly to the bath during whole-cell patch clamp recordings (dilution factor was 10$^{-2}$): menthol (Sigma, 200 mM stock solution in DMSO), icilin (Cayman, 30 mM stock solution in DMSO), allylisothiocyanate (Sigma, 10 mM stock solution in DMSO), or WS-12, CPS-369, and CPS-154 (30 mM stock solutions in DMSO). The compounds WS-12, CPS-369, and CPS-154 were kindly provided by Dr. Edward T. Wei, University of California, Berkeley. If not otherwise stated, the experiments were performed at room temperature (20°C), the holding potential was −60 mV, and the current-voltage relations were obtained during voltage ramps from −90 to +60 mV and back to −90 mV applied over 200 ms.

**RESULTS**

For the characterization of the stimulatory and inhibitory effects of menthol, menthol derivatives, and icilin on TRPM8cation channels, we established two human embryonic kidney cell lines, one stably expressing wild-type (wt) TRPM8 and one an icilin-insensitive variant of TRPM8 (s3-TRPM8). We used a combined expression construct (see “Experimental Procedures”) that allows the selection of positive clones by neomycin selection and expression of enhanced green fluorescent protein. Therefore, expression levels of either channel protein could be estimated from the emission of enhanced green fluorescent protein fluorescence. All five stimuli tested, i.e. menthol (200 μM), icilin (30 μM), WS-12 (30 μM), CPS-369 (30 μM), and CPS-154 (30 μM), induced characteristic cation currents after application to the bath during whole-cell patch clamp experiments (example shown for the stimulation with icilin in Fig. 1). Notably, any effect of the stimuli occurred only after a delay of 0.5–1 min (Fig. 1A) as in previous studies (e.g. Ref. 18). At a holding potential of −60 mV, the current amplitudes were well above 2 nA and showed a strong outward rectification (Fig. 1B). The currents were readily inhibited in the inward direction by the large cation NMDG, whereas the outward currents were moderately reduced by NMDG (Fig. 1B). Similar currents as in TRPM8-expressing cells were activated by menthol (not shown) and by icilin (Fig. 1, C and D) in control experiments using cells stably expressing human TRPA1. Moreover, as in previous studies (e.g. Ref. 23) currents induced by both stimuli receded spontaneously. In TRPM8, the decline in the presence of icilin was considerably faster than in the presence of the other stimuli. As a first test of whether icilin and the menthol derivative WS-12 used different mechanisms of current activation in TRPM8, experiments were performed that are shown in Fig. 2. In the presence of an extracellular Ca$^{2+}$ concentration (1.2 mM) and an intracellular Ca$^{2+}$ concentration weakly buffered to 1 μM and after an initial moderate current had declined, icilin and WS-12 were applied subsequently to the bath (Fig. 2A). Icilin induced a large but transient inward current, whereas WS-12 evoked only a small effect. This would be in line with the previous conclusion that icilin and WS-12 used common mechanisms possibly mediated by common binding sites.
(24). However, this view was challenged when the experiments were repeated in the absence of Ca\(^{2+}\) or, more specifically, under conditions where the extracellular and intracellular Ca\(^{2+}\) concentrations were strongly buffered to below 10 nM (Fig. 2). The icilin-induced currents were abolished, whereas large and sustained currents were induced by WS-12. Thus, the current activation was Ca\(^{2+}\)-dependent when icilin was the stimulus. In contrast, WS-12 was effective in the absence of Ca\(^{2+}\) when icilin had not induced a current. As test for a Ca\(^{2+}\)-dependent desensitization of the TRPM8 channels, the two stimuli WS-12 and menthol were subsequently applied either in the presence of a high/weakly buffered (Fig. 2C) or low/strongly buffered (Fig. 2D) intracellular Ca\(^{2+}\) concentration. At high intracellular Ca\(^{2+}\), the second stimulus menthol had hardly any effect, even though WS-12 had been washed out and the current had returned to base line. In distinct contrast, a sizeable response to menthol was demonstrated at low intracellular Ca\(^{2+}\) even if the second response appeared slightly weaker. These experiments are in line with a previous report that demonstrates the essential role of Ca\(^{2+}\) for desensitization with a different approach (13).

We reasoned that even though the intracellular low Ca\(^{2+}\) solution is strongly buffered, local rises in the Ca\(^{2+}\) concentration close by the membrane might still occur, especially in the presence of large currents. Therefore, we additionally removed extracellular Ca\(^{2+}\) in all further experiments in which the absence of Ca\(^{2+}\) had to be studied. In particular, we were interested in the role of Ca\(^{2+}\) in the process of inactivation or desensitization of TRPM8 and whether differences existed between the various stimuli. Base-line currents were slightly but consistently enhanced by removal of extracellular Ca\(^{2+}\) (Fig. 3). Similar observations were described in a previous study (29). Current activation by icilin was abolished in the absence of Ca\(^{2+}\); icilin even reversed the effects of Ca\(^{2+}\) removal on base-line currents (Fig. 3A, see also Fig. 2B). In contrast, menthol, WS-12, and CPS-369 all induced large and long lasting cation currents under Ca\(^{2+}\)-free conditions (Figs. 3, B–D). Base-line as well as stimulus-induced inward currents were inhibited by NMDG. Notably, the outward rectification of base-line currents persisted in Ca\(^{2+}\)-free solutions (Fig. 3A), ruling out that the rectification might be the result of open channel block by Ca\(^{2+}\). In these experiments, menthol and menthol derivatives behaved alike in terms of Ca\(^{2+}\)-independent current activation and Ca\(^{2+}\)-dependent desensitization. In contrast, the role of Ca\(^{2+}\) in the decline of icilin-induced currents and possible differences between icilin and the other stimuli cannot be clarified in such experiments. Therefore, we exploited a variant of TRPM8 that cannot be activated by icilin. Such a variant has been described by Chuang et al. (18). We generated an alterna-
Inhibiting Effects of Icilin on TRPM8

A tive variant where not only the critical glycine 805 was substi-
tuted but the amino acid sequence 803–808 was exchanged by the corresponding sequence of the icilin insensitive channel TRPM2 (Fig. 4A). Electrophysiological studies on the s3-TRPM8 demonstrate that indeed no currents were induced by icilin (Fig. 4B). However, s3-TRPM8 was not noticeably different from wt-TRPM8 in terms of sensitivity to menthol, voltage (Fig. 4C), and cold temperatures (data not shown). Moreover, the menthol derivatives WS-12, CPS-154, CPS-369 all evoked characteristic currents in s3-TRPM8, demonstrating again that they behave indistinguishably from menthol but distinctly different from icilin (Fig. 5).

Because s3-TRPM8 cannot be activated by icilin, it allows us to study if icilin negatively affects currents induced by other stimuli, especially if Ca2+-dependent current desensi-
tization is prohibited by removal of Ca2+. Representative experiments addressing this question are shown in Fig. 6. Icilin added to s3-TRPM8 exposed to Ca2+-free solution led (after a delay typical for all icilin effects) to a rapid decline of base-line currents (also see Fig. 3A). The addition of WS-12 (Fig. 6A) shortly after icilin failed to induce currents, in contrast to controls where the solvent of icilin was applied alone (Fig. 6B). Surprisingly, the addition of WS-12 after a longer interval of icilin incubation then evoked currents, although these were slower in onset than in the absence of icilin (Fig. 6C, see also Fig. 2B). We supposed that this phenomenon might be caused by a time-dependent loss of activity of icilin such as by degradation in aqueous solution in the presence of cells. Therefore, we applied icilin for a second time just when WS-12-induced currents were strongly growing in amplitude. Indeed, a complete reversal of the currents was observed (Fig. 6D).

In an identical manner as currents induced by WS-12, currents induced by menthol (Fig. 6E) were completely inhibited by icilin in the s3-TRPM8 mutant. The same finding was obtained in a limited number of experiments (n = 2) for currents evoked by CPS-369 (data not shown).

To investigate whether the icilin-evoked inhibition of currents was specific for this agent, the solvent alone (Fig. 6F) or menthol (after stimulation with WS-12, not shown) was tested instead; both substances did not change the long-lasting currents. Moreover, no such current reduction was found when menthol was the initial stimulus, and WS-12 was applied subsequently (data not shown).

After we had demonstrated that icilin inhibits currents through TRPM8 stimulated by menthol and its derivatives, we

FIGURE 2. Repeated stimulation of wt-TRPM8 and Ca2+-mediated desensitization. A, icilin (30 μM) and WS-12 (30 μM) were subsequently applied at the time points indicated by arrows. The intracellular (pipette) solution (in) contained 1 μM Ca2+, and the (standard (ex)) bath contained 1.2 mM Ca2+. W.c., whole cell. B, the Ca2+ of the pipette solution was buffered with EGTA to below 10 nM; the standard bath solution was changed to a DVF during the time period indicated by a horizontal bar. Icilin and WS-12 were applied as in A. C, same pipette and bath solutions as in A; WS-12 (30 μM) was applied (indicated by an arrow) and later removed by a wash as indicated by the horizontal bar. Afterward, menthol (200 μM) was added. D, the protocol of panel C was repeated for another cell with an intracellular (pipette) Ca2+ concentration of <10 nM.
asked whether it would furthermore inhibit currents induced by cold temperatures. Therefore, we designed experiments in which cells expressing the s3-TRPM8 mutant were repeatedly exposed to 4 °C and then allowed to warm up again to room temperature. Ca\textsuperscript{2+}/H\textsubscript{11001}-free conditions were maintained throughout to rule out Ca\textsuperscript{2+}/H\textsubscript{11001}-dependent desensitization that takes place in the course of a temperature-dependent activation of TRPM8 as well (13). Cooling consistently induced characteristic currents which developed much more rapidly than those in response to chemical stimuli and returned to baseline during warm-up (Fig. 7A). Currents could be enhanced by menthol (Fig. 7B). In contrast, icilin completely reversed the effects of cooling (Fig. 7C) once more after a typical delay of about 30 s. After wash-out of icilin, the sensitivity to cooling was readily restored, although the onset of the second response to cooling was somewhat delayed. The inhibitory effects of icilin were similar for all voltage ranges studied (Fig. 7D).

Thus, the studies on the s3-TRPM8 variant demonstrate that icilin inhibits the channel independently of the stimulus by which currents are activated and independently of the structures within S3 where an interaction with or even binding of icilin is generally assumed. Even in wild-type TRPM8, such inhibitory effects could be shown (Fig. 8, A and B) when menthol or its derivatives were used as stimulus and the Ca\textsuperscript{2+}-dependent desensitization was prohibited. Icilin completely suppressed the currents induced by WS-12 (Fig. 8A) or by menthol (not shown). In control experiments, no such inhibitory effect was detected in response to menthol when the current was induced by WS-12 (Fig. 8B) or to WS-12 when the current was induced by menthol (not shown). As additional controls, icilin was tested on TRPA1-expressing cells kept Ca\textsuperscript{2+}/H\textsubscript{11001} free and pre-stimulated with either menthol (200 \mu M; Fig. 8, C and D) or allylisothiocyanate (10 \mu M; Fig. 8, E and F). In contrast to the results in TRPM8, there was no inhibition of currents by icilin in TRPA1. On the contrary, enhancing effects were consistently observed (n = 8) in the presence of the weak stimulus menthol (200 \mu M). Currents induced by 10 \mu M allylisothiocyanate were already large such that enhancing the effects of icilin could not be safely resolved (Fig. 8E), but inhibitory effects were excluded. Thus, current inhibition by icilin is exclusively found for TRPM8 and its mutant.

DISCUSSION

In this study we have demonstrated that the menthol derivatives WS-12, CPS-369, and CPS-154 act identically with menthol in terms of Ca\textsuperscript{2+}-independent current activation and Ca\textsuperscript{2+}-dependent induction of desensitization on wild-type TRPM8 as well as on the S3 mutant. The alternative TRPM8 stimulus i-
On the other hand, proved to act distinctly different with respect to several aspects. As already shown previously, icilin-induced current activation was strictly dependent on the presence of Ca\(^{2+}\), and the current decline after peak stimulation occurred much faster (3, 18). Our studies on the S3 mutant that cannot be activated by icilin allowed us to analyze inhibitory actions of icilin. When the mutant was stimulated either with menthol, menthol-derivatives, or cold temperatures, icilin led to a rapid decline of the currents; this decline was indistinguishable from that observed in icilin-stimulated wild-type TRPM8. Furthermore, the decline occurred completely independent of Ca\(^{2+}\) and was independent of the assumed interaction site of icilin and TRPM8 within the S2-S3 loop of the channel. Finally, icilin could not only reverse but also suppress menthol-induced currents in the S3 mutant. Thus, we have dissected various mechanisms in the processes of current activation and current inhibition of TRPM8 that are specific for its activators, with menthol, its derivatives, and cold temperatures comprising one group and icilin a second one.

The compounds WS-12, CPS-369, and CPS-154 share a common basic structure with menthol, the para-menthane group, but contain a carboxyamide group instead of the hydroxyl group of menthol. The derivatives differ in their substituent attached to the carboxyamide group. Hence, from a

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**FIGURE 4.** Activation of TRPM8 by icilin depends on intracellular Ca\(^{2+}\) and a specifically localized glycine residue within the S2-S3 linker of the channel. A, corresponding amino acid sequences of TRPM2 and TRPM8 enclosing the C-terminal part of the S2-S3 linker and the N-terminal part of the transmembrane segment S3. Amino acid residues conserved between the two channels are indicated with bold letters. The essential glycine 805 is marked by an asterisk. The stretch of amino acid residues that was exchanged between TRPM8 and TRPM2 to generate the icilin-insensitive TRPM8 variant, termed s3-TRPM8, is given in small capitals. B, whole-cell recording of a HEK-293 cell stably expressing the s3-TRPM8 variant. Icilin (30 \(\mu\)M) was present in the bath during the time indicated by a horizontal bar. The intracellular Ca\(^{2+}\) concentration was weakly buffered to 1 \(\mu\)M. C, stimulation of s3-TRPM8 by menthol under conditions as in A. The insets show the corresponding current-voltage relations of the experiments.

**FIGURE 5.** Stimulation of the TRPM8 variant s3-TRPM8 by carboxyamide derivatives. The stimuli WS-12, CPS-154, and CPS-369 (each 30 \(\mu\)M) were present in the bath during time periods as indicated by horizontal bars. The intracellular (pipette) solution contained 1 \(\mu\)M Ca\(^{2+}\). The corresponding current-voltage relations of each experiment are given as insets. w.c., whole cell.
Structural point of view, the functional relationship between menthol and the carboxamide derivatives does not seem surprising even though previous studies have proposed a functional similarity between icilin and WS-12 (23, 24).

When menthol or its derivatives were the stimulus of TRPM8, current activation and current decline can be easily separated by omitting Ca\(^{2+}\)/H\(_{11001}\) from the intracellular (and additionally the extracellular) solution because the decline represents a desensitization most likely mediated by depletion of phosphatidylinositol 4,5-bisphosphate (13). In the case of icilin, however, current activation occurs through different mechanisms that essentially involve Ca\(^{2+}\)/H\(_{11001}\)-dependent steps (3, 18); therefore, it cannot be decided in experiments on wild-type TRPM8 whether the rapid current decline that follows current activation by icilin is Ca\(^{2+}\)-dependent as well. Experiments after menthol stimulation, however, performed in the absence

**FIGURE 6. Icilin inhibits the stimulation of s3-TRPM8 by other agonists.** A–F, the bath solution was DVF during the time periods indicated by horizontal bars to suppress Ca\(^{2+}\)-dependent desensitization. Stimuli or the solvent DMSO were added at the time points indicated by the arrows. The intracellular (pipette) solution always contained <10 nM Ca\(^{2+}\). w.c., whole cell.
of Ca²⁺ either on wild-type or s3-TRPM8 demonstrate an icilin-induced current decline in the complete absence of Ca²⁺. Moreover, an inhibitory effect of icilin can also be demonstrated in the absence of a current because icilin temporarily prevented current activation by menthol. Therefore, the effect of icilin cannot be described as desensitization; in contrast to the desensitization observed after stimulation with menthol, it represents a current inhibition, the mechanisms of which cannot be completely understood at present, but they do not seem to involve interference with menthol binding because icilin inhibits cold-induced currents as well.

Surprisingly, the activity of icilin after the addition to the cells vanished rapidly. This is exemplified in Fig. 6D where preincubation with icilin (30 μM) did not completely abolish the effects of WS-12; rather, these were considerably delayed and slowed down. A second application of icilin then led to the characteristic current inhibition. It should be noted that this finding does not represent a dose-response relation of icilin because full current inhibitions could be achieved with icilin (30 μM, e.g. Fig. 6E). According to the manufacturer, aqueous solutions of icilin are unstable over the course of several hours; thus, our results would be best explained either by cell-dependent degradation or by sequestration of icilin.

Our study reveals some new aspects regarding the putative binding site of icilin within the S2-S3 loop of TRPM8. The s3-TRPM8 mutant used in our experiments is a chimerical channel in which a sequence of six amino acid residues is replaced by the corresponding sequence of the closely related but icilin-insensitive channel TRPM2. The manipulation completely abolished the sensitivity to icilin. Within the exchanged sequence, there are three amino acid residues (Asn-799, Asp-802, and Gly-805) critical for icilin sensitivity of TRPM8 (Ref. 18, see also Fig. 4A). The first two of them are conserved in the corresponding region of TRPM2 (Asn-869 and Asp-872). The third one, a glycine (Gly-874), is shifted to the N terminus by one position in the icilin-insensitive channel TRPM2 and the s3-TRPM8 mutant. Hence, Gly-805 seems to represent the most important determinant for the icilin sensitivity of TRPM8 and is required in its exact position.

In contrast to the stimulating actions of icilin on TRPM8 for which a site critical for the interaction can be defined, no such
site is known for the inhibitory effect. A future search of such a site may use our results on TRPA1 as a starting point because TRPA1 neither contains the motif in the S2-S3 loop nor is inhibited by icilin. The latter finding may be taken as a hint that TRPM8 may indeed possess a further site for an interaction with icilin. If, alternatively, icilin exerted its inhibitory effects through indirect mechanisms, e.g. by interfering with the interaction with membrane lipids considered critical for the activation and regulation of both TRPM8 and TRPA1 (13, 19), icilin would be expected to inhibit TRPA1 as well.

The biological significance of TRPM8 is not confined to the perception of cold and of “cool” agents such as menthol; an additional role has been established in prostatic tumor tissue where the expression level of TRPM8 coincides with malig-

**FIGURE 8.** Inhibitory effects of icilin on TRPM8 but not on TRPA1. A and B, comparison of the effects of menthol and icilin on wt-TRPM8 currents prestimulated with WS-12. A, TRPM8 currents first were stimulated by WS-12 (30 μM). Subsequently, icilin (30 μM) was added during the maximum of the WS-12-evoked currents. B, same as A, but menthol instead of icilin was added after stimulation with WS-12. C–F, control experiments on cells stably expressing TRPA1, prestimulated either with menthol (C; 200 μM) or with allylisothiocyanate (E; 10 μM) before icilin was added during the development of currents. The current voltage relations are shown in D and F. w.c., whole cell.
nancy (12). In contrast, a recent study has revealed that the activation of endogenous TRPM8 channels by menthol suppresses cellular viability of human melanoma cells (30). Therefore, the understanding of the mechanisms of both activation and inhibition of TRPM8 may be of major medical interest. Perhaps, the identification of the dual role of icilin in the gating of TRPM8 may provide us with a novel target of drug development. For the s3-TRPM8 channel, icilin is a pure inhibitor; possibly, derivatives could be developed that do no longer activate wild-type TRPM8 but retain the inhibitory effects.

In conclusion, icilin evokes stimulating effects on TRPM8 by interaction with the S2-S3 loop of the channel; separately, inhibitory effects take place that are preserved in the S3 mutant. Both activating and inactivating actions are different from phenotypically similar effects of menthol and temperature and may help in the future to delineate the complex interactions of the channel proteins and the membrane lipids that take place in the regulation of TRPM8.

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Inhibiting Effects of Icilin on TRPM8