TRPM8 represents an ion channel activated by cold temperatures and cooling agents, such as menthol, that underlies the cold-induced excitation of sensory neurons. Interestingly, the only human tissue outside the peripheral nervous system, in which the expression of TRPM8 transcripts has been detected at high levels, is the prostate, a tissue not exposed to any essential temperature variations. Here we show that the TRPM8 cloned from human prostate and heterologously expressed in HEK-293 cells is regulated by the Ca\(^{2+}\)-independent phospholipase A\(_2\) (iPLA\(_2\)) signaling pathway with its end products, lysophospholipids (LPLs), acting as its endogenous ligands. LPLs induce prominent prolongation of TRPM8 channel openings that are hardly detectable with other stimuli (e.g. cold, menthol, and depolarization) and that account for more than 90% of the total channel open time. Down-regulation of iPLA\(_2\) resulted in a strong inhibition of TRPM8-mediated functional responses and abolished channel activation. The action of LPLs on TRPM8 channels involved either changes in the local lipid bilayer tension or interaction with the critical determinant(s) in the transmembrane channel core. Based on this, we propose a novel concept of TRPM8 regulation with the involvement of iPLA\(_2\) signaling pathway as its endogenous ligands. LPLs cause a prominent rundown of TRPM8 activity in excised patches (4, 5). This would raise a possibility that some endogenous ligands might be necessary for channel activation. Phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) has recently been found to be such a factor, since it was capable not only of restoring menthol-activated TRPM8 current after its rundown in excised patches but also of activating the current independently of menthol (5, 6). This would suggest the importance of this endogenous lipid signaling in sustaining the TRPM8 function. Notably, high concentrations of diC\(_8\) PIP\(_2\) (e.g. 500 \(\mu\)M) allowed channel opening even at 32–37 °C (6). The functional importance of the PIP\(_2\)-dependent TRPM8 gating has thus become evident in the cold sensation, since any of the well known PIP\(_2\) depletion scenarios (e.g. activation of C\(_{\alpha_{11}}\)-PLC-coupled receptors or Ca\(^{2+}\)-dependent activation of some PLC isoforms) would limit TRPM8 activation by shifting its gating toward lower temperatures or higher voltages. However, the possibility of channel activation (rather than desensitization) with the involvement of PIP\(_2\) seems more remote. Experimentally, this can be achieved by co-expression of diC\(_8\) PIP\(_2\) with TRPM8.

Although TRPM8, a member of the transient receptor potential (TRP)\(^6\) channel family, was originally cloned from the prostate (1), recent studies have firmly established its function as a cold/menthol receptor in sensory neurons (2, 3). Thus, an outstanding problem heightened by a significant TRPM8 up-regulation in several tumors (prostate, lung, breasts, skin) concerns the whole spectrum of the physiological and pathophysiological roles of TRPM8 in those tissues devoid of any significant temperature variations. Revealing possible alternative molecular events leading to TRPM8 activation at constant temperature would undoubtedly help to uncover other TRPM8 functions beyond the cold sensation.

Recently, a model ascribing TRPM8 activation to cold/menthol-induced shifts in the channel voltage dependence toward physiological membrane potentials has been proposed (4). Although attractive in its explanation of the temperature sensitivity, this model seems to be insufficient to account for TRPM8 activation in those tissues that are not exposed to any essential temperature variations, such as prostate. Moreover, prominent rundown of TRPM8 activity in excised patches (4, 5) raised a possibility that some endogenous ligands might be necessary for channel activation. Phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) has recently been found to be such a factor, since it was capable not only of restoring menthol-activated TRPM8 current after its rundown in excised patches but also of activating the current independently of menthol (5, 6). This would suggest the importance of this endogenous lipid signaling in sustaining the TRPM8 function. Notably, high concentrations of diC\(_8\) PIP\(_2\) (e.g. 500 \(\mu\)M) allowed channel opening even at 32–37 °C (6). The functional importance of the PIP\(_2\)-dependent TRPM8 gating has thus become evident in the cold sensation, since any of the well known PIP\(_2\) depletion scenarios (e.g. activation of C\(_{\alpha_{11}}\)-PLC-coupled receptors or Ca\(^{2+}\)-dependent activation of some PLC isoforms) would limit TRPM8 activation by shifting its gating toward lower temperatures or higher voltages. However, the possibility of channel activation (rather than desensitization) with the involvement of PIP\(_2\) seems more remote. Experimentally, this can be achieved by co-expression of diC\(_8\) PIP\(_2\) with TRPM8.
of enzymes, which catalyze PIP$_2$ formation (e.g. PIP5K), and this maneuver indeed significantly attenuated TRPM8 desensitization (6), suggesting a physiological relevance of this mechanism in connection with the (over)expression of PIP$_2$-forming enzymes. PIP$_2$ replenishment also requires Ca$^{2+}$ influx (the mechanism remains unknown), and TRPM8 desensitization is also known to be accelerated by Ca$^{2+}$ withdrawal.

Since the prostate has not been shown to be exposed to any temperature variations, the issue of physiological TRPM8 activation in the prostate is even less clear. The problem is complicated by the fact that TRPM8 may be localized not only in the plasma membrane (PM), but also in the membrane of the endoplasmic reticulum (ER), where it can operate as a Ca$^{2+}$ release channel (7–9). Intracellular Ca$^{2+}$ store depletion is known to be causally associated with the stimulation of the Ca$^{2+}$-independent phospholipase A$_2$ (iPLA$_2$) (10), which catalyzes the production of lysophospholipids (11, 12) (LPLs), important lipid messengers, which were implicated in the activation of a number of ion channels (13–15), including the TRPC5 channel (16). Considering this as well as a potential role of lipid messengers in the way TRPM8 functions, in the present study we asked whether or not iPLA$_2$ plays a role in TRPM8 functions and, if so, what the mechanism of this coupling would be.

**EXPERIMENTAL PROCEDURES**

**Cloning of TRPM8 cDNA and Fusion Protein Construction**—
TRPM8 gene from normal human prostate was cloned from 1 µg of human prostate poly(A)$^+$ RNA (Clontech, Palo Alto, CA) by a SMART PCR cDNA synthesis kit (Clontech). Primers for TRPM8 amplification were as follows: 5'-ACGGGGTACAC-CATGTCTTTCGGG-3' (TRPM8-dir) and 5'-AGATCTCGAGTGGATTATATTAGCAATCTCTTTCA-3' (TRPM8-rev). The PCR product was purified by a MinElute PCR Purification kit (Qiagen) and completely digested with KpnI (New England Biolabs) and partially digested with XhoI (New England Biolabs). pcDNA4.TO.A plasmid (Invitrogen) was linearized with EcoRI (Biorad) and completely digested with XhoI (New England Biolabs). pcDNA4.TO.A plasmid (Invitrogen) was linearized with the same restrictases. The vector and digested PCR product was ligated and transformed into bacteria, and recombinant clones were selected by PCR. Plasmid DNA was purified by Nucleospin plasmid (Macherey-Nagel, Düren, Germany), and the insert was sequenced.

**Creation of HEK-293 Cell Line Stably Expressing TRPM8**—
Tetracycline repressor-expressing HEK-293 cells defined as “HEK-TREx” were obtained from Invitrogen. The cells were cultured in a 6-well plate (Nunc) and then transfected with 2 µg (per well) of TRPM8-pcDNA4.TO.A plasmid using Gene PorterTM2 transfection reagent (Gene Therapy Systems, Inc., San Diego, CA). 3 days after transfection, cells were submitted to Zeocin selection (500 µg/ml) for 10 days, and eight positive clones were later isolated and propagated. All eight clones were thoroughly checked for the tetracycline induction of the full-size TRPM8 channel expression and function, and all of them appeared to be equivalent. Therefore, no distinction between the clones was made in the functional studies.

**iPLA$_2$ Activity Assays**—The activity of iPLA$_2$ was assayed as detailed in the original work (15) using a modified kit originally designed for cytosolic phospholipase A$_2$ (cPLA$_2$ assay kit; Cayman Chemicals). The activity of iPLA$_2$ was expressed in units: absorbance/min/mg of total protein. iPLA$_2$ antisense efficiency was calculated on the bromoenol lactone (BEL)-dependant iPLA$_2$ activity (total iPLA$_2$ activity minus iPLA$_2$ activity treated with 5 µM BEL). Thapsigargin-mediated iPLA$_2$ induction was calculated specifically for the iPLA$_2$β (sense iPLA$_2$ activity minus activity with antisense treatment).

**Electrophysiology and Solutions**—Membrane currents were recorded in the whole cell, cell-attached, and inside-out configurations of the patch clamp techniques using the Axopatch 200B amplifier (Molecular Devices, Union City, CA). The resistance of the patch pipettes fabricated from borosilicate glass capillaries (World Precision Instruments, Inc., Sarasota, FL) when filled with the intracellular solution was 2–3 megohms for the whole cell recordings and 5–7 megohms for the single channel recordings. In the whole cell experiments, series resistance was compensated for by about 70%. Currents were filtered at 1 or 2 kHz and sampled at 10 kHz.

Whole cell currents were measured under nearly symmetrical ionic conditions (i.e. standard extracellular solution containing 150 mM NaCl, 1 mM MgCl$_2$, 5 mM glucose, 10 mM HEPES, pH 7.3 (adjusted with NaOH), whereas the pipette was filled with the intracellular solution containing 150 mM NaCl, 3 mM MgCl$_2$, 5 mM EGTA, 10 mM HEPES, pH 7.3 (adjusted with NaOH). In the cell-attached mode, cells were bathed in a modified standard extracellular solution with an equimolar substitution of KCl for NaCl to bring the resting potential close to zero, whereas the patch pipette was filled with standard extracellular solution. The bath was replaced with intracellular solution prior to patch excision. For temperature control and solution exchange, the TC1-SL25 system (Bioscience Tools, San Diego, CA) was used with the temperature probe placed near the patch pipette tip. The system provided a temperature stability of at least 0.2 °C.

**Fluorescence Measurements of [Ca$^{2+}$]$_c$**—Cytosolic Ca$^{2+}$ concentration ([Ca$^{2+}$]$_c$) was measured using the fura-2AM ratiometric dye as detailed previously (17). Temperature and solution changes were performed as described for electrophysiology.

**Data Analysis**—Membrane current recordings obtained from 172 cells were analyzed and plotted using the pCLAMP 9 (Axon Instruments, Inc.) and Origin 5 software (Microcal Software Inc.). Single channel transitions were identified on the basis of the half-amplitude threshold crossing criteria. Shutter durations were generally not analyzed, since two or more active channels were present in a membrane patch. Open time histograms were constructed as distributions of the duration logarithm (in ms, 20 bins/decade) and were fitted with exponentials, using the built-in “compare models” algorithm of the pCLAMP software. Amplitude and dwell time histograms were constructed for fitted levels, ignoring brief transitions that lasted less than 2$T_\mu$, where $T_\mu$ is filter rise time (e.g. 0.165 ms for a 2-kHz filter). Amplitude histograms fitted with Gaussian components showed equal spacing between the peaks (Fig. 2C), indicating multiple openings of the same conductance channel (from 1 to 7 in different patches). Unitary amplitudes at negative potentials were measured in a relatively small number of patches, where sufficiently long openings could be collected. Channel activity was expressed as $N P_o$, where $N$ represents the (unknown) number of channels in the patch and $P_o$ represents open probability determined from idealized traces. Results
were expressed as the means ± S.E. Statistical analysis was performed using Student's t test (differences considered significant when p was <0.05).

Drugs and Treatments—All chemicals were purchased from Sigma, except for thapsigargin and fura-2AM, which were from Calbiochem and Molecular Probes, Inc., respectively.

iPLA2 hybrid depletion was performed by treating HEK-293/ TRPM8 cells for up to 5 days with either 0.5 μM phosphorothioate antisense oligodeoxynucleotides (ODNs) (Eurogentec) targeted at the iPLA2 coding region and 2.5 μM cytofetcin (GS 3815 to DOPE at a 2:1 molar ratio, unsized) (Eurogentec) or sense (for control purposes) ODNs by adding them directly to the culture medium. The 18-mer ODNs used in these studies had the following sequences: 5′-CTCCCTTCACCCCCGGAATGGGT-3′ for antisense ODN and 5′-ACCCATTTCCGGTGTAAGG.

For small interfering RNA-mediated TRPM8, knock-out NPE/BPHE cell cultures were transfected with 15–50 nM small interfering RNA against TRPM8 (small interfering RNA TRPM8 synthesized by Dahracon Inc., Lafayette, CO) using 6 μl of TransIT-TKO transfection reagent (Mirus Inc., Madison, WI) following the manufacturer’s instructions. The small interfering RNA sense sequence used was 5′-UUCUGAGGCCACU-AUUAUCA(dTdT)-3′. This sequence is located at position 894 of the TRPM8 gene (accession number NY32840).

Cell treatments with BEL (50–100 μM) were performed for 30 min at 37 °C in the standard extracellular solution, followed by a thorough washout before using them in the experiments. Menthol was dissolved in ethanol as 1 M stock and was added to the experimental solution by consecutive dilutions to reach the concentration required.

RESULTS

Our study was performed using HEK-293 cells stably expressing human TRPM8 under control of a tetracycline-inducible promoter (HEK-293/TRPM8). HEK-293/TRPM8 cells without tetracycline induction were used as controls (HEK-293/ctrl cells).

Plasmalemmal TRPM8 Can Be Activated by ER Calcium Store Depletion—Recent studies have established dual localization of TRPM8 in the PM as well as in ER membranes (7–9). The functional evidence of the plasma- and endolemmal target- tion of TRPM8 in the PM as well as in ER membranes (7–9).

FIGURE 1. Heterologously expressed human TRPM8 functions, such as Ca2+ entry and Ca2+ release channel. A and B, [Ca2+]i responses in HEK-293/TRPM8 (TRPM8) and HEK-293/ctrl (control) cells evoked by either menthol (100 μM; A) or TG application (1 μM; B). A “classical” protocol was employed, which permits dissociation of Ca2+ release in nominally Ca2+-free external solution (0/Ca) from Ca2+ entry in 2 mM Ca2+ (2/Ca). Note that 10 μM La3+ was co-applied with TG to inhibit SOCE but not TRPM8 channels. C, a summary of the effects of menthol, TG/La3+, and a combination of TG/La3+, plus menthol on the Ca2+ entry phase in HEK-293/TRPM8 (TRPM8) and HEK-293/ctrl (control) cells; mean ± S.E. from 10–42 measurements.

endogenous store-operated Ca2+ channels (SOC) of HEK-293 cells as well as foreign PM-localized TRPM8 channels, which in previous studies have been shown to be Ca2+-permeable (2, 3).

To distinguish between these possibilities, we next proceeded to experiments with combined applications of menthol, thapsigargin (TG), and La3+, of which TG was used as the “classical” store-depleting agent acting independently of TRPM8 via the inhibition of SERCA-mediated Ca2+ uptake, whereas La3+ was used as a specific SOC blocker (17). Fig. 1B shows that the presence of La3+ (10 μM) in the Ca2+-free bathing solution did not interfere with the ability of TG (1 μM; a concentration known to induce maximal SOCs activation (18)) to produce ER store depletion in HEK-293/ctrl cells, but it almost completely eliminated Ca2+ entry following readmission of 2 mM [Ca2+]o, suggesting a reliable blockade of the endogenous SOCs. The same experiment conducted in HEK-293/TRPM8 cells revealed a substantially La3+-insensitive Ca2+ entry (Fig. 1B). Moreover, application of menthol (100 μM) during the TG-evoked Ca2+ entry plateau in HEK-293/TRPM8 cells produced an additional Ca2+ entry (Fig. 1B), which elevated [Ca2+]i to nearly the same maximal level as menthol alone (Fig. 1, B and C).

Taken together, these results can only be explained with the following assumptions: 1) TG-induced store depletion is somehow coupled to a partial activation of the plasmalemmal Ca2+-permeable TRPM8 channels, and 2) menthol can activate plasmalemmal TRPM8 via two mechanisms, one that involves direct action on TRPM8 channel and another one via a functional coupling of store depletion to PM-localized TRPM8.

To test these hypotheses, we undertook patch clamp measurements of menthol- and TG-induced membrane currents in control and TRPM8-expressing cells. To avoid contamination of TRPM8-mediated current with Ca2+-carried store-operated current (ISOC), activated in response to menthol- or TG-induced ER store depletion (although its contribution was expected to be negligible, since ISOC density even at elevated [Ca2+]o typically does not exceed 3 pA/pF (17, 19)), we used nominally Ca2+-free extracellular solutions. The absence of extracellular Ca2+ also minimized spontaneous rundown as well as desensitization of menthol-induced TRPM8-mediated current (2).
The currents recorded in HEK-293/TRPM8 cells were characterized by “classical” temperature and menthol dependence as well as by those biophysical properties that were initially described for the recombinant TRPM8, operating as PM channel (2–4). Indeed, at room temperature (20 °C), HEK-293/TRPM8 cells, but not control cells, showing substantial outwardly rectifying current, which diminished upon heating to 36 °C, could be reactivated at this temperature by menthol (100 μM) and diminished again by further heating to 45 °C in the presence of menthol (Fig. 2A). Consistent with the previous observations, both cooling (from 36 to 20 °C) and menthol (100 μM) shifted the half-maximal activation voltage (V1/2) of the TRPM8-mediated current negatively by 100 ± 19 mV (n = 7) and 156 ± 19 mV (n = 9), respectively, without any measurable change in the Boltzmann slope factor (k), which was about 30 mV in all cases. As evident from Fig. 2A, the density of the cold- or menthol-activated membrane currents (Icold/menthol) in HEK-293/TRPM8 cells was great (i.e. exceeding 200 pA/pF at 120 mV), suggesting significant plasmalemmal expression of the TRPM8 channel. Consistent with this, the cell-attached patches usually contained more than one active channel (on average 3.5 ± 0.6, n = 26; see also Fig. 2C, showing an example of six channels at a high time resolution). Fig. 2B shows that single channel activity was temperature-dependent, as expected for TRPM8. This was further confirmed by the outwardly rectifying shape of the mean patch current I-V relationship (Fig. 2C, squares) and by the 74 ± 4 pS (n = 15) unitary conductance (Fig. 2C, circles), which was quite close to the ~83 pS reported before (2).

Consistent with our hypothesis of functional coupling between plasmalemmal TRPM8 and ER Ca2+ store, pretreatment of HEK-293/TRPM8 cells with TG (1 μM, 10 min) resulted in a significant current response development (termed I_{TC}) at 36 °C, which was characterized by the same hallmark features as Icold/menthol (i.e. prominent outward rectification and close to 0 mV reversal potential (Fig. 2D, squares)). Since TG was unable to induce such a current in HEK-293/ctrl cells (Fig. 2D, circles), we attributed I_{TC} to the activation of plasmalemmal TRPM8 channels due to their coupling to the ER store depletion.

It should be noted that although the whole cell Icold/menthol experienced rundown in the course of cell dialysis with the artificial intracellular solution (data not shown), the rundown of I_{TG} was even faster and more complete (time constant of 83 ± 10 s, n = 5), suggesting that the presumed store-to-TRPM8 coupling may involve a diffusible cytoplasmic messenger that is washed out during cell dialysis. The fact that menthol was able to activate a much larger current than TG in HEK-293/TRPM8 cells (i.e. current density of 350 pA/pF for menthol versus 130 pA/pF for TG at +100 mV (Fig. 2, A and D)), which in addition was less prone to rundown, indicated that plasmalemmal...
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TRPM8 gating by menthol most probably involved ER store-dependent (SD) and store-independent (SI) mechanisms, whereas TRPM8 gating by TG occurred solely via the store depletion. The existence of the two modes of TRPM8 gating allows the whole TRPM8-mediated membrane current \( I_{\text{TRPM8}} \) to be represented as the sum of two components: one that is associated with store depletion \( I_{\text{TRPM8/SD}} \) and another one that is not \( I_{\text{TRPM8/SD}} \). In the framework of such a convention, \( I_{\text{cold/menthol}} = I_{\text{TRPM8/SD}} + I_{\text{TRPM8/SD}} \) where \( I_{\text{TRPM8/SD}} \) is the difference between the conductance derived from such patches was 71 ± 5 pS (\( n = 5 \)), similar to that found in the cold/menthol experiments. In the presence of TG, open channel probability (\( NP_o \)) increased with time and reached a maximum at about 10 min, consistent with the idea of the involvement of a gradual intracellular process (i.e. store depletion) rather than any direct drug action on the channel (Fig. 2F). From a physiological standpoint, the most important feature of the TG-dependent TRPM8 activation was the appearance of channel opening at negative potentials at 36 °C (Fig. 2E), at which conditions no unitary activity, not even brief isolated open events, could be resolved before TG application. Such a negative shift of channel gating on the voltage axis was directly confirmed by measuring \( NP_o \) voltage dependence before and after TG application (Fig. 2E, top right).

Even more strikingly, following TG treatment, long channel openings became apparent, which with time made a progressively larger contribution to the channel activity (Fig. 2E, bottom). Analysis of the relative probabilities based on the distributions of open lifetimes (Fig. 2E) predicted that these longer openings contributed up to 90% of the total channel open time. Notably, the two “classical” stimuli, cooling and menthol, were considerably weaker in promoting long openings, which contributed only 6 and 17% of the total channel open time, respectively, even at strong depolarization (see Fig. 3I, left). Thus, the store-dependent mechanism appears to be potent enough to provide physiologically relevant TRPM8 activation in tissues not experiencing any cooling. We next aimed to establish the mechanism of the store-to-TRPM8 coupling.

**Signaling Pathway Involved in the Store-dependent TRPM8 Activation**—Since, on the one hand, ER store depletion is known to stimulate iPLA2 (10, 20) and, on the other hand, iPLA2 stimulation and resultant generation of lysophospholipids as the active end-products has been implicated in SOCs activation (15, 21), we focused on the iPLA2 pathway as a possible mediator of store-to-TRPM8 coupling. In the framework of this model, store depletion induces displacement of the inhibitory calmodulin (CaM) from iPLA2, thereby turning it into a catalytically active state capable of producing two impor-
tand second messengers, arachidonic acid (AA) and lysophospholipids, of which the latter activate the channels in a "membrane-delimited" fashion (15). Thus, when testing the role of the iPLA$_2$ pathway, our strategy was to show that: 1) iPLA$_2$ activity is stimulated by TG, 2) pharmacological or antisense iPLA$_2$ impairment leads to the down-regulation of menthol- and TG-activated, TRPM8-mediated responses, and finally 3) the products of iPLA$_2$-catalyzed lipid breakdown, lysophospholipids or AA, are capable of TRPM8 activation.

Consistent with the previously demonstrated causal link between ER depletion and iPLA$_2$ stimulation (10, 20), our experiments on assaying iPLA$_2$ activity have shown a 2.5-fold enhancement above the basal level following HEK-293/TRPM8 cell treatment with TG (1 mM, 10 min, n = 4; Fig. 3A). Moreover, using specific iPLA$_2$ inhibitor, BEL, to prevent iPLA$_2$ stimulation by store depletion resulted in about an 80% suppression of the TG-induced, La$^{3+}$-insensitive Ca$^{2+}$ influx in HEK-293/TRPM8 cells (Fig. 3B). BEL also effectively antagonized menthol-induced Ca$^{2+}$ influx in the presence of La$^{3+}$ (Fig. 3C), although in this case, the percentage of suppression was smaller (i.e. 55 ± 4%, n = 25), most probably due to the contribution of the store-independent TRPM8-mediated influx component. Furthermore, S-enantiomer of BEL ((S)-BEL, 10 μM), known to be a selective inhibitor of the CaM-dependent iPLA$_2$β isomorf (22), antagonized menthol-activated current more potently (i.e. 45 ± 6% inhibition, n = 6) than R-enantiomer ((R)-BEL, 10 μM, 5 ± 2% of current inhibition, n = 5), which is selective for CaM-independent iPLA$_2$γ, thereby confirming iPLA$_2$β involvement.

The specificity of the inhibition of menthol-activated Ca$^{2+}$ influx via iPLA$_2$ impairment was also demonstrated in the experiments employing antisense iPLA$_2$ mRNA hybrid depletion. In these experiments, we used antisense ODNs with proven specificity and efficacy (21). In our hands, they inhibited iPLA$_2$ activity (Fig. 3D). The fact that this was paralleled by 39 ± 6% (n = 21) suppression of the menthol-activated Ca$^{2+}$ influx (Fig. 3C, inset) provided further support for the hypothesis of iPLA$_2$ involvement in the store-to-TRPM8 channel coupling (note that the substantial remaining iPLA$_2$-insensitive component is due to $I_{\text{TRPM8/SI}}$ according to our convention).

When activated, iPLA$_2$ catalyzes the production of two important lipid messengers, AA and lysophospholipids (11, 12), which can influence ion channels in a membrane-delimited fashion (12–15). Therefore, next we asked which of these two products is involved in plasmalemmal TRPM8 activation via store depletion. To answer this question, we used single channel recordings in inside-out patches while applying the agents to the cytosolic side of the membrane. Fig. 3E shows that patch excision from a HEK-293/TRPM8 cell maintained at room temperature resulted in a dramatic reduction in the single TRPM8 channel activity from $N_P$ of 0.056 ± 0.023 in the cell-attached configuration to $N_P$, of 0.010 ± 0.002 (i.e. 4.6 ± 0.7-fold if individual patches were compared, n = 13). This effect is explained by PIP$_2$ depletion by lipid phosphatases in isolated membrane fragments (5, 6). However, application of LPLs, lysophosphatidylcholine (LPC; 10 μM) or lysophosphatidylinositol (LPI; 10 μM), not only restored the activity but also augmented it significantly above the cell-attached level (Fig. 3, E and G).

Unitary events during LPC/LPI action were of the same amplitude as those induced by cold or menthol, thereby indicating an enhanced TRPM8 channel activity, since in the above described case of TG action, LPLs were capable of activating TRPM8 at negative potentials (Fig. 3E, bottom traces), pointing toward a physiological relevance of this effect, which is also consistent with our Ca$^{2+}$ measurement tests of the iPLA$_2$ role in unclamped cells (Fig. 3, B and C). The effects of LPLs in inside-out patches were evidently unrelated to a change in TRPM8 temperature sensitivity (compare Figs. 2B and 3E) or to the fact that these patches were presumably PIP$_2$-depleted (5, 6). On the other hand, the fact that the potentiating action of TG on TRPM8 activity was abolished by BEL treatment (Fig. 3H) strongly implies its relation to the iPLA$_2$/LPLs pathway.

Notably, the observed $N_P$, increase mainly occurred via the LPC/LPI-induced promotion of the long TRPM8 channel openings. This was evidenced by the appearance of a second significant exponential component associated with the long openings in the distribution of open times (Fig. 3I, right panels). In contrast, the primary mode of TRPM8 channel activation in response to depolarization, cold, or menthol, was characterized by the dominance of channel openings with a mean open time ($\tau_o$) in the range of 0.4–0.7 ms (depending on the membrane potential, Fig. 3I, left panels), whereas any increases in $N_P$ were associated with an increase in the frequency rather than the duration of an open event. Indeed, at 20°C, long openings of around 3 ms were detectable in only 3 of 12 patches, making a trivial contribution of 1.3 ± 1.0% even at 120 mV. Even after menthol application, although more readily detectable (6 of 6 patches examined), the long $\tau_o$, remained the same (2.74 ± 0.21 ms), whereas its contribution increased to only 6 ± 4% of all events. However, employment of the agents that activated TRPM8 channel via a store-dependent pathway, like TG (see Fig. 2E) and especially LPC and LPI (Fig. 3I), induced a strong shift to the major contribution (up to 50% of all events and up to 90% of the total open time) of the long channel openings, with a simultaneous prolongation of the slow $\tau_o$ to 7.9 ± 2.4 ms (n = 7).

From these results, one can conclude that the store-dependent mechanism of TRPM8 channel gating preferentially recruits the activation mode that relies on long openings, whereas the store-independent mechanism relies on short openings of accelerated frequency (i.e. shortening of the closed state). This also suggests that the two modes of gating probably involve different molecular determinants of the TRPM8 channel and thus justified our separation of the overall TRPM8-mediated whole cell current into $I_{\text{TRPM8/SD}}$ and $I_{\text{TRPM8/SI}}$ components. None of the activating effects observed with LPC/LPI could be detected with AA (data not shown).

Possible Mechanism of LPC/LPI Action on TRPM8 Channel—LPC and LPI are representatives of conical neutral lysophospholipids, which, by having conically shaped molecules when partitioned into a single leaflet of membrane lipid bilayer, tend to stabilize its convex curvature (13). The resulting change in the local tension around the channel can affect channel function (14, 23). Such a mechanism has been implicated in the LPL-mediated activation/modulation of a number of channels, including a thermosensitive member of a novel family of mammalian background two-pore domain K$^+$ channels.
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TREK-1 (14), which was shown to respond not only to thermal stimuli but also to mechanical force arising from the membrane lipid bilayer deformation (13, 14). Thus, we hypothesized that the LPC/LPI stimulatory action mechanism on the TRPM8 channel may at least in part be explained by the change in the local bilayer microcurvature. Such a hypothesis was supported by the fact that LPI, which has a larger polar head than LPC and is therefore capable of producing stronger membrane deformation, was a more potent TRPM8 activator (see Fig. 3H). In verifying this hypothesis, our strategy was to use other interventions known to cause changes in physical membrane properties in the attempt either to mimic or to antagonize LPC/LPI effects.

Application of positive or negative pressure (±60 mm Hg) to the cell-attached patches from HEK-293/TRPM8 cells did not alter the basal TRPM8 unitary activity observed at room temperature (data not shown), suggesting that mechanical membrane stretch is not critical for the channel function. Extracellularly applied AA (10 μM) or anionic amphiphil, trinitrophenol (500 μM), both being potent TREK-1 openers due to membrane crenation (13) (i.e. formation of convex curvature), also failed to affect menthol-activated [Ca^{2+}], rise or membrane current in HEK-293/TRPM8 cells (data not shown). However, cationic amphiphaths, tetracaine (100 μM) and chlorpromazine (CPZ; 1 μM), which promote changes in physical membrane properties, such as formation of cup-shape membrane invaginations (13), antagonized menthol-induced [Ca^{2+}], by more than 50% and menthol-activated current by nearly 100% (Fig. 4, A–C). Inclusion of tetracaine (100 μM) in the patch pipette also prevented single TRPM8 channel activity in the cell-attached patches (Fig. 4D), suggesting that cuplike invaginations might indeed play a role in TRPM8 channel closure.

Extracellular application of LPC activated an outwardly rectifying membrane current in HEK-293/TRPM8 cells, which by its biophysical properties was identical to I_{TRPM8} and could be blocked by CPZ (Fig. 4E), thereby demonstrating that LPLs are able to activate TRPM8 not only from the inside but also from the outside of the membrane.

DISCUSSION

Collectively, the results of our study demonstrate that TRPM8, which was initially viewed as just a molecular counter-part of the cold/menthol receptor in sensory neurons, is in fact characterized by bimodal activation involving store-dependent and store-independent mechanisms. Based on this, we propose a novel concept of TRPM8 activation involving iPLA_2 stimulation (Fig. 5). Such an activation may play a role at least in the prostate, which is not exposed to any essential temperature variations. The link of iPLA_2 to TRPM8 may be important in the establishment of the proapoptotic roles of iPLA_2 (12, 24). Indeed, iPLA_2 activation has been implicated in ER stress-induced apoptosis, whereas menthol has been shown to promote apoptosis of LNCaP prostate cancer cells (7). Therefore, it is likely that promotion of apoptosis by menthol in addition to the ER depletion-related mechanism may also include the Ca^{2+}...
entry component associated with the activation of TRPM8 and SOC channels as a result of iPLA₂ stimulation.

We have found that the active substances in the store-dependent mechanism of TRPM8 activation were LPC/LPI, lysophospholipids, which are produced upon iPLA₂ stimulation. It is interesting that recently yet another lipid messenger, PIP₂, has been established as a crucial element in maintaining TRPM8 channel function (5, 6). PIP₂ serves as a substrate for PLC, which, in response to the stimulation via PLC-coupled TRPM8 channel function (5, 6), while LPLs provide the main chemical messengers inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol. Of these two messengers, IP₃ is the major store-releasing agent acting via the ER IP₃ receptors. The fact that receptor-mediated stimulation of PLC will potentially produce oppositely directed effects on TRPM8 (inhibition via decreasing IP₃ levels and activation via IP₃-mediated store depletion) seems puzzling. It can be explained assuming differential roles for the two lipid messenger pathways, the PIP₂ primarily controlling TRPM8 desensitization (6), while LPLs provide the main chemical activation input. In connection with this, it is important that TRPM8 activation by LPLs can occur after channel run-down in PIP₂-depleted inside out patches (Fig. 3, E and F), obviously indicating that the activation mechanism is powerful enough to override the PIP₂-dependent desensitization. Furthermore, it is important to note that the existence of a number of ER subcompartments has been suggested (25). Therefore, it is quite possible that IP₃ receptors and TRPM8 deplete the ER from different subcompartments, and physiologically, iPLA₂ is activated by store depletion via TRPM8 (but not via IP₃ receptors). In our work, we used TG as a pharmacological tool for ER store depletion and iPLA₂ activation. Further studies are needed to understand this ER-plasma membrane TRPM8 coupling. Overall, these two regulatory lipid-mediated pathways appear to act in parallel, and their physiological significance would be primarily determined by the input signal. In the case of cold sensation, the interplay between voltage, temperature, and PIP₂ levels (6) is the main determinant of the channel activity, but in the case of store-dependent activation at physiological temperature, PIP₂ levels would be irrelevant.

When considering the various mechanisms of TRPM8 activation, our single channel recordings have revealed a unique difference in the modes of TRPM8 N_PS increase. While cooling, depolarization, menthol, and PIP₂ primarily act by accelerating channel openings (i.e. the main effect is to destabilize the closed state of the channel), the effect of LPLs is to stabilize the open state of the channel. This effect was mimicked by TG-induced store depletion, providing additional biophysical evidence for the common ion channel mechanism. Our single channel analysis showing differential effects on varying channel conformational states adds to the recent significant developments concerning the requirement of different TRPM8 residues for activation by cold, PIP₂, and menthol (6, 26). It should be noted that store-dependent mechanisms have recently been shown to contribute to the activation of such "apparently store-independent" TRP members as TRPM3 (27) and noxious cold-sensitive ANKTMI (28), although the mediators in this mode of activation remain unknown.

The surface potential of biological membranes varies according to their lipid composition (29). Most lipids are electrically neutral; however, PIP₂ is highly negatively charged (for a review, see Ref. 30). It has recently been shown that positively charged residues in the highly conserved TRP domain of the TRPM8 channels are important for their activation by PIP₂ (6). Here, we show that both conic neutral lysophospholipid (LPC) and conic anionic lysophospholipid (LPI) are potent openers of TRPM8. Therefore, the potential role of alterations in charge distribution in TRPM8 activation by LPLs is not clear.

Interestingly, extracellular application of LPC activated TRPM8 current that could be blocked by CPZ. Thus, LPLs seem to be able to activate TRPM8 not only from the inside but also from the outside of the membrane. This argues against a simple mechanistic hypothesis that LPLs might exert their action on TRPM8 channel by promoting a certain type of membrane deformation, since intra- and extracellular LPLs would favor the formation of the opposite deformations, cuplike and convex curvatures, respectively. Inefficiency of anionic amphipaths, which facilitate convex membrane deformation, although via a different mechanism than extracellular LPLs (i.e. due to the negative molecular charge, as opposed to the molecule shape in the case of LPLs), also contradicts such a hypothesis. Therefore, it is likely that the action of LPLs on TRPM8 may involve more complex membrane deformations, leading to the changes in the local compression, surface tension, and/or splay around the channel core (23) that do not depend on the side of the agent’s application. Alternatively, they may directly interact with some determinants of the channel located in its transmembrane core, which is equally accessible from both sides of the membrane. Likewise, the blocking action of cup-forming cationic amphipaths on TRPM8 may also be unrelated to their cup-forming ability.

**Gating of TRPM8 by Lysophospholipids**

**FIGURE 5.** Schematic depiction of TRPM8 activation modes evident from our study. Cold/menthol by acting directly on PM-localized TRPM8 via an as yet unknown mechanism in the case of cold and via TRP and S2 domains in the case of menthol (26) causes its partial activation resulting in a conduction of the store-independent current component (I_{TRPM8/SI}). Simultaneous activation by cold/menthol of TRPM8 localized in the endoplasmic reticulum membrane (ERM) evokes store depletion and stimulation of the iPLA₂, via displacement of the inhibitory CaM. It results in the generation of lysophospholipids, LPC/LPI, which activate PM-localized TRPM8 as well as SOC in a "membrane-delimited" fashion. The store/iPLA₂/LPC/LPI pathway leads to the activation of a store-dependent TRPM8-carried current component (I_{TRPM8/SP}). The same store/iPLA₂/LPC/LPI pathway for TRPM8 activation can be recruited by store depletion via ER leak channels as a result of SERCA pump inhibition by thapsigargin.
Indeed, the precise mechanism(s) by which LPLs activate TRPM8 is still not fully understood and should be addressed in future studies. It is now strongly suggested (for a review, see Ref. 31) that a number of so-called nonbilayer lipids (including LPLs) could affect peripheral and membrane proteins according to a “lateral pressure” model. It has been demonstrated that the asymmetrical insertion of LPC into sonicated vesicles (which are spheres with the highest curvature that can withstand a phospholipid bilayer) did not change the membrane curvature but was able to induce the formation of surface tension (lateral pressure) (32). The authors suggested that this strain in the membrane can be a physiological way of modulating the conformation of membrane proteins. Lipid asymmetry can also mean a difference in surface density resulting from a difference in the number of lipids between the two coupled monolayers. Such a difference in lipid surface density can be achieved artificially by the addition of LPC to the external surface of a biological membrane. Because of the very slow flip-flop of lipids, the asymmetrical surface density is stable. However, the mismatch causes a difference in lateral pressure or tension between the two leaflets. In biological membranes, the lateral pressure formed by asymmetrical lipid distribution could affect the structure and function of intrinsic membrane proteins, such as TRPM8. It is therefore quite possible that the intracellular addition of LPC also leads to a difference in lateral pressure, which in turn activates TRPM8.

Several members of the TRP family are emerging as candidates as mechanosensors (for review, see Ref. 33). Some of them (TRPY and TRPC1) are likely to be directly activated by force conveyed through membrane lipid tension. Others, including TRPN1 (NOMPC), TRPA1, the invertebrate TRPVs Nanchung and Inactive, and perhaps PKD2, are probably directly activated by mechanical force delivered through structural proteins. However, indirect activation by force conveyed to a mechanically sensitive protein that does not form the channel has also been demonstrated for some types of TRP channels, including osmotically activated channels, such as the mammalian TRPV4. In this case, a second messenger carries the signal to a ligand-activated TRP channel. It is therefore quite possible that TRPM8 activation by lysophospholipids could be explained by one of these mechanisms. Future work focused on the one hand on determining the specific mechanisms of TRPM8 activation and, on the other, on potential activation of already known mechano-sensitive TRP channels by LPLs will probably show which activation mechanisms are shared between all of these channels.

Our data show the involvement of the iPLA₂ pathway in a previously unsuspected chemical regulation of the TRPM8 channel and suggest that variable degrees of store dependence may be a common feature in the activation of different TRP channels.

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