Introduction

Members of the transient receptor potential (TRP) family of ion channels function as sensors of the physical and chemical environment (Clapham, 2003). Light, chemicals, touch, temperature, and osmolarity are examples of stimuli that can activate different TRP channels. Several TRP channels are thermosensitive and together they confer the ability to sense temperature throughout the range from noxious cold to noxious heat (Jordt et al., 2003; Patapoutian et al., 2003). Thus, TRPV1 is activated near the threshold for noxious heat pain, whereas TRPV2 is stimulated by even higher temperatures (Caterina et al., 1997, 1999). TRPV3 and TRPV4 both sense “warm” temperatures around and below body temperature (Guler et al., 2002; Peier et al., 2002b; Smith et al., 2002; Xu et al., 2002). Transient receptor potential (melastatin)-8 (TRPM8) is activated by cold with a threshold temperature of ~25°C (McKemy et al., 2002; Peier et al., 2002a; Story et al., 2003).

TRPM8 is expressed in a subpopulation of small cold-sensitive dorsal root ganglion (DRG) neurons consistent with its proposed function as a thermosensor. TRPM8 is also expressed in sensory nerves innervating deeper tissues that, under normal circumstances, never experience the low temperatures (<25°C) necessary to activate the channel. In these tissues, it is likely that TRPM8 is activated or modulated by endogenous agonists or mechanisms other than temperature.

The activities of many TRP channels are regulated by lipids. PIP2, diacylglycerol, and polyunsaturated fatty acids have been shown to activate or inhibit various TRP channels directly (Runnels et al., 2002; Clapham, 2003; Hardie, 2003; Liu and Liman, 2003; Liu and Qin, 2005; Rohacs et al., 2005). Polyunsaturated fatty acids (PUFAs) and arachidonic acid metabolites constitute a distinct group of lipids that can act as endogenous agonists at some TRP channels. TRPV3 is activated by unsaturated fatty acids, TRPV1 by anandamide and lipoxigenase products, and TRPV4 by epoxyeicosatrienoic acids produced by cytochrome P450 mono-oxygenases (Zygmun et al., 1999; Hwang et al., 2000; Watanabe et al., 2003; Hu et al., 2006).

In this study, we have examined the role of phospholipase A2 (PLA2) in the activation of TRPM8. All PLA2 enzymes hydrolyze the sn-2 ester of glycerophospholipids to release a free polyunsaturated fatty acid (PUFA) and a lysophospholipid (LPL). Two main groups of intracellular PLA2 enzymes exist, cytosolic PLA2 (cPLA2) and calcium-insensitive PLA2 (iPLA2), also called group IV and group VI PLA2, respectively. Although cPLA2 is selective for phospholipids with arachidonic acid in the sn-2 position, this is not the case for iPLA2, which releases other fatty acids as well (Balsinde and Balboa, 2005). Here we report the discovery of a key role for iPLA2 in the regulation of TRPM8 and show that lysophospholipids, produced by PLA2 activity, positively modulate TRPM8 and can act as endogenous agonists to activate the channel at normal physiological temperatures. In contrast, PUFAs such as arachidonic, eicosapentaenoic, and docosahexaenoic acid inhibit TRPM8. Although the two groups of PLA2 products exert opposing modulatory effects on TRPM8, the net bal-

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Modulation of the Cold-Activated Channel TRPM8 by Lysophospholipids and Polyunsaturated Fatty Acids

David A. Andersson,1,2 Mark Nash,2,3 and Stuart Bevan1,2

1Wolfson Centre for Age-Related Diseases, King’s College London, London SE1 1UL, United Kingdom, 2Novartis Institute for Biomedical Sciences, London WC1E 6BS, United Kingdom, and 3Novartis Institute for Biomedical Sciences, Horsham, West Sussex RH12 5AB, United Kingdom

We investigated the role of phospholipase A2 (PLA2) and the effects of PLA2 products (polyunsaturated fatty acids and lysophospholipids) on the cold-sensitive channel transient receptor potential (melastatin)-8 (TRPM8), heterologously expressed in Chinese hamster ovary cells. TRPM8 responses to cold and the agonist icilin were abolished by inhibitors of the calcium-independent (iPLA2) form of the enzyme, whereas responses to menthol were less sensitive to iPLA2 inhibition. Inhibition of PLA2 similarly abolished the cold responses of the majority of cold-sensitive dorsal root ganglion neurons. The products of PLA2 had opposing effects on TRPM8. Lysophospholipids (LPLs) (lysophosphatidylcholine, lysophosphatidylinositol, and lysophosphatidylserine) altered the thermal sensitivity of TRPM8, raising the temperature threshold toward normal body temperature. Polyunsaturated fatty acids (PUFAs), such as arachidonic acid, inhibited the activation of TRPM8 by cold, icilin, and menthol. The relative potencies of lysophospholipids and PUFAs are such that lysophosphatidylcholine is able to modulate TRPM8 in the presence of an equimolar concentration of arachidonic acid. Positive modulation by LPLs provides a potential physiological mechanism for sensitizing and activating TRPM8 in the absence of temperature variations.

Key words: TRPM8; phospholipase A2; lysophospholipid; arachidonic acid; menthol; icilin
ance of equimolar concentrations of the PL\(_A\) products favors TRPM8 activation. These findings introduce lysophospholipids as novel modulators of thermosensitive TRP channel activity.

**Materials and Methods**

**Cell culture.** Chinese hamster ovary (CHO) cells expressing mouse TRPM8 (Peier et al., 2002a) were grown in MEM \(\alpha\) medium without ribonucleosides and deoxyribonucleosides, supplemented with penicillin (100 U/ml), streptomycin (100 \(\mu\)g/ml), \(\alpha\)-l-glutamine (2 mM), FCS (10%), and hygromycin B (200 \(\mu\)g/ml).

**DRG cultures.** DRG neurons were prepared from adult (200 g) male or female Wistar rats as described previously (Bevan and Winter, 1995). Rats were asphyxiated with CO\(_2\), as approved by the United Kingdom Home Office, and spinal ganglia were removed aseptically from all levels of the spinal cord. Ganglia were incubated in 0.125% collagenase type IV (Worthington Biochemical, Lake-wood, NJ) for 3 h in DMEM (Invitrogen, Paisley, UK) with 2 mM \(\alpha\)-l-glutamine at 37°C in a humidified incubator gassed with 5% CO\(_2\) in air. Neurons were dissociated mechanically by trituration with a flame-polished Pasteur pipette. The DRG neurons were centrifuged through 2 ml of 15% bovine albumin in DMEM media, and the pellet was resus-
pended in DMEM in 10% fetal bovine serum (Invitrogen), 2 mM \(\alpha\)-l-glutamine, 10 \(\mu\)g/ml penicillin and streptomycin, and 50 ng/ml NGF (Promega, Madison, WI). The neurons were plated onto sterile 13 mm glass coverslips previously coated with 10 \(\mu\)g/ml poly-n-lysine and 5 \(\mu\)g/ml laminin and maintained at 37°C in a humidified incubator gassed with 5% CO\(_2\).

**Imaging of intracellular calcium levels.** CHO cells and DRG neurons were grown on glass coverslips covered with poly-n-lysine and loaded with 2 \(\mu\)M fura-2 AM (Invitrogen, Leiden, The Netherlands) for 1 h in the presence of 1 mM probenecid and 0.01% pluronic F-127 at 37°C before the experiments. The dye loading and the subsequent experiments were performed in an assay buffer containing the following (in mM): 140 NaCl, 5 KCl, 10 glucose, 10 HEPES, 2 CaCl\(_2\), and 1 MgCl\(_2\) buffered to pH 7.4 (NaOH). Compounds were applied to cells by local microperfusion of solution through a fine tube placed very close to the cells being studied. The temperature of the perfusion buffer was controlled by a regulated Peltier device (Marlow Industries, Dallas, TX) and monitored by a ther-
mocouple positioned in the tip of the perfusion tube. Images of a group of cells were captured every 2 s at 340 and 380 nm excitation wavelengths with emission measured at 520 nm with a microscope-based imaging system (PTI, Birmingham, NJ). Analyses of emission intensity ratios at 340/380 nm excitation (R, in individual cells) were performed with the ImageMaster suite of software.

Neurons in DRG cultures were identified at the end of the experiment by stimulation with an assay buffer supplemented with 50 mM KCl. The resultant depolarization opened voltage-gated calcium channels in the neurons and evoked a calcium influx that was detected by a change in the 340/380 emission ratio.

**Electrophysiology.** Cells expressing TRPM8 were grown on glass covers-
lips covered with poly-n-lysine and studied by whole-cell voltage-clamp recordings using an Axopatch 200A amplifier and pClamp 8.0 software (Molecular Devices, Union City, CA). Experiments were performed at a holding potential of −60 mV, unless stated otherwise. Borosilicate glass pipettes (2–5 M\(\Omega\)) were filled with the following (in mM): 140 KCl, 0.05 CaCl\(_2\), 1 MgATP, 0.1 EGTA, and 10 HEPES. The external assay buffer was the same as described above for imaging of intracellular Ca\(^{2+}\) \(\cdot\). The cells were superfused locally through a thin tube positioned very close to the cells.

*Ninety-six-well plate-based intracellular calcium assays.* In some experiments, changes in intracellular calcium were determined in TRPM8 expressing CHO cells grown in 96-well black-walled plates (Costar, Cam-
bridge, MA) using a Flexstation (Molecular Devices). Cells were loaded with fura-2 AM as described above and washed with assay buffer. Assays were performed at 30°C. Basal emission ratios (340/380 nm) were mea-
sured for 17 s, and then compounds were injected, and the change in dye emission ratio was determined after 60 s.

**Drugs.** The specific cPLA\(_2\) inhibitor ACA (\(N\)-[2-(2,4-R)-4-(biphenyl-2-ylmethyl-isobutyl-amino)-1-(2,4-difluorobenzoyl)-benzoyl]-pyrrolidin-2-ylmethyl]-3-[4-(2,4-dioxothiazolidin-5-ylidenemethyl)-phenyl]acrylamide HCl (cPLA), \(N\)-[p-amylcinnamoyl]anthranilic acid (ACA), 5,8,11,14-eicosatet-

![Figure 1](image1.png)

**Figure 1.** The PL\(_A\) inhibitor ACA inhibits TRPM8. A–C. Cold (\(A\)), icilin (\(B\)), and menthol (\(C\)) evoked large [Ca\(^{2+}\)] responses in CHO cells expressing TRPM8. Superfusing the cells with ACA before agonist challenge abolished responses induced by a cold ramp (\(A\)) and icilin (\(B\)) and significantly reduced responses to menthol (\(C\)). Traces are the mean responses of groups of cells monitored individually (\(n = 28–41\)). Veh, Vehicle; temp, temperature.

**Table 1. Effect of PL\(_A\) inhibitors on [Ca\(^{2+}\)] responses evoked by cold, icilin, and menthol**

| Inhibitor | Cold (13°C) | Icilin (1 \(\mu\)M) | Menthol (1 \(\mu\)M) |
|-----------|-------------|---------------------|---------------------|
| Veh       | 113 ± 28    | 97 ± 2.2            | 92 ± 8.8            |
| ACA 10 \(\mu\)M | −3.6 ± 0.9*** | 0.5 ± 1.7***        | 41 ± 12**           |
| BEL 20 \(\mu\)M | 1.0 ± 0.8*** | 11 ± 7.3***         | 71 ± 12             |
| NDGA 20 \(\mu\)M | 0.6 ± 1.1*** | 0.5 ± 1.0***        | 52 ± 1.6*           |
| ETYA 10 \(\mu\)M | 5.9 ± 2.5*** | 28 ± 2.6**          | 66 ± 8.9            |

*All values are mean ± SEM of \(n = 3–5\) experiments. *\(p < 0.05\), **\(p < 0.01\), ***\(p < 0.001\), compared to control.

![Figure 2](image2.png)

**Figure 2.** ACA and BEL concentration–response curves. A, B. The nonselective PL\(_A\) inhibitor ACA (\(A\)) and the selective iPLA\(_2\) inhibitor BEL (\(B\)) concentration-dependently reduced [Ca\(^{2+}\)] responses evoked by icilin and menthol. Both inhibitors were significantly more potent against icilin than menthol. Data points are mean ± SEM measured in triplicates, and the data shown are representative of \(n = 3\) separate experiments.
Arachidonic acid inhibits TRPM8. Figure 3. Induced by a cold ramp from 37°C to 12°C (shown in We used fura-2 to monitor the effect of PLA2 inhibition on TRPM8 responses evoked by 1 mM menthol (Fig. 1). ACA does not distinguish between different PLA2 isozymes. To identify the enzyme targeted by ACA in CHO cells, we examined the effects of PLA2 inhibitors selective for iPLA2 and cPLA2 on TRPM8 activity. A potent and selective cell-permeable cPLA2 inhibitor, cPI (100 nM) (Seno et al., 2000), had no effect on the responses to menthol, icilin, or cold (Table 1). In contrast, 45 min of incubation with a selective irreversible and cell-permeable iPLA2 inhibitor, BEL (20 µM), almost completely inhibited the [Ca2+]i responses evoked by cold and icilin but only produced a small inhibition of the response to menthol (Table 1). ETYA, a general inhibitor of arachidonic acid metabolism, and the lipoxigenase inhibitor nordihydroguaiaretic acid (NDGA), are two compounds that also inhibit PLA2 (Lanni and Becker, 1985). Both compounds had the same profile as BEL and ACA. ETYA (10 µM) and NDGA (20 µM) effectively inhibited the responses to icilin and cold but had only a small effect on the response to menthol (Table 1). Because TRPM8 was sensitive to BEL and nonselective inhibitors of PLA2, but not to the cPLA2 selective inhibitor cPI, our results strongly implicate iPLA2 in the maintenance and regulation of TRPM8 activity.

Menthol-induced [Ca2+]i responses showed a different sensitivity to PLA2 inhibition than those evoked by icilin and cold (Table 1). Conditions that completely inhibited responses to cold and icilin (ACA, 10 µM; BEL, 20 µM for 45 min) only partially reduced the responses to menthol. This parallels previous findings that responses to menthol are less sensitive to the intracellular Ca2+ concentration and pH than those elicited by cold and icilin (Andersson et al., 2004; Chuang et al., 2004). To characterize the abilities of PLA2 inhibitors to inhibit the responses evoked by menthol and icilin in more detail, we constructed inhibition–response curves studying the effects of different concentrations of ACA and BEL on TRPM8 responses evoked by fixed concentrations of agonist (Fig. 2). In these experiments, we used concentrations of icilin (40 nM) and menthol (40 µM) that elicited submaximal responses of similar amplitude (~EC50 concentrations). ACA and BEL completely inhibited responses to icilin (IC50 values of 0.46 ± 0.1 and 8.6 ± 2 µM, respectively), whereas much higher concentrations of these inhibitors were required to inhibit the [Ca2+]i responses to menthol (IC50 values of 6.9 ± 3 and 30 ± 4 µM with ACA and BEL).

Although BEL is regularly used to determine the involvement of iPLA2, it is also a well-documented inhibitor of phosphatidic acid phosphohydrolase 1 (PAP-1). We addressed the possibility that the effect of BEL was attributable to PAP-1 inhibition by using propranolol, which at high concentrations is an inhibitor of PAP-1 (Fuentes et al., 2003). At a relevant
high concentration (250 μM), propranolol failed to inhibit [Ca^{2+}]_i responses to cold or icilin, ruling out the involvement of PAP-1 (data not shown).

Effects of PLA2 inhibition on native TRPM8 in DRG neurons

TRPM8 is expressed in sensory neurons in trigeminal and dorsal root ganglia. In good agreement with previous studies (Peier et al., 2002a), we found that 6.8% of DRG neurons in culture were sensitive to stimulation with menthol and 9% to icilin (Table 2). Cold stimulated a slightly larger cell population (11.6%) (Table 2). Two distinct populations of cold-sensitive DRG neurons have been described based on their sensitivity or insensitivity to menthol and differences in threshold temperatures (Story et al., 2003; Babes et al., 2004). Story et al. (2003) found that menthol-sensitive neurons had a temperature threshold of ~23°C, whereas the threshold for the menthol-insensitive neurons was lower, ~15°C. A smaller difference in temperature threshold for menthol-sensitive (24.9°C) and menthol-insensitive (22.9°C) neurons was noted by Babes et al. (2004). Nevertheless, both studies showed that TRPM8 expression was associated with higher temperature thresholds. We noted that treatment with either ACA (10 μM) or BEL (25 μM) reduced the population of cold-sensitive neurons to <4%. ACA and BEL also significantly reduced the number of menthol-sensitive neurons and inhibited the effect of icilin almost completely (Table 2). The percentage of cold-sensitive neurons that were resistant to inhibition of PLA2 was the same as the percentage of menthol-insensitive, cold-sensitive neurons. Interestingly, the temperature threshold for cold-sensitive DRG neurons after PLA2 inhibition was 4–7°C lower than in the overall population (Table 2). Thus, our data are consistent with the conclusion that cold and icilin activation of TRPM8 in DRG neurons, as in CHO cells, was abolished by iPLA2 inhibition and that the small remaining population of (non-TRPM8) cold-sensitive DRG neurons require lower temperatures for activation. In addition, menthol responses in DRG neurons were more resistant to PLA2 inhibition than those to cold and icilin, similar to our results in CHO cells.

TRPM8 is inhibited by polyunsaturated fatty acids

All PLA2 enzymes hydrolyze the sn-2 ester of glycerophospholipids to release a free fatty acid and a LPL. Arachidonic acid and endogenous metabolites of arachidonate are known activators of other thermo-sensitive TRP channels: TRPV1, TRPV3, TRPV4, and TRPA1 (Zygmunet et al., 1999; Hwang et al., 2000; Watanabe et al., 2003; Bandell et al., 2004; Hu et al., 2006). TRPM2, the ion channel with greatest homology to TRPM8, can also be activated by arachidonic acid (Hara et al., 2002; Togashi et al., 2006). We therefore examined the potential regulatory role of arachidonic acid and related fatty acids on TRPM8.

Unlike the findings with other thermo-sensitive TRP channels, arachidonic acid reduced the cold, icilin, and menthol sensitivity of CHO cells expressing TRPM8. Arachidonic acid (10 μM) depressed the [Ca^{2+}]_i responses to icilin (1 μM) by 97 ± 1% and to cold stimulation (measured at 13°C) by 96 ± 2%. A smaller (76 ± 2%), but highly significant, inhibition of the response to 100 μM menthol was recorded in the presence of arachidonic acid (Fig. 3). To discern whether the observed inhibition was specific for arachidonic acid (20:4) or a more general property of polyunsaturated fatty acids, we also tested the effects of eicosapentaenoic acid (EPA) (Fig. 4A) and docosahexaenoic acid (DOHA) (Fig. 4B) prevented [Ca^{2+}]_i responses to stimulation with a cold ramp (from 37°C to 12°C). The traces in A and B are average responses in groups of cells (n = 25–50 cells). C, Average peak amplitude from experiments like those in A, B, and Figure 34 (the arachidonic acid (AA) data are the same as those shown in Fig. 3D).

### Table 3. IC50 values for polyunsaturated fatty acids as inhibitors of icilin and menthol

| Inhibitor      | IC50 (μM) | IC50 (1 μM) | IC50 (50 μM) |
|----------------|-----------|-------------|--------------|
| AA (20:4)      | 1.3 ± 0.1 | 3.2 ± 0.6   |              |
| EPA (20:5)     | 2.4 ± 0.1 | 6.3 ± 0.9   |              |
| DOHA (22:6)    | 1.6 ± 0.2 | 2.0 ± 0.3   |              |

All values are mean ± SEM of n = 3–5 experiments.

Figure 4. TRPM8 is sensitive to polyunsaturated fatty acids. A, B, Eicosapentaenoic acid (EPA) (A) and docosahexaenoic acid (DOHA) (B) prevented [Ca^{2+}]_i responses to stimulation with a cold ramp (from 37°C to 12°C). The traces in A and B are average responses in groups of cells (n = 25–50 cells). C, Average peak amplitude from experiments like those in A, B, and Figure 34 (the arachidonic acid (AA) data are the same as those shown in Fig. 3D).

Figure 5. TRPM8 is activated by lysophospholipids. A, Lysophosphatidylcholine elicited [Ca^{2+}]_i responses in cells expressing TRPM8 but not in untransfected CHO cells. After a longer initial delay, LPC also activated TRPM8 at 37°C (traces are averages of 20–40 cells monitored individually). B, TRPM8 was activated by lysophospholipids with different head groups. The negatively charged LPI and LPS evoked [Ca^{2+}]_i responses of amplitude similar to the zwitterionic LPC, whereas SPC was much less effective (all at a concentration of 3 μM). Experiments were performed at 29°C, and the data in B are mean ± SEM of the number of experiments indicated.
inhibition of iPLA₂ as well as treatment with arachidonic acid prevented activation of TRPM8, we next tested whether LPLs could act as endogenous activators or positive modulators of TRPM8 (Fig. 5).

Application of 3 μM lysophosphatidylcholine (LPC) (16:0) produced large [Ca²⁺]ᵢ responses in CHO cells transfected with TRPM8 (Δratio 2.6 ± 0.2) but not in untransfected control cells (Δratio 0.1 ± 0.1). LPC (3 μM) was able to induce [Ca²⁺]ᵢ responses in TRPM8 at 37°C although with a longer delay than at 29°C (Fig. 5A). PLA₂ activity in vivo is likely to produce a mixture of LPLs with different head groups and acyl chains. To explore whether LPLs containing head groups with different properties can activate TRPM8, we used the anionic lysophosphatidylinositol (LPI) and lysophosphatidylserine (LPS) as well as the zwitterionic LPC. We also tested the effects of a spongolipid, sphingosyl-phosphorylcholine (SPC). The [Ca²⁺]ᵢ responses evoked by 3 μM LPI and LPS were similar to those elicited by LPC, whereas SPC was much less effective at this concentration (Fig. 5B). We were unable to construct full agonist concentration–response curves for the lysophospholipids acting at TRPM8 because higher concentrations (≥5 μM) evoked a second type of cellular [Ca²⁺]ᵢ response that was evident in untransfected CHO cells (data not shown). Nevertheless, we noted that lower (1 and 2 μM) concentrations of LPC, LPI, and LPS produced mean [Ca²⁺]ᵢ responses that showed little difference in amplitude between the lipid species (data not shown). We investigated the importance of the fatty acid chain length for LPL activity by examining the effects of an LPC with a much shorter acyl chain (6:0). This short-chain LPC failed to activate TRPM8 even at concentrations as high as 40 μM (data not shown).

To further demonstrate that LPC can activate TRPM8, we compared the single-channel activity evoked by menthol and LPC in cell-attached membrane patches (Fig. 6). Before application of menthol or LPC, no channel activity was recorded in the patches. Application of menthol (50 μM) to the cells elicited brief channel openings within a few seconds with a mean single-channel current amplitude of 2.9 ± 0.1 pA at +60 mV. LPC (3 μM) evoked channel activity similar to menthol (single-channel current amplitude 3.1 ± 0.1 pA at +60 mV) but after a much longer delay (Fig. 6C,D). This delay may reflect slow penetration of the cell membrane attributable to the amphiphatic character of LPC. In a different set of experiments, LPC (3 μM) was applied in cell-attached mode, and when channel activity had developed, we excised the membrane patch in the continued presence of LPC.

Lyso phospholipids stimulate TRPM8
PLA₂ liberates LPLs as well as fatty acids, and iPLA₂ has been suggested to regulate store-operated calcium entry through production of LPLs (Smani et al., 2004). Because we discovered that

of 10 μM eicosapentaenoic acid (20:5) and docosahexaenoic acid (22:6) on the cold responses of TRPM8. The results shown in Figure 4 clearly demonstrate that cold activation of TRPM8 was almost completely inhibited by all three polyunsaturated fatty acids. This was also the case with [Ca²⁺]ᵢ responses induced by menthol (50 μM) and icilin (1 μM). IC₅₀ values determined from concentration–response curves showed that all three fatty acids inhibit responses to icilin and menthol with similar potencies (Table 3). The results suggest that TRPM8 can be regulated by variations in the cellular concentrations of PUFAs.

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to record channel activity in the inside-out patch configuration (Fig. 6G). After excision, P(open) decreased as reported previously (Reid and Flonta, 2002; Voets et al., 2004), but there was still marked channel activity in the patch, suggesting that LPC is able to activate TRPM8 in a membrane-delimited manner.

Menthol potentiates the effect of cooling on TRPM8 by raising the temperature threshold (Reid and Flonta, 2001; McKemy et al., 2002; Peier et al., 2002a). We found that this was also the case with LPC. LPC strongly potentiated the whole-cell currents and [Ca2+], responses evoked by cooling. In electrophysiology experiments, the threshold for temperature activation in the presence of 3 μM LPC was raised by 3–5°C and the amplitude of the cold-evoked current at 11°C was increased sixfold from 190 ± 90 to 1280 ± 190 pA (Fig. 7A). The effect of LPC on [Ca2+]i responses elicited by cooling was much more dramatic. Application of increasing concentrations of LPC (1–3 μM) progressively elevated the temperature activation threshold. At 3 μM, TRPM8 was activated at 37°C in many cells. The augmented cold-activated [Ca2+]i, responses in the presence of LPC were caused by a calcium influx through TRPM8 because it was dependent on the presence of extracellular Ca2+ (Fig. 7B). In addition, LPC had no effect on [Ca2+]i in untransfected CHO cells (data not shown).

If PLA2 is important for TRPM8 activation because it produces LPLs, TRPM8 activation by LPLs should be resistant to PLA2 inhibitors. We tested this by applying LPC to cells incubated with 10 μM ACA, a concentration that inhibits responses to menthol, icilin, and cold (Figs. 1, 2). As predicted, ACA failed to affect responses to LPC (Fig. 8A). TRPM8 activation by cold and icilin, but not menthol, is inhibited by reducing the external and internal pH (Andersson et al., 2004). LPC shared this feature because application of LPC (3 μM) failed to evoke any responses in an external solution of pH 6 (Fig. 8A).

One apparent paradox is that two products of iPLA2 activity, LPLs and PUFAs, have opposite actions on TRPM8. To address this balance between the excitatory and inhibitory effects of LPLs and PUFAs, we applied equimolar concentrations (3 μM) of arachidonic acid and LPC to TRPM8-expressing cells at 31°C. Under these conditions, the amplitude of [Ca2+]i responses induced by LPC were only marginally reduced by the presence of arachidonic acid (Fig. 8B). However, a higher concentration of arachidonic acid (10 μM) was able to inhibit activation of TRPM8 by 3 μM LPC (Fig. 8C).

Discussion
Our results have demonstrated a key role for iPLA2 in modulating TRPM8 activity. Furthermore, we have demonstrated that the products of PLA2, LPLs and PUFAs, can modulate TRPM8 activity in a reciprocal manner. Lysophospholipids act as positive modulators, whereas PUFAs inhibit TRPM8 activity. Our findings are consistent with a very recent study demonstrating that
brane patches were excised into the inside-out configuration suggesting that TRPM8 could be activated by LPC at 37°C. LPC had a more dramatic effect on temperature sensitivity in experiments monitoring [Ca\(^{2+}\)]\(_i\) than in voltage-clamp experiments. The reason for this difference is unclear, but it may reflect a loss of regulatory factor when the intracellular contents are dialysed by the pipette solution in the whole-cell configuration. LPLs acted relatively slowly over many tens of seconds, which suggests that the site of action was intracellular and that activation required translocation across the plasma membrane. The speed of action of LPLs on TRPM8 differs from the almost immediate effects of PLs on TWIK-related K\(^+\) channels (TREK) in which the site of action is thought to be extracellular (Maingret et al., 2000). Because iPLA\(_2\) is an intracellular enzyme, the supply of endogenous LPLs to the inner surface of the plasma membrane and subsequent modulation of TRPM8 is likely to be a faster process than suggested by the slow responses to extracellular LPLs observed in our studies.

LPLs are known to modulate the activities of other membrane channels including TREK-1 (Lesage et al., 2000; Maingret et al., 2000) and TRPC5 (Flemming et al., 2006) and can regulate store-operated calcium entry (Smahi et al., 2004; Singaravelu et al., 2006). TRPC5 activation by LPLs is also relatively slow and probably requires translocation of the LPL to the internal membrane surface. The absence of LPL responses in uncontected cells at the concentrations studied and the similar properties of LPC- and menthol-activated single-channel currents indicate that the responses to LPLs are mediated by TRPM8 and not by a nonspecific action on membrane lipids (cf. Wilson-Ashworth et al., 2004). How LPLs regulate TRPM8 is unclear. The observation that LPC evoked single-channel current activity was retained when membrane patches were excised into the inside-out configuration suggests that LPLs act on TRPM8 in a membrane-delimited manner. They could have an indirect effect on channel function by interacting with either plasma membrane lipids or channel-associated proteins or a direct effect by binding to TRPM8. We did note that there was no significant difference in the activity of the anionic LPI (16:0) and the zwitterionic LPC (16:0). However, a short-chain species of LPC (16:0) lacked activity altogether, whereas the sphingolipid SPC (16:0) was less active than LPI and LPC. These results suggest that both the length of the fatty acyl chain and the properties of the head-group influence their actions at TRPM8.

Arachidonic acid and two other PUFAs (docosahexaenoic acid and eicosapentaenoic acid) inhibited TRPM8 with approximately equal potency. This inhibition contrasts with the activation of other thermosensitive TRP channels by PUFAs and arachidonic acid metabolites. Once again it is unclear whether the inhibitory effect of the PUFAs is mediated by an interaction with the plasma membrane or TRPM8 itself.

Continued iPLA\(_2\) activity was essential for the maintenance of cold and icilin sensitivity and the ability of these stimuli to activate TRPM8 was lost within minutes of adding the enzyme inhibitors. iPLA\(_2\) is considered to have a housekeeping role in cells regulating the turnover of membrane lipids (Balsinde and Balboa, 2005). Consequently, the concentration of LPLs will decrease when iPLA\(_2\) is inhibited as the existing LPLs continue to be acylated by acyltransferases. The loss of cold and icilin sensitivity a few minutes after adding PLA\(_2\) inhibitors is therefore consistent with a tonic regulatory role for LPLs generated by PLA\(_2\).

The pharmacology of icilin and cold differs from that of menthol with respect to pH sensitivity, dependence on extracellular Ca\(^{2+}\), and iPLA\(_2\) activity (Andersson et al., 2004; Chuang et al., 2004) (Fig. 2). LPC shared the pH sensitivity with cold and icilin, suggesting that there is a common step or cofactor requirement for cold, icilin, and LPL activation that is not shared by menthol. Importantly, the action of LPC was resistant to a high concentration of ACA.

LPLs and PUFAs are both released by PL\(_2\), which raised the possibility that the stimulatory effect of LPLs might be cancelled out by the coincident inhibitory action of the PUFAs. However, this scenario is unlikely. First, the reduction in icilin and cold sensitivity after iPLA\(_2\) inhibition is consistent with an overall stimulatory effect of the PL\(_2\) products. Second, when we applied LPC and arachidonic acid at equimolar concentrations, LPC still evoked large [Ca\(^{2+}\)]\(_i\) responses. Third, iPLA\(_2\) lacks substrate specificity for phospholipids containing particular fatty acids in sn-2 position (unlike cPL\(_2\)), which is selective for arachidonic acid and therefore releases a mixture of saturated and unsaturated fatty acids (Balsinde and Balboa, 2005).

The ability of LPLs to act as positive modulators of TRPM8 function raises the possibility that treatments that increase their concentration will alter the thermal threshold for activation and may even open the channels at normal body temperature (37°C). There is growing evidence that iPLA\(_2\) is involved in some cell functions.
signaling pathways in addition to its role in homeostatic phospholipid deacylation/reacylation reactions (Smani et al., 2004; Balsinde and Balboa, 2005; Singaravelu et al., 2006). The other major intracellular PLA2 enzyme, cPLA2, is activated by micro- molar levels of intracellular calcium and plays a central role in lipid mediator production in pathological conditions, including inflammation, primarily through the release of arachidonic acid and its metabolites (Kita et al., 2006). Although our data indicate that cPLA2 does not have a role in maintaining basal TRPM8 activity, stimuli that stimulate cPLA2 to generate LPLs could sensitize or activate TRPM8.

The phospholipid PIP2 can control the activity and desensitization of TRPM8, and a reduction in the PIP2 levels has been shown previously to explain the rundown of TRPM8 channels in isolated patches (Fig. 6) (Liu and Qin, 2005; Rohacs et al., 2005). This channel rundown and associated decrease in PIP2 concentrations is dramatically accelerated in the presence of Mg2+ (Huang et al., 1998; Liu and Qin, 2005). Because LPC (3 μM)- evoked channel activity was maintained in excised patches in the presence of Mg2+ (1 mM in all recording solutions), PIP2 does not seem to be required for the actions of LPLs on TRPM8.

TRPM8 is a physiological sensor of cold and cool temperatures (McKemy et al., 2002; Peier et al., 2002a). However, temperature alone is unlikely to account for activation of TRPM8 in all tissues because this channel is expressed in sensory nerves innervating visceral organs such as the bladder (Mukerji et al., 2006) and lower gastrointestinal tract (Zhang et al., 2004). The other innervating visceral organs such as the bladder (Mukerji et al., 2006) and lower gastrointestinal tract (Zhang et al., 2004).

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