Cellular/Molecular

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

David A. Andersson,[1,2] **Mark Nash,**[2,3] **and Stuart Bevan**[1,2]

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

We investigated the role of phospholipase A_2 (PLA_2) and the effects of PLA_2 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 ($iPLA_2$) form of the enzyme, whereas responses to menthol were less sensitive to $iPLA_2$ inhibition. Inhibition of PLA_2 similarly abolished the cold responses of the majority of cold-sensitive dorsal root ganglion neurons. The products of PLA_2 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**

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. PIP_2, 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 lipoxygenase products, and TRPV4 by epoxyeicosatrienoic acids produced by cytochrome P450 mono-oxygenases (Zygmunt 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 A_2 (PLA_2) in the activation of TRPM8. All PLA_2 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 PLA_2 enzymes exist, cytosolic PLA_2 ($cPLA_2$) and calcium-insensitive PLA_2 ($iPLA_2$), also called group IV and group VI PLA_2, respectively. Although $cPLA_2$ is selective for phospholipids with arachidonic acid in the sn-2 position, this is not the case for $iPLA_2$, which releases other fatty acids as well (Balsinde and Balboa, 2005). Here we report the discovery of a key role for $iPLA_2$ in the regulation of TRPM8 and show that lysophospholipids, produced by PLA_2 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 PLA_2 products exert opposing modulatory effects on TRPM8, the net bal-

Received Nov. 7, 2006; revised Feb. 16, 2007; accepted Feb. 19, 2007.

This work was supported in part by a grant from the Medical Research Council. We thank Isabelle Garnier for her contribution to the 96-well plate assays.

Correspondence should be addressed to David Andersson, Wolfson Centre for Age-Related Diseases, Guy's Campus, King's College London, London SE1 1UL, UK. E-mail: david.andersson@kcl.ac.uk.

DOI:10.1523/JNEUROSCI.4846-06.2007

 0270-6474/07/273347-09$15.00/0

ance of equimolar concentrations of the PLA_2 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 α medium without ribonucleosides and deoxyribonucleosides, supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), FCS (10%), and hygromycin B (200 μg/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, Lakewood, NJ) for 3 h in DMEM (Invitrogen, Paisley, UK) with 2 mM 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 resuspended in DMEM with 10% fetal bovine serum (Invitrogen), 2 mM L-glutamine, 10 μg/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 μg/ml poly-D-lysine and 5 μg/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-D-lysine and loaded with 2 μM 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 thermocouple 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 coverslips covered with poly-D-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 MΩ) 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+}. 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, Cambridge, 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 measured 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 *N*-{(2*S*,4*R*)-4-(biphenyl-2-ylmethyl-isobutyl-amino)-1-[2-(2,4-difluorobenzoyl)-benzoyl]-pyrrolidin-2-ylmethyl}-3-[4-(2,4-dioxothiazolidin-5-ylidenemethyl)-phenyl]acrylamide HCl (cPI), *N*-(p-amylcinnamoyl)anthranilic acid (ACA), 5,8,11,14-eicosatet-

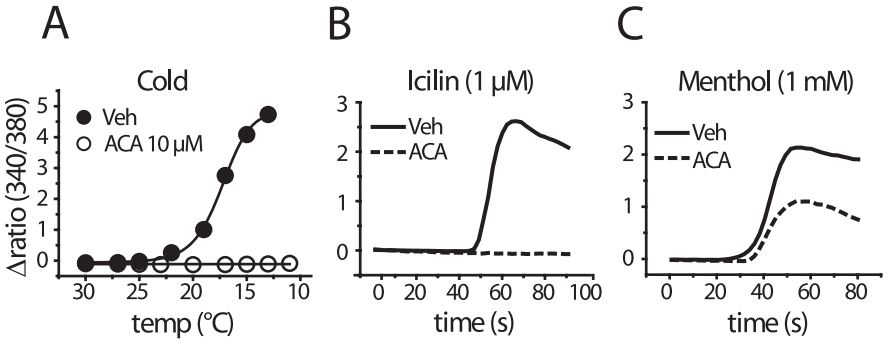

Figure 1. The PLA_2 inhibitor ACA inhibits TRPM8. ***A–C***, Cold (***A***), icilin (***B***), and menthol (***C***) evoked large $[Ca^{2+}]_i$ responses in CHO cells expressing TRPM8. Superfusing the cells with ACA before agonist challenge abolished responses induced by a cold ramp (***A***) and icilin (***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 PLA_2 inhibitors on $[Ca^{2+}]_i$ responses evoked by cold, icilin, and menthol

	$[Ca^{2+}]_i$ response (% of control)		
Inhibitor	Cold (13°C)	Icilin (1 μM)	Menthol (1 mM)
cPI 100 nM	113 ± 28	97 ± 2.2	92 ± 8.8
ACA 10 μM	−3.6 ± 0.9***	0.5 ± 1.7***	41 ± 12**
BEL 20 μM	1.0 ± 0.8***	11 ± 7.3***	71 ± 12
NDGA 20 μM	0.6 ± 1.1***	0.5 ± 1.0***	52 ± 1.6*
ETYA 10 μM	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

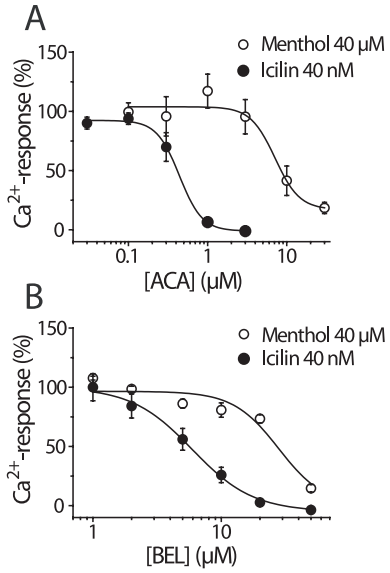

Figure 2. ACA and BEL concentration–response curves. ***A***, ***B***, The nonselective PLA_2 inhibitor ACA (***A***) and the selective $iPLA_2$ inhibitor BEL (***B***) concentration-dependently reduced $[Ca^{2+}]_i$ responses evoked by icilin and menthol. Both inhibitors were significantly more potent against icilin than menthol. Data points are mean ± SEM measured in triplicates, and the data shown are representative of $n = 3$ separate experiments.

Table 2. PLA_2 inhibitors reduce the proportion of cold-sensitive cultured DRG neurons

	Cold [threshold (°C)]	Menthol	Icilin
Control	27 of 232 (11.6%) (25.7 ± 1.0 °C)	71 of 1039 (6.8%)	57 of 634 (9.0%)
ACA 10 μM	17 of 448*** (3.8%) (21.5 ± 2.2 °C)	24 of 565* (4.2%)	5 of 365*** (1.4%)
BEL 25 μM, 45 min	11 of 301*** (3.7%) (18.4 ± 1.6 °C)	10 of 372** (2.7%)	2 of 374*** (0.5%)

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared with control

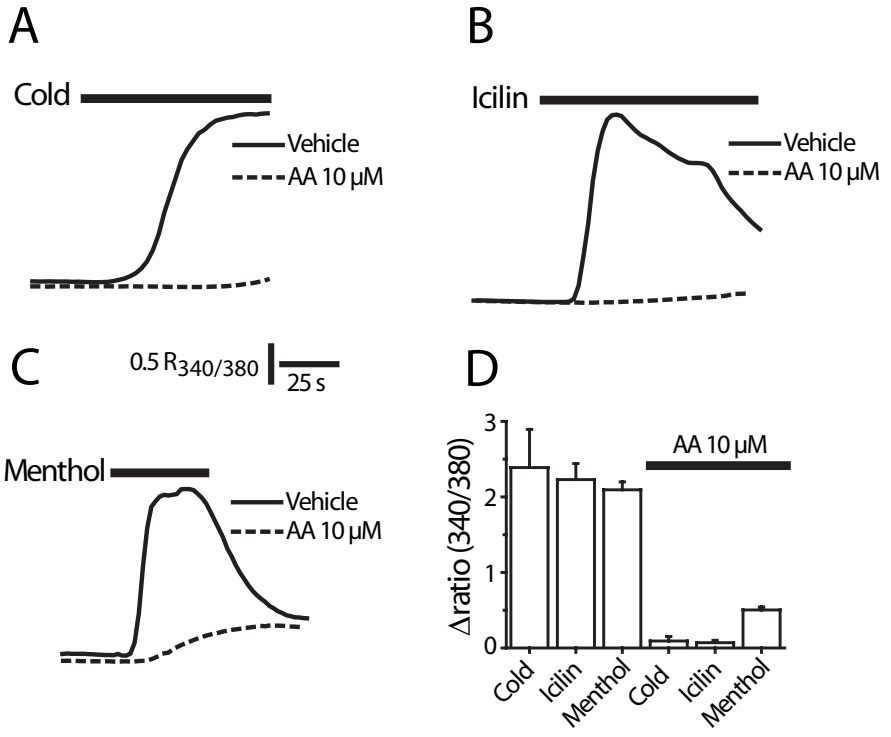

Figure 3. Arachidonic acid inhibits TRPM8. ***A***, ***B***, Arachidonic acid (AA) (10 μM) almost completely inhibited $[Ca^{2+}]_i$ responses induced by a cold ramp from 37°C to 12°C (***A***), 100 nM icilin (***B***), and 100 μM menthol (***C***). Each trace is the average response from a group of cells ($n = 25–50$) monitored individually. The average peak response amplitude of experiments like those in ***A–C*** is shown in ***D*** ($n = 3–4$).

raynoic acid (ETYA), and E-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2*H*-pyran-2-one (BEL) were obtained from Calbiochem (La Jolla, CA). Icilin was obtained from Tocris Bioscience (Ellisville, MO) and arachidonic acid, lysophospholipids, fatty acids, and all other reagents were purchased from Sigma (St. Louis, MO).

Calculation and statistics. Data are presented as means ± SEM for the number of experiments indicated (n). Curve fitting was performed in Origin 7 (OriginLab, Northampton, MA). Statistical significance was calculated using an unpaired t test or ANOVA, followed by Bonferroni's *post hoc* test.

Results

Inhibition of PLA_2 prevents activation of TRPM8

We used fura-2 to monitor the effect of PLA_2 inhibition on $[Ca^{2+}]_i$ responses induced by cold, icilin, and menthol in CHO cells expressing TRPM8. The PLA_2 inhibitors were applied by superfusion for 2 min before the cells were challenged with cold, menthol, or icilin.

ACA, a cell-permeable, nonselective PLA_2 inhibitor, completely inhibited responses to stimulation with icilin (1 μM) or cold (cold ramp to 13°C) and reduced the amplitude of $[Ca^{2+}]_i$ responses evoked by 1 mM menthol (Fig. 1). ACA does not distinguish between different PLA_2 isoforms. To identify the enzyme targeted by ACA in CHO cells, we examined the effects of PLA_2 inhibitors selective for $iPLA_2$ and $cPLA_2$ on TRPM8 activity. A potent and selective cell-permeable $cPLA_2$ 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 $iPLA_2$ inhibitor, BEL (20 μM), almost completely inhibited the $[Ca^{2+}]_i$ 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 lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA), are two compounds that also inhibit PLA_2 (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 PLA_2, but not to the $cPLA_2$ selective inhibitor cPI, our results strongly implicate $iPLA_2$ in the maintenance and regulation of TRPM8 activity.

Menthol-induced $[Ca^{2+}]_i$ responses showed a different sensitivity to PLA_2 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 Ca^{2+} concentration and pH_i than those elicited by cold and icilin (Andersson et al., 2004; Chuang et al., 2004). To characterize the abilities of PLA_2 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 elicit submaximal responses of similar amplitude (~EC_{80} concentrations). ACA and BEL completely inhibited responses to icilin (IC_{50} values of 0.46 ± 0.1 and 8.6 ± 2 μM, respectively), whereas much higher concentrations of these inhibitors were required to inhibit the $[Ca^{2+}]_i$ responses to menthol (IC_{50} values of 6.9 ± 3 and 30 ± 4 μM with ACA and BEL).

Although BEL is regularly used to determine the involvement of $iPLA_2$, 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 PLA_2 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 PLA_2 was the same as the percentage of menthol-insensitive, cold-sensitive neurons. Interestingly, the temperature threshold for cold-sensitive DRG neurons after PLA_2 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 $iPLA_2$ 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 PLA_2 inhibition than those to cold and icilin, similar to our results in CHO cells.

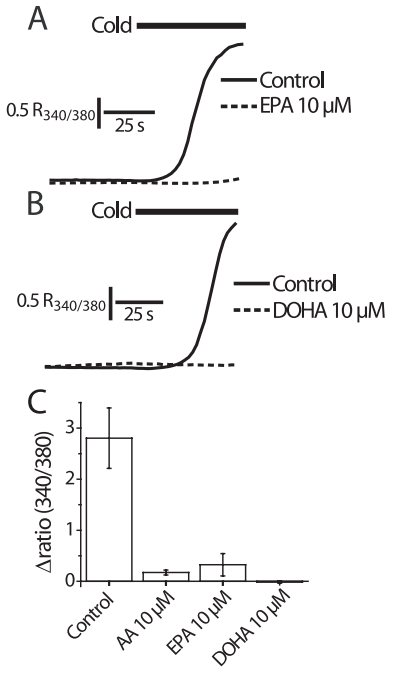

Figure 4. TRPM8 is sensitive to polyunsaturated fatty acids. ***A***, ***B***, Eicosapentaenoic acid (EPA) (***A***) and docosahexaenoic acid (DOHA) (***B***) prevented $[Ca^{2+}]_i$ responses to stimulation with a cold ramp (from 37°C to 12°C). The traces in ***A*** and ***B*** are average responses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

Table 3. IC_{50} values for polyunsaturated fatty acids as inhibitors of icilin and menthol

	IC_{50} (μM)	
Inhibitor	Icilin (1 μM)	Menthol (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.

TRPM8 is inhibited by polyunsaturated fatty acids

All PLA_2 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 thermosensitive TRP channels: TRPV1, TRPV3, TRPV4, and TRPA1 (Zygmunt 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.

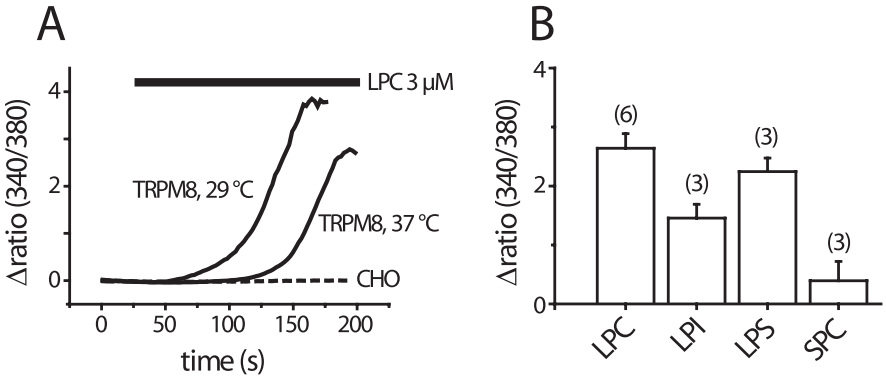

Figure 5. TRPM8 is activated by lysophospholipids. ***A***, Lysophosphatidylcholine elicited $[Ca^{2+}]_i$ responses in cells expressing TRPM8 but not in untransfected CHO cells. After a longer initial delay, LPC also activated TRPM8 at 37°C (traces are averages of 25–40 cells monitored individually). ***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.

Unlike the findings with other thermosensitive 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

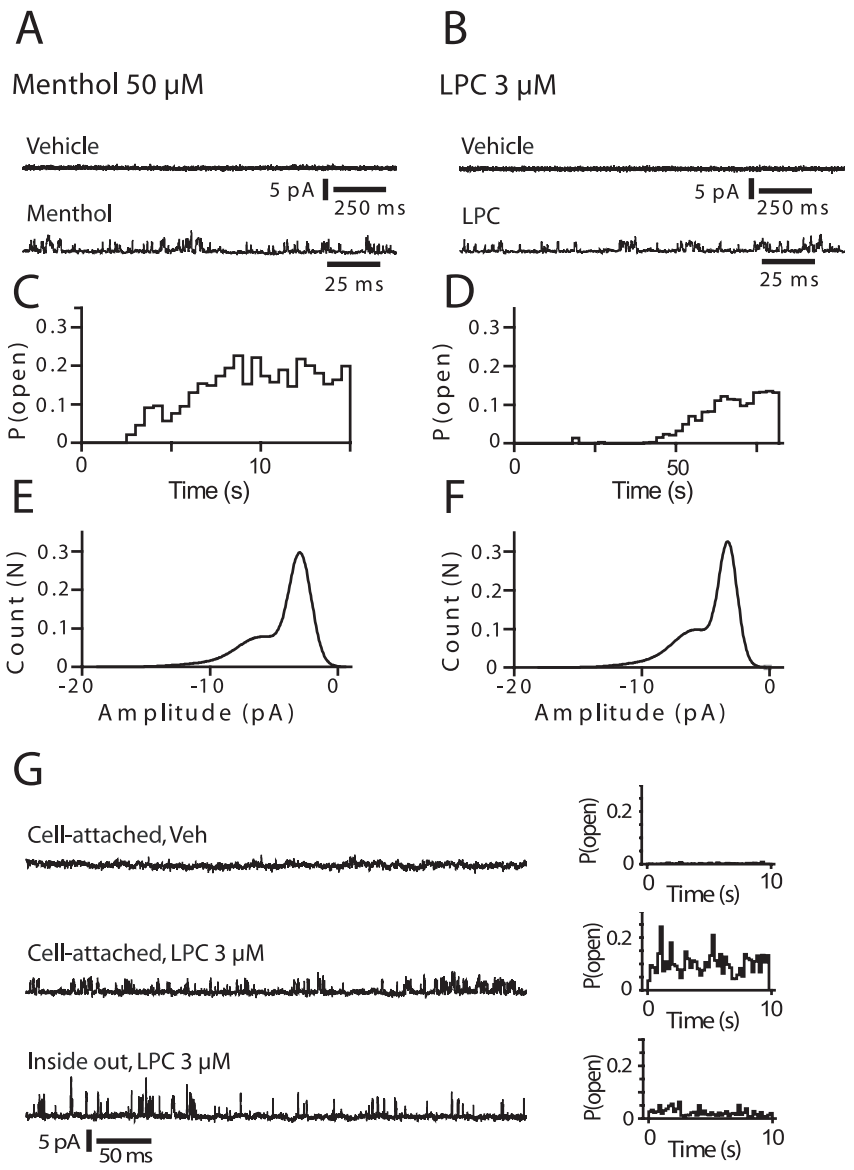

Figure 6. Menthol and LPC stimulate TRPM8 single-channel activity. ***A***, ***C***, In cell-attached patches, menthol (50 μM) stimulated channel activity within a few seconds of application. ***B***, ***D***, LPC (3 μM) elicited very similar single-channel activity but after a much longer initial delay. ***E***, ***F***, All point histograms from traces recorded in the presence of menthol (***E***) and LPC (***F***) show a similar distribution. Both histograms have been fitted with the sum of three Gaussian functions. ***G***, Traces were recorded in cell-attached configuration in the absence and presence of LPC (3 μM). When channel activity had developed, the patch was excised to inside-out configuration. After patch excision, the channel activity stimulated by LPC persisted. Data were recorded at a holding potential of +60 mV. Veh, Vehicle.

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 $[Ca^{2+}]_i$ responses induced by menthol (50 μM) and icilin (1 μM). IC_{50} 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.

Lysophospholipids stimulate TRPM8

PLA_2 liberates LPLs as well as fatty acids, and $iPLA_2$ has been suggested to regulate store-operated calcium entry through production of LPLs (Smani et al., 2004). Because we discovered that inhibition of $iPLA_2$ 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 $[Ca^{2+}]_i$ responses in CHO cells transfected with TRPM8 (Δratio 2.6 $\pm$ 0.2) but not in untransfected control cells (Δratio 0.1 $\pm$ 0.1). LPC (3 μM) was able to induce $[Ca^{2+}]_i$ responses in TRPM8 at 37°C although with a longer delay than at 29°C (Fig. 5*A*). PLA_2 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 sphingolipid, sphingosyl-phosphorylcholine (SPC). The $[Ca^{2+}]_i$ 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. 5*B*). We were unable to construct full agonist concentration–response curves for the lysophospholipids acting at TRPM8 because higher concentrations ($\geq$5 μM) evoked a second type of cellular $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ 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 $\pm$ 0.1 pA at +60 mV. LPC (3 μM) evoked channel activity similar to menthol (single-channel current amplitude 3.1 $\pm$ 0.1 pA at +60 mV) but after a much longer delay (Fig. 6*C*,*D*). This delay may reflect slow penetration of the cell membrane attributable to the amphiphathic 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

to record channel activity in the inside-out patch configuration (Fig. 6*G*). 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 $[Ca^{2+}]_i$ 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. 7*A*). The effect of LPC on $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ responses in the presence of LPC were caused by a calcium influx through TRPM8 because it was dependent on the presence of extracellular Ca^{2+} (Fig. 7*B*). In addition, LPC had no effect on $[Ca^{2+}]_i$ in untransfected CHO cells (data not shown).

If PLA_2 is important for TRPM8 activation because it produces LPLs, TRPM8 activation by LPLs should be resistant to PLA_2 inhibitors. We tested this by applying LPC to cells incubated with 10 μM ACA, a concentration that inhibits its responses to menthol, icilin, and cold (Figs. 1, 2). As predicted, ACA failed to affect responses to LPC (Fig. 8*A*). 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. 8*A*).

One apparent paradox is that two products of $iPLA_2$ 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 $[Ca^{2+}]_i$ responses induced by LPC were only marginally reduced by the presence of arachidonic acid (Fig. 8*B*). However, a higher concentration of arachidonic acid (10 μM) was able to inhibit activation of TRPM8 by 3 μM LPC (Fig. 8*C*).

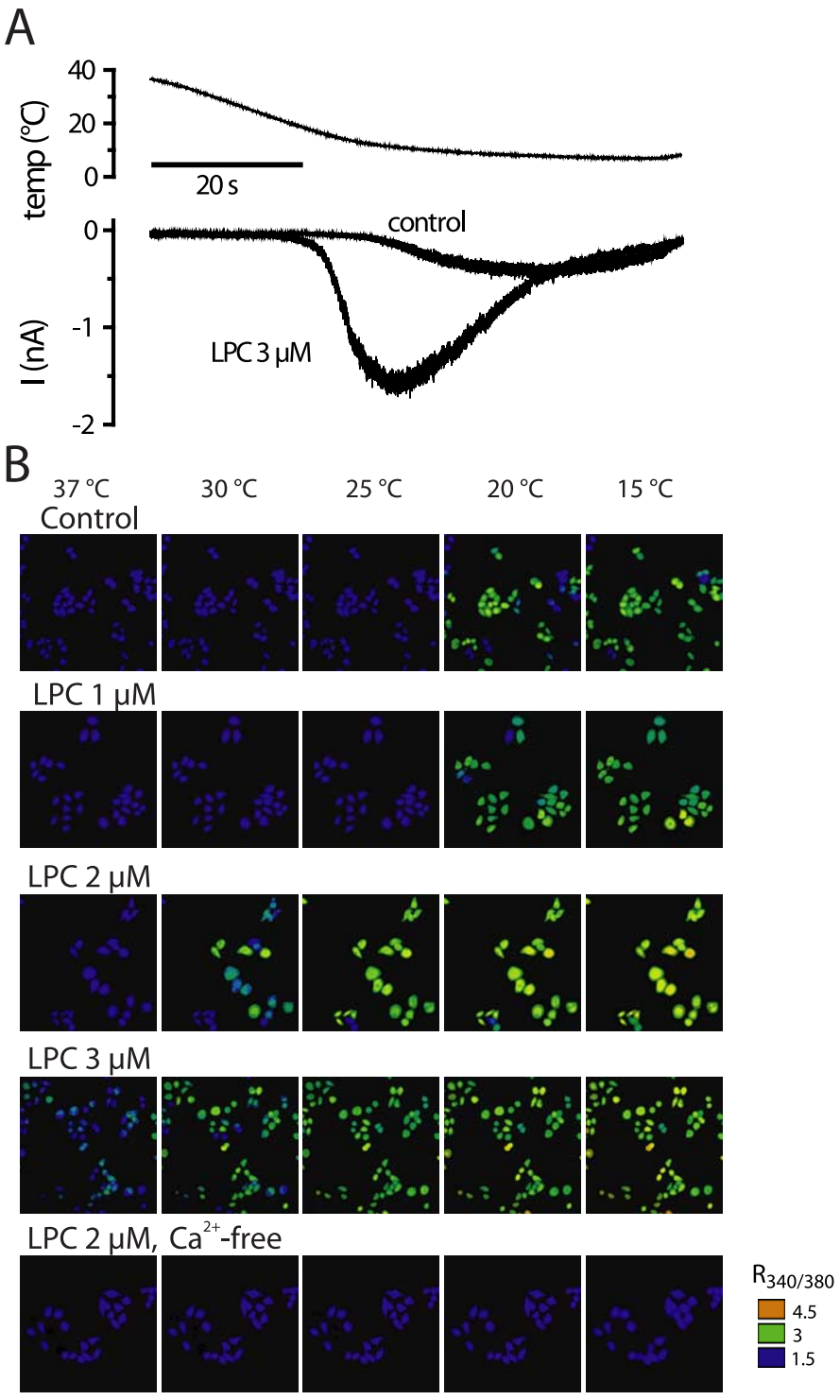

Figure 7. LPC modulates the cold sensitivity of TRPM8. ***A***, LPC potentiates responses to cold by increasing the current amplitude and by shifting the activation threshold to higher temperatures (temp). ***B***, Pseudocolored ratio images from $[Ca^{2+}]_i$ imaging experiments illustrating that LPC shifts the temperature activation threshold of TRPM8 closer to physiologically relevant values. No response was seen in the absence of extracellular Ca^{2+}.

Discussion

Our results have demonstrated a key role for $iPLA_2$ in modulating TRPM8 activity. Furthermore, we have demonstrated that the products of PLA_2, 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

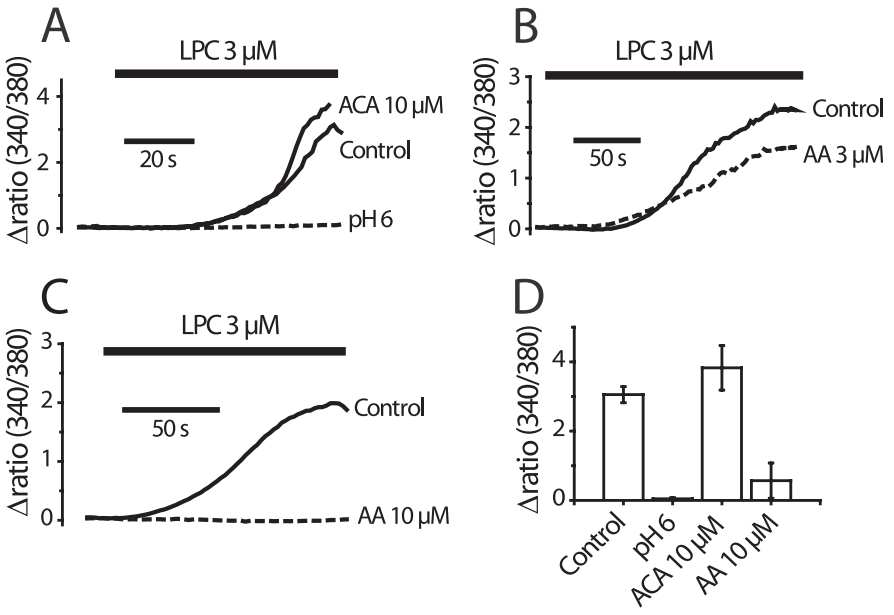

Figure 8. LPC is resistant to inhibition of PLA_2 but sensitive to low pH. ***A***, The agonist effect of LPC (3 μM) on TRPM8 is unaffected by the presence of 10 μM ACA. Cells incubated in pH 6 for 1 min before stimulation failed to respond to LPC. ***B***, Application of equimolar concentrations of LPC and arachidonic acid (AA) only produced a small reduction of the LPC induced $[Ca^{2+}]_i$ responses. ***C***, However, 10 μM arachidonic acid completely inhibited the response to 3 μM LPC. ***D***, Average of the peak amplitude in three determinations in groups of 20–40 cells.

$iPLA_2$ inhibition partially reduced menthol sensitivity (Abeele et al., 2006). These authors also showed that LPC and LPI could activate TRPM8 expressed in human embryonic kidney 293 cells.

Addition of LPLs raised the thermal threshold for activation so that TRPM8 could be activated by 3 μM 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 a 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 LPLs 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 (Smani 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 untransfected 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 (6:0) 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 headgroup 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_i 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 PLA_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 PLA_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 $cPLA_2$, 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

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 PLA_2 enzyme, $cPLA_2$, is activated by micromolar 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 $cPLA_2$ does not have a role in maintaining basal TRPM8 activity, stimuli that stimulate $cPLA_2$ to generate LPLs could sensitize or activate TRPM8.

The phospholipid PIP_2 can control the activity and desensitization of TRPM8, and a reduction in the PIP_2 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 PIP_2 concentrations is dramatically accelerated in the presence of Mg^{2+} (Huang et al., 1998; Liu and Qin, 2005). Because LPC (3 μM)-evoked channel activity was maintained in excised patches in the presence of Mg^{2+} (1 mM in all recording solutions), PIP_2 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). TRPM8 is also expressed in non-neuronal cells in prostate and bladder (Tsavaler et al., 2001; Stein et al., 2004). These neurons and non-neuronal cells rarely, if ever, experience the low temperatures generally considered to activate TRPM8 (<25°C). In these cells, endogenous modulators, such as LPLs and PIP_2, would raise the temperature threshold so that TRPM8 can operate at physiological body temperatures. These lipid modulators could therefore have two roles: setting the temperature sensitivity of cold receptors in the skin and other tissues that are exposed to the external environment and regulating the activity of TRPM8-expressing neurons that innervate deeper tissues and organs.

References

Abeele FV, Zholos A, Bidaux G, Shuba Y, Thebault S, Beck B, Flourakis M, Panchin Y, Skryma R, Prevarskaya N (2006) Ca(2+)-independent phospholipase A(2)-dependent gating of TRPM8 by lysophospholipids. J Biol Chem 281:40174–40182.

Andersson DA, Chase HW, Bevan S (2004) TRPM8 activation by menthol, icilin, and cold is differentially modulated by intracellular pH. J Neurosci 24:5364–5369.

Babes A, Zorzon D, Reid G (2004) Two populations of cold-sensitive neurons in rat dorsal root ganglia and their modulation by nerve growth factor. Eur J Neurosci 20:2276–2282.

Balsinde J, Balboa MA (2005) Cellular regulation and proposed biological functions of group VIA calcium-independent phospholipase A2 in activated cells. Cell Signal 17:1052–1062.

Bandell M, Story GM, Hwang SW, Viswanath V, Eid SR, Petrus MJ, Earley TJ, Patapoutian A (2004) Noxious cold ion channel TRPA1 is activated by pungent compounds and bradykinin. Neuron 41:849–857.

Bevan S, Winter J (1995) Nerve growth factor (NGF) differentially regulates the chemosensitivity of adult rat cultured sensory neurons. J Neurosci 15:4918–4926.

Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D (1997) The capsaicin receptor: a heat-activated ion channel in the pain pathway. Nature 389:816–824.

Caterina MJ, Rosen TA, Tominaga M, Brake AJ, Julius D (1999) A capsaicin-receptor homologue with a high threshold for noxious heat. Nature 398:436–441.

Chuang HH, Neuhausser WM, Julius D (2004) The super-cooling agent icilin reveals a mechanism of coincidence detection by a temperature-sensitive TRP channel. Neuron 43:859–869.

Clapham DE (2003) TRP channels as cellular sensors. Nature 426:517–524.

Flemming PK, Dedman AM, Xu SZ, Li J, Zeng F, Naylor J, Benham CD, Bateson AN, Muraki K, Beech DJ (2006) Sensing of lysophospholipids by TRPC5 calcium channel. J Biol Chem 281:4977–4982.

Fuentes L, Perez R, Nieto ML, Balsinde J, Balboa MA (2003) Bromoenol lactone promotes cell death by a mechanism involving phosphatidate phosphohydrolase-1 rather than calcium-independent phospholipase A2. J Biol Chem 278:44683–44690.

Guler AD, Lee H, Iida T, Shimizu I, Tominaga M, Caterina M (2002) Heat-evoked activation of the ion channel, TRPV4. J Neurosci 22:6408–6414.

Hara Y, Wakamori M, Ishii M, Maeno E, Nishida M, Yoshida T, Yamada H, Shimizu S, Mori E, Kudoh J, Shimizu N, Kurose H, Okada Y, Imoto K, Mori Y (2002) LTRPC2 Ca2+-permeable channel activated by changes in redox status confers susceptibility to cell death. Mol Cell 9:163–173.

Hardie RC (2003) Regulation of TRP channels via lipid second messengers. Annu Rev Physiol 65:735–759.

Hu HZ, Xiao R, Wang C, Gao N, Colton CK, Wood JD, Zhu MX (2006) Potentiation of TRPV3 channel function by unsaturated fatty acids. J Cell Physiol 208:201–212.

Huang CL, Feng S, Hilgemann DW (1998) Direct activation of inward rectifier potassium channels by PIP2 and its stabilization by Gbetagamma. Nature 391:803–806.

Hwang SW, Cho H, Kwak J, Lee SY, Kang CJ, Jung J, Cho S, Min KH, Suh YG, Kim D, Oh U (2000) Direct activation of capsaicin receptors by products of lipoxygenases: endogenous capsaicin-like substances. Proc Natl Acad Sci USA 97:6155–6160.

Jordt SE, McKemy DD, Julius D (2003) Lessons from peppers and peppermint: the molecular logic of thermosensation. Curr Opin Neurobiol 13:487–492.

Kita Y, Ohto T, Uozumi N, Shimizu T (2006) Biochemical properties and pathophysiological roles of cytosolic phospholipase A(2)s. Biochim Biophys Acta 1761:1317–1322.

Lanni C, Becker EL (1985) Inhibition of neutrophil phospholipase A2 by p-bromophenylacyl bromide, nordihydroguaiaretic acid, 5,8,11,14-eicosatetraynoic acid and quercetin. Int Arch Allergy Appl Immunol 76:214–217.

Lesage F, Terrenoire C, Romey G, Lazdunski M (2000) Human TREK2, a 2P domain mechano-sensitive K+ channel with multiple regulations by polyunsaturated fatty acids, lysophospholipids, and Gs, Gi, and Gq protein-coupled receptors. J Biol Chem 275:28398–28405.

Liu B, Qin F (2005) Functional control of cold- and menthol-sensitive TRPM8 ion channels by phosphatidylinositol 4,5-bisphosphate. J Neurosci 25:1674–1681.

Liu D, Liman ER (2003) Intracellular Ca2+ and the phospholipid PIP2 regulate the taste transduction ion channel TRPM5. Proc Natl Acad Sci USA 100:15160–15165.

Maingret F, Patel AJ, Lesage F, Lazdunski M, Honore E (2000) Lysophospholipids open the two-pore domain mechano-gated K(+) channels TREK-1 and TRAAK. J Biol Chem 275:10128–10133.

McKemy DD, Neuhausser WM, Julius D (2002) Identification of a cold receptor reveals a general role for TRP channels in thermosensation. Nature 416:52–58.

Mukerji G, Yiangou Y, Corcoran SL, Selmer IS, Smith GD, Benham CD, Bountra C, Agarwal SK, Anand P (2006) Cool and menthol receptor TRPM8 in human urinary bladder disorders and clinical correlations. BMC Urol 6:6.

Patapoutian A, Peier AM, Story GM, Viswanath V (2003) ThermoTRP channels and beyond: mechanisms of temperature sensation. Nat Rev Neurosci 4:529–539.

Peier AM, Moqrich A, Hergarden AC, Reeve AJ, Andersson DA, Story GM, Earley TJ, Dragoni I, McIntyre P, Bevan S, Patapoutian A (2002a) A TRP channel that senses cold stimuli and menthol. Cell 108:705–715.

Peier AM, Reeve AJ, Andersson DA, Moqrich A, Earley TJ, Hergarden AC, Story GM, Colley S, Hogenesch JB, McIntyre P, Bevan S, Patapoutian A (2002b) A heat-sensitive TRP channel expressed in keratinocytes. Science 296:2046–2049.

Reid G, Flonta ML (2001) Physiology. Cold current in thermoreceptive neurons. Nature 413:480.

Reid G, Flonta ML (2002) Ion channels activated by cold and menthol in cultured rat dorsal root ganglion neurones. Neurosci Lett 324:164–168.

Rohacs T, Lopes CM, Michailidis I, Logothetis DE (2005) PI(4,5)P(2) regulates the activation and desensitization of TRPM8 channels through the TRP domain. Nat Neurosci 8:626–634.

Runnels LW, Yue L, Clapham DE (2002) The TRPM7 channel is inactivated by PIP(2) hydrolysis. Nat Cell Biol 4:329–336.

Seno K, Okuno T, Nishi K, Murakami Y, Yamada K, Nakamoto S, Ono T (2000) Pyrrolidine inhibitors of human cytosolic phospholipase A(2). J Med Chem 43:1041–1044.

Singaravelu K, Lohr C, Deitmer JW (2006) Regulation of store-operated calcium entry by calcium-independent phospholipase A2 in rat cerebellar astrocytes. J Neurosci 26:9579–9592.

Smani T, Zakharov SI, Csutora P, Leno E, Trepakova ES, Bolotina VM (2004) A novel mechanism for the store-operated calcium influx pathway. Nat Cell Biol 6:113–120.

Smith GD, Gunthorpe MJ, Kelsell RE, Hayes PD, Reilly P, Facer P, Wright JE, Jerman JC, Walhin JP, Ooi L, Egerton J, Charles KJ, Smart D, Randall AD, Anand P, Davis JB (2002) TRPV3 is a temperature-sensitive vanilloid receptor-like protein. Nature 418:186–190.

Stein RJ, Santos S, Nagatomi J, Hayashi Y, Minnery BS, Xavier M, Patel AS, Nelson JB, Futrell WJ, Yoshimura N, Chancellor MB, De Miguel F (2004) Cool (TRPM8) and hot (TRPV1) receptors in the bladder and male genital tract. J Urol 172:1175–1178.

Story GM, Peier AM, Reeve AJ, Eid SR, Mosbacher J, Hricik TR, Earley TJ, Hergarden AC, Andersson DA, Hwang SW, McIntyre P, Jegla T, Bevan S, Patapoutian A (2003) ANKTM1, a TRP-like Channel Expressed in Nociceptive Neurons, Is Activated by Cold Temperatures. Cell 112:819–829.

Togashi K, Hara Y, Tominaga T, Higashi T, Konishi Y, Mori Y, Tominaga M (2006) TRPM2 activation by cyclic ADP-ribose at body temperature is involved in insulin secretion. EMBO J 25:1804–1815.

Tsavaler L, Shapero MH, Morkowski S, Laus R (2001) Trp-p8, a novel prostate-specific gene, is up-regulated in prostate cancer and other malignancies and shares high homology with transient receptor potential calcium channel proteins. Cancer Res 61:3760–3769.

Voets T, Droogmans G, Wissenbach U, Janssens A, Flockerzi V, Nilius B (2004) The principle of temperature-dependent gating in cold- and heat-sensitive TRP channels. Nature 430:748–754.

Watanabe H, Vriens J, Prenen J, Droogmans G, Voets T, Nilius B (2003) Anandamide and arachidonic acid use epoxyeicosatrienoic acids to activate TRPV4 channels. Nature 424:434–438.

Wilson-Ashworth HA, Judd AM, Law RM, Freestone BD, Taylor S, Mizukawa MK, Cromar KR, Sudweeks S, Bell JD (2004) Formation of transient non-protein calcium pores by lysophospholipids in S49 Lymphoma cells. J Membr Biol 200:25–33.

Xu H, Ramsey IS, Kotecha SA, Moran MM, Chong JA, Lawson D, Ge P, Lilly J, Silos-Santiago I, Xie Y, DiStefano PS, Curtis R, Clapham DE (2002) TRPV3 is a calcium-permeable temperature-sensitive cation channel. Nature 418:181–186.

Zhang L, Jones S, Brody K, Costa M, Brookes SJ (2004) Thermosensitive transient receptor potential channels in vagal afferent neurons of the mouse. Am J Physiol Gastrointest Liver Physiol 286:G983–G991.

Zygmunt PM, Petersson J, Andersson DA, Chuang H, Sorgard M, Di M, V, Julius D, Hogestatt ED (1999) Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. Nature 400:452–457.