TRPC calcium channels are emerging as a ubiquitous feature of vertebrate cells, but understanding of them is hampered by limited knowledge of the mechanisms of activation and identity of endogenous regulators. We have revealed that one of the TRPC channels, TRPC5, is strongly activated by common endogenous lysosphospholipids including lysosphatidylcholine (LPC) but, by contrast, not arachidonic acid. Although TRPC5 was stimulated by agonists at G-protein-coupled receptors, TRPC5 activation by LPC occurred downstream and independently of G-protein signaling. The effect was not due to the generation of reactive oxygen species or because of a detergent effect of LPC. LPC activated TRPC5 when applied to excised membrane patches and thus has a relatively direct action on the channel structure, either because of a phospholipid binding site on the channel or because of sensitivity of the channel to perturbation of the bilayer by certain lipids. Activation showed dependence on side-chain length and the chemical head-group. The data revealed a previously unrecognized lysosphospholipid-sensing capability of TRPC5 that confers the property of a lipid ionotropic receptor.

TRPC channels are mammalian homologues of the transient receptor potential (TRP) protein found in photoreceptors of Drosophila melanogaster (1–5). There are seven isoforms (TRPC1–7), with all but one being expressed in human. They form mixed conductance cationic (Ca$^{2+}$-, Na$^+$-, and K$^+$-) permeable) channels and are expressed in a wide variety of cell types. The channels have received considerable attention over the past decade, but their mechanisms of activation and endogenous regulators remain quite poorly understood. In this regard, we have focused on studies of TRPC5, an isoform that generates a readily recorded functional signal on overexpression and that has a distinct role demonstrating TRPC5 and TRPM2 expression has been reported previously (7). Some experiments (see Fig. 1c) were on HEK-293 cells stably expressing tetracycline-regulated human TRPC5 (accession number AF054568), as described previously (7). Some experiments (see Fig. 1c) were on HEK-293 cells stably expressing tetracycline-regulated human TRPM2 (accession number NM_003307), as described previously (19). Biochemical evidence demonstrating TRPC5 and TRPM2 expression has been reported previously (7, 19). Cells were grown in Dulbecco’s modified Eagle’s medium–F12 (Invitrogen) medium containing 10% fetal calf serum, 100 units ml$^{-1}$ penicillin, and 100 µg ml$^{-1}$ streptomycin. Cells were maintained at 37°C under 95% air and 5% CO$_2$ and replated on coverslips prior to experiments. The expression of TRPC5 or TRPM2 was induced by 1 µg ml$^{-1}$ tetracycline (Tet+) for 24–72 h before recording. Non-induced cells without addition of tetracycline (Tet–) were used as control.

Arterial Smooth Muscle Cell Isolation—8-week-old male C57/BL6 mice were killed in accordance with the Code of Practice, UK Animals Scientific Procedures Act 1986. Aortae were dissected and incubated in Hanks’ solution containing 0.25 mg ml$^{-1}$ collagenase A, 0.13 mg ml$^{-1}$ protease, 0.18 mg ml$^{-1}$ hyaluronidase, and 0.09 mg ml$^{-1}$ elastase for 2 h at 4°C followed by 10 min at 37°C. Enzymes were removed, and tissue was agitated. Smooth muscle cells were stored at 4°C and used within 5 h.

Calcium (Ca$^{2+}$) and Ionic Current Measurements—Intracellular Ca$^{2+}$ was measured using fura-PE3 and ratiometric fluorescence microscopy as described previously (7) or fluo-4 and single wavelength excitation on a real-time fluorescence 96-well plate reader (Flexstation II 3841, Molecular Devices). For whole-cell patch clamp on HEK-293 cells, the patch pipette contained (in mM): 135 CsCl, 2 MgCl$_2$, 1 EGTA, 10 HEPES, 5 Na$^+$-ATP, and 0.1 Na$^+$-GTP (when specified), titrated to pH 7.2 with CsOH. For outside-out patch experiments and for whole-cell experiments on smooth muscle cells, the pipette contained (in mM): 35 CsCl, 30 EGTA, 10 HEPES, 22.5 CaCl$_2$ (200 nm free), 8.05 MgCl$_2$ (2 mm free), and 0.1 or 1 lithium GDP-$\beta$-S (pH 7.4). Whole-cell dialysis
occurred for at least 10 min prior to testing an external agent. The recording medium for Ca$^{2+}$ measurements, whole-cell recordings, and outside-out patch recordings was “standard bath solution” containing (in mM): 130 NaCl, 5 KCl, 8 D-glucose, 10 HEPES, 1.2 MgCl$_2$, 1.5 CaCl$_2$, titrated to pH 7.4 with NaOH. For inside-out patch experiments, the bath solution contained (in mM): 130 CsCl, 1 EGTA, 2 MgCl$_2$, 10 HEPES, 5 sodium ATP, titrated to pH 7.2 with CsOH; standard bath solution was in the pipette. All recordings were made at room temperature (22–28°C). Gadolinium (Gd$^{3+}$) was included to block background signals (7) where indicated, although the contribution from these signals was generally small (e.g. see Fig. 3f). The recording chamber was continuously perfused, and complete bath exchange took 1–2 min after turning the valve to the reservoir containing the test substance. In the figures, the horizontal bars indicate application of a substance starting at the time of switching the valve.

Anti-TRPC5 Antibody Labeling—T5Chk antibody was custom-made in chicken to peptide CVFETWGEACDLLMHKWGDGQ and affinity-purified. Specificity was demonstrated by Western blotting. Tissue was fixed in 4% paraformaldehyde, frozen, and stained using standard methods and a Cy3-conjugated secondary antibody.

Lipid Preparation—The solvent was methanol for LPCs, palmitoleic acid, and 1-α-lyso phosphatidylinositol (LPI) and ethanol for arachidonic acid and vitamin E. The final bath solvent concentration was ≤0.1% and was present in the control period prior to application of the lipid molecule. No effects of solvents were evident (e.g. see Fig. 1a). 1-α-caproyl lysophosphatidylcholine (LPC C6:0), 1-α-lauroyl lysophosphatidylcholine (LPC C12:0), 3-sn-1-myristoyl-lysophosphatidylcholine (LPC C14:0), 1-α-palmitoyl lysophosphatidylcholine (LPC C16:0), 3-sn-1-oleoyl-lysophosphatidylcholine (LPC C18:1), LPI (soybean), natural lysophosphatidylcholine from bovine brain, palmitoleic acid, choline (2-hydroxyethyl)trimethylammonium hydroxide), arachidonic acid, and fatty acid-free bovine serum albumin were from Sigma or Avanti Lipids.

Data Analysis—All mean Ca$^{2+}$ imaging data are from at least three independent experiments (coverslips), and numbers of cells are given in the figure legends. For patch clamp data, n is the number of independent cells. In each case, the error bars are S.E. Statistical comparisons of two sets of data were made using Student’s t test for paired or unpaired data, as appropriate. Statistically significant difference is indicated by probability (p) values of <0.01 (*) or <0.05 (**).

RESULTS AND DISCUSSION

Phospholipid Sensitivity of Ca$^{2+}$ Signals in TRPC5-expressing Cells—The tetracycline (Tet)-inducible cDNA expression system enables comparison of the same batch of cells with (Tet+) and without (Tet−) TRPC5 (7). Ca$^{2+}$ permeability of TRPC5 allows its activity to be detected by the fluo-4 intracellular Ca$^{2+}$ indicator dye on a 96-well real-time fluorescence platform. Here we see that LPC and the related lipid LPI evoke responses in TRPC5-expressing cells but not the controls and that the solvent for LPC and LPI, methanol, has no effect (Fig. 1, a and b). LPC and LPI responses have the same magnitude, but the LPI response has faster onset and some subsequent decay. There is specificity for TRPC5 because the hydrogen peroxide- and arachidonic acid-activated TRPM2 channel (19, 20) is not stimulated by the lysophospholipids (Fig. 1c). For further and more quantitative analysis, we used microscopy-based single-cell imaging with the ratiometric Ca$^{2+}$ indicator dye fura-PE3. Similarly, LPC is an activator. Responses developed over a period of a few minutes (Fig. 2a) and were sustained for up to 20 min (data not shown). LPC and LPI have different head-groups, choline and inositol, but evoke responses of the same magnitude (Fig. 2b). The above experiments used LPC C16:0. In contrast, LPC with a shorter side chain (LPC C6:0) has no effect (Fig. 2b). Phospholipase A$_2$ enzymes hydrolyze phosphatidylcholine to yield LPC and arachidonic acid. This other product, arachidonic acid, fails to activate TRPC5 (Fig. 2b).

Basic Characteristics of the Effect of LPC—The response of TRPC5-expressing cells to LPC was lost in the absence of extracellular Ca$^{2+}$, showing that the effect relates to TRPC5-dependent Ca$^{2+}$ influx (Fig. 2, c and d). LPC contains a choline group, choline is an agonist at muscarinic receptors, and muscarinic agonists activate TRPC5 in HEK-293 cells. However, choline has only a minor role in the effect of LPC because LPI mimics the effect of LPC (Figs. 1 and 2; see also Fig. 4a), and there is substantial resistance of the LPC response to the muscarinic antagonist atropine (Fig. 2d). LPC may generate reactive oxygen species (17) and act via such intermediates. However, TRPM2 is activated by reactive oxygen species (20) but not LPC (Fig. 1c), and activation of TRPC5 by LPC is unaffected by vitamin E, a scavenger of reactive oxygen species (Fig. 2d). The prefix “lyso” in LPC refers to the property of...
this substance to cause cell lysis by what is presumed to be a detergent effect on the membrane. The primary evidence against the LPC effect reflecting such a detergent action is that responses did not occur in the control (Tet –) cells (Figs. 1–5). Secondly, for comparison, the expected nonspecific chemical detergent effect can be demonstrated by using Triton X-100 (Fig. 2e). These experiments show the detergent response in control (Tet –) cells and also reveal that Tet + cells have a weaker response, which is opposite to that found for LPC. The mechanism for this protective effect of TRPC5 is unknown, but the data showed that TRPC5 does not inadvertently increase sensitivity of the cells to chemical detergent.

**Electrophysiology, TRPC5 Character and Chemical Requirements**—Patch clamp is a powerful analytical technique for ion channel studies, allowing observation of the signature characteristics of TRPC5. Whole-cell recordings in voltage clamp mode show that LPC activates current in TRPC5-expressing cells with a time course (Fig. 3a) similar to that found in Ca²⁺ imaging experiments (Fig. 2c). Fig. 3b shows that the evoked current has the fingerprint-inflected TRPC5 current-voltage relationship (I-V) (7, 8, 11, 12). Given the ability to definitively identify activated TRPC5, we used this approach to further elucidate chemical requirements for activation. Consistent with data in Fig. 2b, LPC chain length is important; TRPC5 activation occurs with chain lengths greater than 12 (Fig. 3c). Choline (the head-group of LPC) has no effect (Fig. 3d). Without the head-group, LPC is palmitic acid, but because of poor solubility, we used palmityl acid, which is the same except for one unsaturated bond. Palmitoleic acid had a weak and statistically insignificant activating effect on TRPC5 (Fig. 3e). These data suggest that the combination of the head-group and side chain of sufficient length is important for activation. The dependence on chain length implies that lipid solubility is a requirement.

**LPC Acts Independently of G-protein Signaling**—Because TRPC5 is activated by agonists at G-protein-coupled receptors (8, 10, 11) and there are G-protein-coupled receptors for LPC (14), it is necessary to explore whether LPC acts via one of these receptors endogenously expressed in HEK-293 cells. However, the amplitude of the response to LPC is much bigger than that to activation of endogenous muscarinic receptors by carbachol (Fig. 3f), and robust LPC responses occur without GTP or ATP in the patch pipette, a condition unfavorable for G-protein signaling; for example, with such a pipette solution, carbachol responses failed in five of nine cells within one batch of experiments, whereas the LPC C16:0 response occurred in every cell. Furthermore, we had observed in Ca²⁺ imaging experiments that LPC responses are unaffected by pretreatment of cells with pertussis toxin, an inhibitor of Gαi/o proteins (p > 0.05, n = 30 cells and six independent experiments). Therefore, we focused on the hypothesis that LPC is acting on TRPC5 independently of G-protein signaling. To investigate the role of G-proteins, LPC responses were compared with and without GDP-β-S in the pipette because GDP-β-S is a hydrolysis-resistant analogue of GDP that locks G-proteins in the inactive state. Consistent with our hypothesis, the LPC response is unaffected by intracellular dialysis with GDP-β-S (Fig. 3f). The response to carbachol is, however, abolished, showing a marked contrast between the effects of the two agonists (Fig. 3f). There
associated with the channel. We therefore explored whether LPC is also effective at the inner face of the membrane, using inside-out patch recording. There is activation (Fig. 4, c and d). The small physical size of inside-out patches has the advantage of enabling single channel recordings, and these revealed LPC-evoked unitary current events with conductance as reported previously for TRPC5 (Fig. 4, e and f) (10). Because outside-out and inside-out patches are detached from the cell, the presence of LPC responses in these patches not only suggests a relatively direct action on the channels but also rules out forward trafficking of vesicles (6) as the mechanism underlying the action of LPC.

**Activation by Physiological Lipid**—We hypothesized that activation of TRPC5 by LPC C16:0 might be physiologically important because C16:0 is a common chain length of natural LPC (14, 15). Determination of the concentration-response curve for LPC C16:0 shows effects in the concentration-range of 0.1–10 μM (Fig. 5a) and thus shows that LPC is effective at the lower end of the 5–180 μM concentration range reported for physiological systems (14, 16). To further explore the relevance, we considered that native LPC is not only LPC C16:0 but a mixture of chain lengths. Testing an isolate of natural LPC shows that it too is a strong TRPC5 activator (Fig. 5b). Native LPC, in some contexts, is also in dynamic equilibrium between free and protein-bound forms. To mimic this effect, natural LPC was tested in the continuous presence of fatty acid-free albumin. The effect of LPC was retained, although reduced in amplitude (Fig. 5c), consistent with there being a lower free LPC concentration. Therefore, the data support the notion of LPC as a physiological activator of TRPC5.

**Native Cells Endogenously Expressing TRPC5**—We further explored the potential relevance of the LPC effect by focusing on freshly isolated vascular smooth muscle cells because of the roles phospholipids play in cardiovascular diseases such as atherosclerosis (17). Complementary to mRNA studies (4, 21), we show that TRPC5 protein exists in smooth muscle cells of native mouse aorta (Fig. 5, d and e). We also show that these cells have a previously unrecognized Gd3⁺-resistant and G-protein-independent response to LPC C16:0 (Fig. 5f), occurring with a time course similar to that found for TRPC5 activation in HEK cells (Fig. 3a). Current occurred in 7 out of 12 smooth muscle cells and averaged $-207.3 \pm 83.4$ pA at $-80$ mV, reversing near 0 mV, as for TRPC5. The I-V is relatively linear (data not shown), which suggests that it is not explained by TRPC5 alone but by a heteromultimeric assembly, consistent with expression of additional TRP channels in smooth muscle cells (4). Native HEK-293 cells do not share the Gd3⁺-resistant LPC response (Fig. 3f), possibly because there is lack of native TRPC5 expression (22). It is an attractive idea that lysophospholipids might represent a point of convergence of the diverse general signals already known to stimulate TRPC5, including muscarinic receptor-activation and store depletion (7), both of which have the capability to stimulate phospholipase A₂ and generate LPC (23, 24). Consistent with this idea, study of native store-operated channels has shown activation by LPC or LPI and suggested that these lipids are downstream of phospholipase A₂ in the coupling mechanism between stores and store-operated channels (25).

**Mechanism of Action of LPC**—Primary findings of this study are that LPC is a novel activator of TRPC5 and that this occurs through a relatively direct action on the channel. There is a strong suggestion that the effect does not occur via a G-protein-coupled receptor or free radicals and does not require significant co-factors because the effect is preserved in the sparse environment of excised membrane patches. The effect has specificity because TRPM2 channels are not activated, and chemical detergent and arachidonic acid do not mimic the effect of LPC on TRPC5. Therefore, it is our hypothesis either that LPC is a direct agonist at the TRPC5 channel complex or that TRPC5 has sensitivity to

### FIGURE 3. Chemical requirements and G-protein-independence

Experiments were on HEK-TRPC5 cells with or without induction by Tet studied by whole-cell patch clamp in voltage clamp mode. a, typical time series plot for the response to bath-applied 10 μM LPC C16:0. b, current-voltage relationships (I-V) were constructed using a 1-s voltage ramp applied every 10 s from a holding voltage of −60 mV. Currents are shown for just before (0 min) and 2 min after starting application of 10 μM LPC C16:0, c, mean maximum responses to specified agents (n = 6–10). LPC C18:1 is poorly soluble, and so LPC C18:1 was used. d, paired comparison showing the lack of effect of 10 μM choline but effect of 10 μM LPC in the same cells (n = 5). e, paired comparison showing the lack of effect of 10 μM palmitoleic acid (palmit acid) but effect of 10 μM LPC in the same cells (n = 10). f, mean data for 10 μM LPC C16:0-evoked current at −80 mV: (i) without 1 mM GDP-β-S in the pipette or 10 μM GDP-β-S in the bath and with or without induction of TRPC5 expression by tetracycline (Tet+/-, 9/12 cells); (ii) without GDP-β-S, with Gd3⁺ and with or without TRPC5 expression (Tet+/-, 11/5 cells); (iii) with GDP-β-S and Gd3⁺ and with or without TRPC5 expression (Tet+/-, 5/5 cells). Carbachol (CCh, 100 μM) responses were measured in Tet+ cells with 1 mM GDP-β-S (5 cells) or 0.1 mM GTP (5 cells) in the patch pipette.
FIGURE 4. Effect of lysophospholipids on TRPC5 in excised membrane patches. a and b, outside-out patch with GDP-β-S in the pipette; c–f, inside-out patches all from TRPC5-induced cells; a–d, patch pipettes had low resistance to enable detection of the macroscopic I-V, rather than single channel currents; e and f, patch pipettes had higher resistance to enable detection of single channel, unitary current, events. a, out of four recordings from Tet+ cells, an example of the TRPC5 I-V activated after 2 min of bath-applied 10 μM LPI. b, mean data showing current amplitude induced by 10 μM LPC C16:0 at −80 mV. The bath solution contained 10 μM Gd3+, and patches were pulled from cells with (Tet+, 5 cells) or without (Tet−, 5 cells) induction of TRPC5 expression. c, out of nine recordings, an example time series plot showing activation by bath-applied 10 μM LPC C16:0, as in c, but showing the characteristic TRPC5 I-V. e, example of original single channel events evoked by 10 μM LPC C16:0, as for e, but mean unitary current amplitudes from three patches fitted with a straight line indicating a channel conductance of 39 pS, which is characteristic of TRPC5 (8, 10).

FIGURE 5. Activation by physiological lysophospholipids and in native cells. a–c, mean fura-PE3 Ca2+ imaging data for TRPC5-expressing (Tet+) or control (Tet−) cells. a–c and f, gadolinium (10 μM) was continuously present. a, for Tet+ cells, concentration response curve for LPC C16:0 responses after 10 min (24–130 cells per point); >10 μM LPC was not tested because such concentrations risk cell lysis. b and c, mean responses to 10 μM natural LPC from bovine brain with (b, 149/129 Tet+ cells) or without (c, 98/147 Tet− cells) preincubation and the continuous presence of 5 μM fatty-acid-free bovine serum albumin. d and e, cross-section of part of the mouse aorta (lumen to the left) stained with T5Chk anti-TRPC5 antibody (d) or as a control in the absence of primary antibody (e), showing endogenous expression of TRPC5 in the smooth muscle cells (red staining, arrow). f, typical whole-cell recording from a freshly isolated aortic smooth muscle cell showing the effect of bath-applied 10 μM LPC C16:0 starting 10 min after breaking the cell-attached patch to start whole-cell recording. The patch pipette contained 1 mM GDP-β-S.
the structure of the lipid bilayer such that it is activated when the concentration of certain lipids, such as LPC, increases in the bilayer. Distinguishing between these two concepts is a considerable challenge. Nevertheless, our data have raised intriguing questions about the underlying mechanism of activation. The poor effects of arachidonic and palmitoleic acids indicated that it may not be a simple matter of insertion of a long carbon side chain in the bilayer. That LPC and LPI are more effective suggests that the side chain needs a bulky head-group, implying that physical distortion of the bilayer is involved. Such a hypothesis could be reconciled with the excised patch data if, in this context, LPC has the capacity to flip to the other side of the bilayer surprisingly rapidly; that is, within a few minutes. Alternatively, the mechanisms of the intracellular and extracellular effects may be different. Future detailed studies of the chemical requirements will resolve such issues.

**General Conclusion**—We suggest a novel concept for TRPC5 as a direct sensor of lysophospholipids, a lipid ionotropic receptor, stepping it aside from the mystique surrounding its controversial store- and receptor-operated properties (1, 7) and into the expanding picture of TRP channels as sensors of lipid signals: TRPV1 and anandamide (26), TRPV4 and 5′,6′-epoxyeicosatrienoic acid (27), TRPM8 and menthol (28), and TRPA1/ANKT1M1 and cannabinoids (29).

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