A Sphingosine-1–Phosphate–Activated Calcium Channel Controlling Vascular Smooth Muscle Cell Motility

Shang-Zhong Xu,* Katsuhiko Muraki,* Fanning Zeng,* Jing Li,* Piruthivi Sukumar,* Samir Shah, Alexandra M. Dedman, Philippa K. Flemming, Damian McHugh, Jacqueline Naylor, Alex Cheong, Alan N. Bateson, Christopher M. Munsch, Karen E. Porter David J. Beech

Abstract—In a screen of potential lipid regulators of transient receptor potential (TRP) channels, we identified sphingosine-1–phosphate (S1P) as an activator of TRPC5. We explored the relevance to vascular biology because S1P is a key cardiovascular signaling molecule. TRPC5 is expressed in smooth muscle cells of human vein along with TRPC1, which forms a complex with TRPC5. Importantly, S1P also activates the TRPC5–TRPC1 heteromeric channel. Because TRPC channels are linked to neuronal growth cone extension, we considered a related concept for smooth muscle. We find S1P stimulates smooth muscle cell motility, and that this is inhibited by E3-targeted anti-TRPC5 antibody. Ion permeation involving TRPC5 is crucial because S1P-evoked motility is also suppressed by the channel blocker 2-aminoethoxydiphenyl borate or a TRPC5 ion-pore mutant. S1P acts on TRPC5 via two mechanisms, one extracellular and one intracellular, consistent with its bipolar signaling functions. The extracellular effect appears to have a primary role in S1P-evoked cell motility. The data suggest S1P sensing by TRPC5 calcium channel is a mechanism contributing to vascular smooth muscle adaptation. (Circ Res. 2006;98:0-0.)

Key Words: vascular smooth muscle ■ vein ■ sphingosine-1–phosphate ■ transient receptor potential ■ calcium channel

Sphingosine-1-phosphate (S1P) has emerged as a major endogenous signaling phospholipid with diverse roles in yeast, plants, and mammals.1 Proposed functions include the regulation of cell proliferation, migration, programmed death, and pathological processes including cancer, asthma, inflammation, and trauma. There has been particular interest in the role of S1P in the cardiovascular system, where it accumulates in atherosclerotic lesions and plays a role in ischemic preconditioning of the heart.2–4 S1P is derived from the phosphorylation of sphingosine catalyzed by sphingosine kinase, sphingosine being from ceramide and ceramide from sphingomyelin, a constituent lipid of signaling microdomains of plasma membrane lipid rafts and caveolae.5 S1P is detected in serum at almost 1 mol/L, although protein binding impacts on the available concentration and local concentrations may vary substantially.6

S1P is quite unusual among signaling molecules in having separate intracellular and extracellular effects.1,4,7,8 It affects vascular smooth muscle cell migration,9,10 evokes contraction of rat mesenteric artery,11 and slows pacemaker activity of the sino-atrial node of the heart.12 The underlying mechanisms are only partially worked out, but vascular smooth muscle cells respond to S1P with transient followed by sustained elevation of the cytosolic Ca2+ concentration,10,11,13,14 whereas cardiac myocytes show activation of potassium current and S1P-evoked “Ca2+ deregulation,” depending on extracellular Ca2+.12,15 Despite positive effects on Ca2+ signaling, the molecular basis of a Ca2+ channel stimulated by S1P is unknown. L-type voltage-gated Ca2+ channels are inhibited by S1P.12

The Drosophila transient receptor potential (TRP) channel has provided the foundation for discovery of many novel Ca2+- or Na+-permeable plasma membrane channels,16,17 which are candidates for the less well understood cationic channels of the mammalian cardiovascular system.18–23 Searches for activation mechanisms are revealing TRP channels as sensors of temperature, pheromones, osmolarity, and gustatory stimuli.24,25 However, some TRP channels are expressed outside sensory systems, and activation mechanisms are elusive.17,20 TRPC5 has been associated with the central nervous system and is a regulator of growth cone formation.26–28 There is rapid vesicular insertion regulated by growth factors,29 but this is not the mechanism causing channel opening. TRPC5 may be important outside the

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nervous system because its mRNA species is detected in a range of animal tissues, including human heart and blood vessels.\textsuperscript{20,30–32} Furthermore, downregulation of Ca\textsuperscript{2+}-ATPase in cardiac myocytes leads to compensatory upregulation of TRPC5.\textsuperscript{33} However, activation signals for TRPC5 remain uncertain. One possibility is that TRPC5 exists to respond to passive depletion of Ca\textsuperscript{2+} stores because human TRPC5 activity is enhanced by store depletion,\textsuperscript{34} and vascular smooth and cardiac muscle cells exhibit store-operated Ca\textsuperscript{2+} entry.\textsuperscript{20,35,36} However, in some instances, TRPC5 is unresponsive to store depletion,\textsuperscript{37} and the biological relevance of the often strong passive store depletion in experimental situations remains uncertain. On the assumption that key endogenous regulators of TRPC5 were yet to be discovered, we searched for novel activators.

**Materials and Methods**

**Human Tissue**

Freshly discarded human tissue samples were obtained anonymously and with informed consent from patients undergoing open heart surgery in the general infirmary at Leeds. Approval was granted by the Leeds teaching hospitals local research ethics committee. Saphenous vein was transported to the laboratory in Hanks’ solution (in mmol/L): 137 NaCl, 5.4 KCl, 0.01 CaCl\textsubscript{2}, 0.34 NaH\textsubscript{2}PO\textsubscript{4}, 0.44 K\textsubscript{2}HPO\textsubscript{4}, 8 D-glucose, and 5 HEPES) and processed on the day of the operation. For RNA isolation and cell culture, medial layer was homogenized (Polytron; 2 minute) and 1% BSA with 0.1% Triton X-100 for 1 hour. Incubation in primary antibody was for 2 hours and secondary antibody (goat anti-rabbit IgG-Cy3) for 1 hour. Western blotting protocols were similar to those described.\textsuperscript{18} Small pieces of tissue were placed in PBS containing protease inhibitor cocktail (Sigma) and lysed in Laemmli buffer containing 320 mM dithiothreitol at 80°C to 100°C (15 minutes). Proteins were separated on 8% SDS-PAGE gels, transferred to nitrocellulose membrane (Millipore), and probed with primary antibody and secondary antibody conjugated with horseradish peroxidase. Membranes were washed with PBS and labeling detected by ECL plus (Amersham Pharmacia Biotech). Except for T5Chk, anti-TRPC antibodies were custom-made in rabbit (Sigma-Genosys) to unique peptides. T5Chk antibody was made in chicken to the C-terminal peptide CVFETWGEACDLMHKWGDDQ. T5C3 was made to the C-terminal peptide KLQDDSDYFYETWGE and T5E3 to CYETRAIDEPNNCKG. Unless indicated, antibodies were affinity purified on columns containing immobilized peptides and dialysed in PBS. T1E3 antibody is described.\textsuperscript{18} Rabbit anti-protein S100 and the monoclonal antibody anti-CD31 were from Dako Ltd. For immunoprecipitation (IP) of saphenous vein, medial layer was homogenized (Polytron; 2 minute) and 1% BSA with 0.1% Triton X-100 for 1 hour. Incubation with primary antibody was for 2 hours and secondary antibody conjugated with horseradish peroxidase. Membranes were washed with PBS and labeling detected by ECL plus (Amersham Pharmacia Biotech). Except for T5Chk, anti-TRPC antibodies were custom-made in rabbit (Sigma-Genosys) to unique peptides. T5Chk antibody was made in chicken to the C-terminal peptide CVFETWGEACDLMHKWGDDQ. T5C3 was made to the C-terminal peptide KLQDDSDYFYETWGE and T5E3 to CYETRAIDEPNNCKG. Unless indicated, antibodies were affinity purified on columns containing immobilized peptides and dialysed in PBS. T1E3 antibody is described.\textsuperscript{18} Rabbit anti-protein S100 and the monoclonal antibody anti-CD31 were from Dako Ltd. 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**cDNA Expression**

Full-length human TRPC5 cDNA (accession number AF054568) was cloned and stably expressed in human embryonic kidney 293 cells (HEK 293 cells; T-Rex cells; Invitrogen).\textsuperscript{34} Cells were grown in DMEM–F12 media (Invitrogen) and supplemented with 10% FBS and penicillin/streptomycin at 37°C in a 5% CO\textsubscript{2} incubator. Where indicated, cells were incubated with tetracycline (Tet; 1 μg/mL) for 24 to 48 hours to induce the expression of TRPC5. Human TRPC1 cDNA (accession number X89066; a gift from C. Montell) was subcloned into EcoRV site of the bicistronic vector pRES (E) yellow fluorescent protein (YFP; BD Clontech), transiently transfected into cells with FuGene 6 (Roche), and subcultured onto coated glass coverslips 24 hours later. Cells were then cultured for an additional 24 hours with or without the presence of 1 μg/mL of tetracycline. Dominant-negative (DN) TRPC5 is a triple alanine mutation of the conserved LFW sequence in the ion-pore.\textsuperscript{26} DN function was confirmed in HEK–TRPC5 cells (supplemental Figure I, available online at http://circres.ahajournals.org).

**Immunofluorescence, Western Blotting, and Immunoprecipitation**

Cells adhered to polylysine-coated slides were fixed in 2% paraformaldehyde (30 minutes) and immersed in −20°C methanol (1 minute) and 1% BSA with 0.1% Triton X-100 for 1 hour. Incubation in primary antibody was for 2 hours and secondary antibody (goat anti-rabbit IgG-Cy3) for 1 hour. Western blotting protocols were similar to those described.\textsuperscript{18} Small pieces of tissue were placed in PBS containing protease inhibitor cocktail (Sigma) and lysed in Laemmli buffer containing 320 mM dithiothreitol at 80°C to 100°C (15 minutes). Proteins were separated on 8% SDS-PAGE gels, transferred to nitrocellulose membrane (Millipore), and probed with primary antibody and secondary antibody conjugated with horseradish peroxidase. Membranes were washed with PBS and labeling detected by ECL plus (Amersham Pharmacia Biotech). Except for T5Chk, anti-TRPC antibodies were custom-made in rabbit (Sigma-Genosys) to unique peptides. T5Chk antibody was made in chicken to the C-terminal peptide CVFETWGEACDLMHKWGDDQ. T5C3 was made to the C-terminal peptide KLQDDSDYFYETWGE and T5E3 to CYETRAIDEPNNCKG. Unless indicated, antibodies were affinity purified on columns containing immobilized peptides and dialysed in PBS. T1E3 antibody is described.\textsuperscript{18} Rabbit anti-protein S100 and the monoclonal antibody anti-CD31 were from Dako Ltd. For immunoprecipitation (IP) of saphenous vein, medial layer was homogenized (Polytron; 2 minute) and 1% BSA with 0.1% Triton X-100, 150 mM NaCl, and protease inhibitor cocktail. Homogenate was centrifuged at maximum speed in a microfuge for 20 minutes to remove cell debris. The

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**Figure 1. Identification of S1P as a novel activator of human TRPC5.** a, Tetracycline-inducible expression of human TRPC5 in HEK 293 cells. T5C3 antibody detected (red labeling) TRPC5 in induced (Tet+) but not control (Tet−) cells. Cell nuclei were counterstained with 4’,6-diamidino-2-phenylindole DAPI (blue). For clarity, the Tet− anti-TRPC5 stain is shown without the DAPI overlay. Bar=10 μm. b, Typical single cell responses to S1P at 1, 3, and 10 μmol/L in cells expressing TRPC5 (Tet+). Control cell (Tet−) failed to respond to 3 μmol/L S1P. c, As in b, but mean data as the change in Ca\textsuperscript{2+} indicator fluorescence ratio from the baseline. d, Mean data for responses 5 and 11 minutes after starting the application of 0.1 μmol/L S1P.
supernatant was washed with Protein A (Sigma) or G (Pierce) sepharose (10 μg) and centrifuged again at maximum speed for 20 minutes. The supernatant (200 μg soluble protein) was used for IP with 2 μg antibody and the mixture rotated overnight at 4°C.

Reverse Transcription–Polymerase Chain Reaction
For RT-PCR, see the online supplement, available at http://circres.ahajournals.org.

Ca2+ Imaging
HEK 293 cell recordings were made using fura-PE3 AM and Ca2+ imaging, whereas smooth muscle cell recordings used a 96-well fluorimetry. Recordings were in standard bath solution containing (in mmol/L): 130 NaCl, 5 KCl, 8 d-glucose, 10 HEPES, 1.2 MgCl2, and 1.5 CaCl2, pH titrated to 7.4 with NaOH. For Ca2+-free solution, CaCl2 was omitted (also see online supplement).

Electrophysiology
For whole-cell experiments and outside-out patches, the patch pipette contained (in mmol/L): 135 CsCl, 2 MgCl2, 1 EGTA, 10 HEPES, 5 sodium ATP, titrated to pH 7.2 with CsOH (0.1 mmol/L sodium GTP or 1 mmol/L GDP–S was added when specified). Standard bath solution was used. For inside-out patch experiments, the patch pipette solution contained standard bath solution and the bath (superfusion) solution (in mmol/L): 135 CsCl, 2 MgCl2, 1 EGTA, 10 HEPES, and 5 sodium ATP, titrated to pH 7.4 with NaOH (also see online supplement).

Cell Motility
Human saphenous vein smooth muscle cells were prepared by explant technique and passaged up to 4 times. Cells were grown to confluence in 24-well plates and DMEM supplemented with 10% FBS and penicillin/streptomycin. Cultures were maintained at 37°C in 5% CO2. Cells were harvested with PBS, and a linear scrape of 0.3 mm width was made through the monolayer with a pipette tip. The cells were washed twice with PBS and a linear scrape of ~0.3 mm width was made through the monolayer with a pipette tip. The cells were cultured with DMEM containing 1% serum and with or without S1P (1 μmol/L). TSE3 antibody (10 μg/mL), 75 μmol/L 2-aminoethoxydiphenyl borate (2-APB), or vehicle (methanol or PBS), as specified. After 24 hours, the linear wound was delineated and the number of cells moving into the wound counted. For DN-TRPC5 studies, 3 μg of DNA was delivered to cells 48 hours before making the linear wound using the Basic Nucleofector Kit for primary smooth muscle cells (Amazx Biosystems). Transfection efficiency was ≥70%. For pertussis toxin (1 μg/mL) experiments, preincubation was for 4 hours before application of S1P. As a control, pertussis toxin was boiled for 10 minutes before use.

Reagents
Unless indicated, salts and reagents were from Sigma or BDH (British Drug House). Sphingosine-1–phosphate (S1P) was purchased from Sigma or Biomol Research Laboratories. The solvent was methanol, and stock concentrations were 10 mmol/L. The final methanol concentration in the bath (superfusion) solution was ≤0.1% (v/v), and this was kept constant throughout (before, during, and after S1P application). No effects of methanol were observed. Lithium GDP–β-S, pertussis toxin, and 2-APB were from Sigma. U73122 (1-β-[6-[(17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino]hexyl]-1H-pyrollo-2,5-dione) and U73343 were from Calbiochem.

Data Analysis
Human tissue/cell experiments were repeated on samples from at least three patients, yielding similar results. Averaged numerical data are presented as mean±SEM. Data sets with two groups were compared by unpaired Student t test and three groups by Tukey–Kramer multiple comparison, with significance indicated by P<0.05 (*), 0.01 (**), or 0.001 (**). For Ca2+ imaging, all mean data were based on four independent experiments (coverslips) and measurements from >30 cells on each coverslip. For patch-clamp experiments, n is the number of independent experiments on cells or patches. For the injury assay, n is the number of images used for analysis. Direct comparisons were made on the same batch of cells, with test and control experiments on the same day.
**Results**

**S1P Is a Novel Activator of TRPC5**

To test for novel activators, human TRPC5 was stably expressed in HEK 293 cells under a tetracycline-dependent promoter. The system gave a defined TRPC5 signal in which TRPC5-expressing cells (tetracycline-induced; Tet+) were compared directly with control cells from the same batch: cells that do not express TRPC5 (Tet−) as shown by anti-TRPC5 antibody (Figure 1a). We noticed stimulation by S1P in Tet+ but not Tet− cells (Figure 1b through 1d). Within a 5-minute application period, S1P was effective at 1 to 10 μmol/L. S1P (0.1 μmol/L) was near the threshold for activation, producing a slow but significant effect (Figure 1d).

**TRPC5-TRPC1 Heteromultimer in Human Vein and Activation by S1P**

Because S1P is a cardiovascular signal, we looked for relevant expression of TRPC5, focusing on human saphenous vein, which is a coronary artery bypass graft prone to failure attributable to unwanted smooth muscle cell growth. RNA encoding TRPC5 was detected, as in human brain (Figure 2a). Because there is strong evidence TRPC1 forms a functional heteromultimeric channel with TRPC5,27 we also looked for RNA encoding TRPC1 in the same samples; both RNA species were detected (Figure 2b). Furthermore, TRPC5 and TRPC1 proteins were present in subendothelial smooth muscle cells of the pre-existing venous intima and in smooth muscle cells of vaso vasorum (Figure 2c and 2d; supplemental Figure II).38 This is important because ion channels form independently of Ca^{2+} release (Figure 4b). To explore cell motility, we made a linear deletion in the culture and measured the number of cells moving into the vacated space over a 24-hour period (Figure 4c). S1P strongly enhanced the movement of cells (Figure 4d).

**E3 targeting can be used to design antibodies that block channels specifically when added to extracellular solution.**42 Such an antibody was described for TRPC5 (T5E3).42 T5E3 inhibited the effect of S1P on motility (Figure 4d), showing endogenous TRPC5 is involved.

Highly specific chemical blockers of TRPC5 have not been discovered, but 2-APB is probably the best option39 and importantly had an inhibitory effect like T5E3 (Figure 4d).
2-APB blocks ion permeation in TRPC5, and thus effectiveness of this agent suggests S1P-evoked motility depends critically on ion flux through the TRPC channel complex. As an independent test of this idea, we made an ion-pore mutant of TRPC5 that fails to pass current and acts as a DN (supplemental Figure I), presumably because it damages ion permeation by entering in the heteromultimeric complex. Transfection of this mutant into vascular smooth muscle cells inhibited S1P-evoked motility (Figure 4e) consistent with the necessity of ion permeation.

Mechanism of Action of Extracellular S1P

To further understand the effect of S1P, we explored its mechanism of action, initially hypothesizing that it might act relatively directly, like lysophosphatidylcholine. To make a definitive analysis of TRPC5, we focused on the TRPC5-expressing (Tet+) HEK 293 cells. In the absence of extracellular Ca\(^{2+}\), there was little response to S1P, consistent with S1P activating TRPC5-mediated Ca\(^{2+}\) influx (Figure 5a and 5f). Furthermore, the S1P effect was not prevented by depletion of calcium stores by thapsigargin (Figure 5b and 5f). In control cells in the absence of a lanthanide, we detected small S1P-evoked Ca\(^{2+}\) release signals (data not shown), suggesting that the lanthanide diminished the Ca\(^{2+}\) release event, but also that the cells express G-protein–coupled receptors for S1P. Often, such receptors couple to their effectors via pertussis toxin-sensitive Gi/o GTP-binding proteins. Treatment of cells with pertussis toxin inhibited the S1P response (Figure 5c; compare with Figure 5d; Figure 5f). In contrast, TRPC5 activation by carbachol acting at endogenous muscarinic receptors was unaffected. S1P receptors also couple to phospholipase C (PLC). Consistent with PLC involvement, the PLC inhibitor U73122 inhibited the S1P response (Figure 5e and 5f), whereas the chemically related U73343 (a poor inhibitor of PLC) was ineffective (2 independent experiments).

S1P also activates TRPC5 in whole-cell voltage-clamp recordings, showing the characteristic double-rectifying I-V relationship (Figure 5g). Consistent with G-protein involvement, the effect of S1P was prevented when GDP-β-S replaced GTP in the patch pipette (Figure 5h). Therefore, extracellular S1P activates TRPC5 via a pertussis toxin-sensitive G-protein pathway, indicating receptor activation of TRPC5. The signal after PLC is unknown.
Ionotropic Receptor for Intracellular S1P

Based on the concept of S1P as both an extracellular and intracellular signaling molecule,7,8 we explored whether there is also an intracellular effect on TRPC5. Inside-out membrane patches were used, enabling exposure of only the intracellular face of the channel to the test agent. Unitary events were detected in response to S1P in Tet/H11001 but not Tet/H11002 cells (Figure 6a). Amplitude histograms show the unitary current events evoked by S1P were indistinguishable in size from those evoked by gadolinium (Figure 6b), and the I-V relationship, which is linear for the single channel current, was the same (Figure 6c). An example time series plot for single channel activity shows stimulation by S1P (Figure 6d). Some patches contained multiple channels and thus exhibited macroscopic currents in response to S1P with the characteristic double-rectifying I-V (supplemental Figure IV). Mean data show an effect in Tet+/H11001 but not Tet-/H11002 cells (Figure 6e). In contrast, and also in the absence of GTP, S1P had no effect when bath-applied to outside-out patches, whereas lanthanum, which acts externally,37 activated the channels (Figure 6f).

Effect of Pertussis Toxin on S1P-Evoked Motility

The above data suggest S1P can activate TRPC5 through two mechanisms. Because activation of S1P receptors can lead to accumulation of intracellular S1P and S1P may be transported across the membrane,3 the effect of exogenous S1P (Figure 4c through 4e) could reflect extracellular or intracellular actions of S1P. However, we found pertussis toxin
inhibited the effect of S1P on cell motility (Figure 7), suggesting requirement for the extracellular action of S1P.

**Discussion**

The study identifies a calcium channel activated by S1P. The channel contains TRPC5 protein, which is sufficient for S1P sensitivity and contributes to ion permeation. The finding led us to discover that S1P evokes vascular smooth muscle cell motility via a mechanism involving TRPC5. TRPC5 is natively associated with TRPC1 in these cells, and S1P activates channels formed by heteromultimerization of TRPC5 and TRPC1. This finding does not exclude involvement of additional TRP channels linked to TRPC5/TRPC1 and expressed in vascular smooth muscle.20 The relationship with cell motility may be a general concept for TRPC channels because they also regulate neuronal growth cone extension and turning.28 TRPC5 sensitivity to other regulators34,37,41 suggests these might in turn act via TRPC5 to regulate cell motility. Intriguingly, and paralleling with the dual signaling function of S1P, TRPC5 is a bipolar target for S1P, showing activation by extracellular and intracellular pathways. The extracellular effect involves G-protein–coupled receptor activation, whereas the intracellular effect survives in inside-out membrane patches, suggesting TRPC5 is an ionotropic receptor for intracellular S1P, and providing one of the few known intracellular targets for S1P. Intrigu-
ingly, the extracellular effect of S1P is pertussis toxin sensitive, unlike the carbachol effect. This suggests the agonists act via different signaling cascades, although both would seem to require PLC. Another sphingolipid positively regulating a calcium channel is extracellular sphingosine (but not S1P) activating TRP3.15 This effect is mechanistically distinct from that of S1P on TRPC5 because the lipid and channel are different, the sphingosine effect on TRP3 occurs without involvement of G-proteins, and intracellular sphingosine is ineffective.16

In some studies, TRPC5 shows sensitivity to calcium store depletion,17 and vascular smooth muscle cells have store-operated calcium entry linked to TRPC channels.18,20 Therefore, activation of TRPC5 by store depletion and intracellular S1P could be linked. Indeed, a previous study suggested S1P as a signal coupling depleted calcium stores to channels.44 Similarly, S1P could be an intracellular messenger contributing to TRPC5 activation by extracellular S1P or carbachol because stimulation of G-protein-coupled receptors elevates intracellular levels of S1P.35,46

Through screening TRP channels for sensitivity to lipid signaling molecules, we reveal a previously unappreciated activator of TRPC5 channels as well as a mechanism contributing to cell motility evoked by the widely studied signaling phospholipid S1P. Vascular smooth muscle cell motility has a central role in the formation and adaptation of new arteries and veins, as well as in progression of vascular diseases including atherosclerosis. Therefore, the data identify a novel and potentially important functional component and sensing mechanism in vascular biology.

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References


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Supplementary Information

A sphingosine-1-phosphate activated calcium channel controlling vascular smooth muscle cell motility

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Additional Materials and Methods

Immunoprecipitation (IP) using HEK 293 (tsA201) cells. The lysate was prepared as for saphenous vein except cells were disrupted by sonication in IP buffer with PIC.

Mouse monoclonal anti-FLAG antibody was from Sigma. The tsA-201 cells were co-transfected with N-terminal FLAG™ epitope tagged human TRPC1 (fT1) and mouse TRPC5 (T5, accession number AF029983; a gift from Y Mori) using a calcium phosphate protocol. Cells were harvested for experiments 36 h after transfection. tsA-201 cells are a subclone of HEK-293 cells that expresses the simian virus 40 T antigen (a gift from WA Catterall).

Staining of isolated smooth muscle cells. To isolate single smooth muscle cells from saphenous vein the medial layer was cut into small pieces (~2×3 mm) and incubated at 37 °C for 1 hr in Hanks’ solution containing 2 mg/ml collagenase (Sigma) and 4 mg/ml
papain (Sigma). After 3 washes in Hanks’ solution the mixture was mechanically agitated with a fire-polished glass Pasteur pipette to release cells, which were then fixed and stained. For immunofluorescence staining, cells adhered to polylysine–coated slides were fixed in 2% paraformaldehyde (30 min) and immersed in -20°C methanol (1 min) and 1% BSA with 0.1% Triton X-100 for 1 hour. Incubation in primary antibody was for 2 hr, and secondary antibody (Donkey anti-chicken IgG-CY3, 1:200, Chemicon) for 1 hr. Fluorescent microscope images were processed with Openlab software (Improvision, UK).

**Block of background signals in HEK 293 cells.** Previously we described the use of extracellular gadolinium (Gd$^{3+}$) to reduce background signals\(^1\). In this study, some experiments included Gd$^{3+}$ at 10 µmole/L (whole-cell patch-clamp and 0.1 µmole/L S1P Ca$^{2+}$-imaging experiments) or 25 µmole/L (other Ca$^{2+}$-imaging experiments). The lower Gd$^{3+}$ concentration was used in some experiments in an effort to ensure tonic or Gd$^{3+}$-facilitated TRPC5 activity did not obscure the S1P response. Gd$^{3+}$ was not included for excised patch experiments, unless specified.

**RT-PCR.** Total RNA was isolated from snap-frozen tissue using a standard TriReagent protocol and treated with DNAse I (Ambion). One aliquot of 0.5 µg RNA was used for cDNA synthesis with oligo-dT primed AMV reverse transcriptase (“+RT”) and another was processed in parallel except for the omission of reverse transcriptase (“-RT”). Human brain RNA was from Ambion. PCR primer sequences were (forward and reverse, 5’-3’): TRPC1 (TTAGCGCATGTGGA and
CCACTTACTGAGGCTACTAAT); TRPC5 (i) (GTCATCAAGCAAACGCT and AGGCTAGAGGCATTC); TRPC5 (ii) (ACAACCGACTGAAGGG and CGTAGCACTGAATGGC). Primers were used at 0.5 µmole/L and with 1.5 mmole/L Mg\(^{2+}\). Thermal cycling was 40 cycles of: 94 °C (30 s); 55 °C (45 s); 72 °C (45 s). PCR products were electrophoresed on a 4 % agarose gel containing ethidium bromide.

**Ca\(^{2+}\) imaging.** HEK 293 cells were preincubated with 1 µmole/L fura PE3-AM (Molecular Probes) at 37 °C for 1 h in standard bath solution, followed by a 30 min wash period at room temperature. The equipment was previously described\(^2\). Images were sampled every 10 s and analyzed off-line using regions of interest to select individual cells (Openlab 2 software, Improvision, UK). \([\text{Ca}^{2+}]_i\) is expressed as the ratio of the fluorescence emission intensities for 345 and 380 nm excitation (F ratio). Recordings were made alternately from test and control cells. For saphenous vein smooth muscle cells, Ca\(^{2+}\) was recorded using a 96-well FlexStation (Molecular Devices). Cells were loaded with 2 µmole/L fura 2-AM and 0.01% pluronic acid in standard bath solution for 1 h at 37 °C. Fura 2 was excited at 340/380 nm every 4 s. Where indicated, Ca\(^{2+}\)-stores were depleted by a 0.5-hr pretreatment with 1 µmole/L thapsigargin.

**Electrophysiology.** Voltage clamp was performed at room temperature with the whole-cell or excised patch configuration. Signals were amplified with an Axopatch 200A patch clamp amplifier, controlled with pClamp software 6.0 (Axon Instruments, USA), and sampled at 3 kHz and filtered at 1 kHz. The recording chamber had a volume of 150 µl and superfusion was continuous at 2-4 ml.min\(^{-1}\).
Figure S1: Dominant negative (DN) effect of the TRPC5 LFW mutant. HEK-TRPC5 cells were co-transfected with DN-TRPC5 and pDsRed2 (BD Clontech) 48 h prior to Ca\textsuperscript{2+}-imaging using fura-PE3AM. Expression of wild-type TRPC5 (Tet+) was induced with tetracycline 24 h prior to imaging. Cells were store-depleted in Ca\textsuperscript{2+} free solution containing 1 µM thapsigargin. The store-operated signal (‘store’) was the increase in intracellular Ca\textsuperscript{2+} in response to adding 1.5 mM Ca\textsuperscript{2+} back to the bath solution. The receptor-operated signal (‘receptor’) was the additional response on subsequent addition of 0.1 mM carbachol to activate endogenous muscarinic receptors. Cells without red fluorescence (DsRed2) were assumed to lack DN-TRPC5 and were used as controls. Data are for 9 independent experiments.
Figure S2. Expression of TRPC5 in human saphenous vein smooth muscle cells. (a) Western blotting on proteins from human saphenous vein, showing labeling of the same size of protein by two different anti-TRPC5 antibodies: T5-Chk or T5E3. The protein is slightly larger than the predicted mass of TRPC5 (110 kDa), which we suggest results from endogenous glycosylation of TRPC5. We find brain TRPC5 has a similar higher mass and deglycosylation reduces the mass to 110 kDa (data not shown). (b) Examples of immunofluorescence (red) from two freshly isolated human saphenous vein (SV) smooth muscle cells labeled with (anti-TRPC5) or without (control) T5-Chk antibody.
Figure S3. Immunoprecipitation of over-expressed TRPC5 and TRPC1. Western blot for immunoprecipitation (IP) results from tsA-201 cells mock-transfected (-) or co-transfected with TRPC5 (T5) and FLAG epitope-tagged TRPC1 (fT1) (+). The IP used anti-FLAG antibody to bind over-expressed TRPC1, T5C2 antibody to bind TRPC5, or an unrelated antiserum as a control (unrel. Ab, which was anti-Kir6.2 antiserum generated in rabbit, as described⁴). Blots were probed with anti-TRPC5 (T5C2) or anti-TRPC1 (T1C1; a gift from G Barritt) antibody. Strong bands just under 50 kDa are IgG from the IP.
Figure S4. Current-voltage relationship for TRPC5 activated by intracellular S1P.

Inside-out excised patch recording from a cell expressing TRPC5 (Tet+). S1P was bath-applied at 10 µmole/L. A 1-s ramp change in voltage from -100 to +100 mV was applied every 10 s from a holding potential of -60 mV. Current was evoked by S1P and control (pre-S1P) current subtracted. The I-V is characteristic of TRPC51,5.
REFERENCES


