Acute stimulation of calcium-permeable TRPC5-containing channels by oxidized phospholipids

Eman AL-Shawaf[1,2], **Jacqueline Naylor**[1,2], **Hilary Taylor**[1,2], **Kirsten Riches**[1,3], **Carol J Milligan**[1,2], **David O'Regan**[1,4], **Karen E Porter**[1,3], **Jing Li**[1,2], and **David J Beech**[*,1,2]

[1]Multidisciplinary Cardiovascular Research Centre, University of Leeds, Leeds, LS2 9JT, UK.

[2]Institute of Membrane & Systems Biology, Faculty of Biological Sciences, University of Leeds, Leeds, LS2 9JT, UK.

[3]Faculty of Medicine & Health, University of Leeds, Leeds, LS2 9JT, UK.

[4]Department of Cardiac Surgery, Leeds General Infirmary, Great George Street, Leeds, LS1 3EX, UK.

Abstract

Objective—Oxidized phospholipids are key factors in inflammation and associated diseases including atherosclerosis yet the initial reception mechanisms for cellular responses to the factors are poorly understood. The objective of this study was to determine if calcium-permeable channels are targets for the oxidized phospholipids, 1-palmitoyl-2-glutaroyl-phosphatidylcholine (PGPC) and 1-palmitoyl-2-oxovaleroyl-phosphatidylcholine (POVPC).

Methods and Results—Low micromolar concentrations of PGPC and POVPC evoked rises in intracellular calcium in HEK 293 cells over-expressing human Transient Receptor Potential Canonical 5 (TRPC5) but not human Transient Receptor Potential Melastatin 2 or 3. Electrophysiological experiments confirmed stimulation of TRPC5. To investigate relevance to endogenous channels we studied proliferating vascular smooth muscle cells from patients undergoing coronary artery bypass surgery. PGPC and POVPC elicited calcium entry that was inhibited by anti-TRPC5 or anti-TRPC1 antibodies or dominant-negative mutant TRPC5. Calcium-release did not occur. The effect was functionally relevant because it enhanced cell migration. Actions of PGPC and POVPC depended on $G_{i/o}$ proteins but not previously identified G protein coupled receptors for oxidized phospholipids.

Conclusions—The data suggest that stimulation of calcium-permeable TRPC5-containing channels is an early event in cellular responses to oxidized phospholipids that couples to cell migration and requires an unidentified G protein coupled receptor.

Keywords

Oxidized phospholipids; Calcium channels; Transient Receptor Potential; G proteins; Vascular smooth muscle cells

[*] Author for correspondence: Institute of Membrane & Systems Biology, Garstang Building, Mount Preston Street, Faculty of Biological Sciences, University of Leeds, Leeds, LS2 9JT, England, UK; d.j.beech@leeds.ac.uk; Tel +44 (0) 113 34 34323/4727; Fax +44 (0) 113 34 34228. .

INTRODUCTION

1-Palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (PAPC) is a phospholipid that is a common component of mammalian cell membranes and lipoproteins[1,2]. The susceptibility of PAPC and other constituent lipids to oxidation through myeloperoxidase, lipoxygenase and other enzyme activities leads to bioactive oxidation products (oxidized phospholipids)[3,4]. Two important products of PAPC are POVPC (1-palmitoyl-2-oxovaleroyl-phosphatidylcholine) and PGPC (1-palmitoyl-2-glutaroyl-phosphatidylcholine)[1,2,5]; they are two examples of oxidized phospholipids that are now recognized to constitute a diverse family of signaling lipids that accumulate during oxidative stress, apoptosis and necrosis, and are often associated with inflammatory conditions such as rheumatoid arthritis and atherosclerosis. There are also suggestions of physiological roles for these lipids that include leukocyte-endothelial interactions and pattern recognition in innate immunity[6,7]. Both pro- and anti-inflammatory effects of oxidized phospholipids have been detected[8], suggesting context dependent actions and critical importance of the free lipid concentration. Although the importance of oxidized phospholipids is increasingly established, understanding of the initial reception and signaling mechanisms is limited. Perhaps surprisingly, given the pivotal roles of Ca^{2+}-signaling in mammalian cells, information is lacking on the effects of oxidized phospholipids on Ca^{2+} channels or other Ca^{2+} transport mechanisms.

Mammalian transient receptor potential (TRP) proteins are ion pore-forming subunits of cationic channels with wide-ranging roles in physiology and disease[9]. The Canonical (C) subfamily has seven members that are generally considered to form heteromultimeric assemblies in native cells[9-12]. While the channels may have constitutive activity they are also stimulated by various chemical factors, potentially acting as polymodal integrators of Ca^{2+} movement across cell membranes[10,11]. TRPC5 is a commonly studied example of the TRPC subfamily[11-13]. It is stimulated by factors that include lanthanide ions, protons, reduced thioredoxin, and sphingosine-1-phosphate (S1P)[11-14]. It is also stimulated by lysophosphatidylcholine[15], a major phospholipid on oxidized low-density lipoproteins. Because of the effect of lysophosphatidylcholine we investigated chemically-related phospholipids with relevance to cardiovascular disease. Here we report on effects of POVPC and PGPC on TRPC5 over-expressed in the HEK 293 cell-line and on endogenous TRPC5-containing channels in vascular smooth muscle cells (VSMCs), which are targets for the actions of oxidized phospholipids[16-18].

METHODS

Conditional channel expression in HEK 293 cells

HEK 293 cells stably expressing tetracycline-regulated human TRPC5 have been described[10]. Cells were grown in DMEM-F12 medium containing 10 % fetal calf serum (FCS), 100 units.ml^{-1} penicillin and 100 μg.ml^{-1} streptomycin. Cells were maintained at 37 °C in 95 % air and 5 % CO_2 and selected with 250 μg.ml^{-1} zeocin and 10 μg.ml^{-1} blasticidin. TRP channel expression was induced by 1 μg.ml^{-1} tetracycline (Tet+). Non-induced cells without addition of tetracycline were controls (Tet−). Cells were replated on poly-D-lysine-coated black 96-well plates (Corning or BD Biosciences) or 13 mm glass coverslips 24 hr prior to experiments.

Vascular smooth muscle cells (VSMCs)

Freshly discarded human saphenous vein segments 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. The investigation conforms to the principles outlined in the Declaration of Helsinki. Proliferating VSMCs were prepared using an explant technique[19] and grown in DME medium supplemented with 10 % FCS, penicillin/streptomycin and L-glutamine at 37 °C in a 5 % CO_2 incubator. Experiments were performed on cells at passage 3-4.

Intracellular Ca^{2+} measurement

Cells were incubated with 2 μM fluo4-AM or fura2-AM for 1 hr at 37 °C followed by 0.5 hr wash at room temperature. The loading buffer was standard bath (extracellular) solution (SBS), which contained (mM): 130 NaCl, 5 KCl, 8 D-glucose, 10 HEPES, 1.5 $CaCl_2$, 1.2 $MgCl_2$, titrated to pH 7.4 with NaOH. Loading buffer included 2 μM probenicid (fluo4-AM only) and 8 μM pluronic acid. Measurements were at room temperature (21±2 °C) on an inverted fluorescence microscope (see Supplemental Material) or a 96-well fluorescence plate reader (FlexStation II[384], Molecular Devices). All Ca^{2+} data derived from the 96-well system except those of Fig 3c. With either system, changes in intracellular calcium (Ca^{2+}_i) concentration were indicated by the change (Δ) in fluo4 fluorescence (F, in arbitrary units) or the ratio of fura2 emission intensities for 340 nm and 380 nm excitation (F ratio). Fluo4 fluorescence values were divided by 10^4 (F*). Wells within columns of 96-well plates were loaded alternately for test and control conditions. The extracellular solution was SBS unless indicated otherwise; Ca^{2+}-free solution was SBS without Ca^{2+} but with 0.4 mM EGTA.

Membrane current recording

Recordings were made under voltage-clamp using the whole-cell configuration of the patch-clamp technique at room temperature. Signals were amplified and sampled using an Axopatch 200B amplifier and pCLAMP software (Molecular Devices). The extracellular solution was SBS and the patch pipette solution contained (mM): 135 CsCl, 2 $MgCl_2$, 1 EGTA, 10 HEPES, 5 Na_2ATP and 0.1 Na_2GTP; titrated to pH 7.2 with NaOH and filtered using a 0.2-μm filter. The voltage paradigm was a 200-ms ramp protocol (−100 to +100 mV) applied every 10 s from a holding potential of 0 mV. Data were filtered at 2 kHz and digitally sampled at 4 kHz.

Linear wound assay

After transfection, cells were serum-starved for 16 hr, wounded, washed and photographed, and then treated with vehicle or PGPC before being photographed and analysed. For more details see the Supplemental Material.

Reagents

Unless indicated otherwise, reagents were from Sigma UK. Stocks of chemicals were reconstituted in an appropriate vehicle: fura2-AM and fluo4-AM (Invitrogen) were dissolved at 1 mM in dimethylsulphoxide (DMSO); lysophosphatidylcholine (LPC) was dissolved at 50 mM in methanol; 1-palmitoyl-2-glutaroyl-phosphatidylcholine (PGPC), 1-palmitoyl-2-oxovaleroyl-phosphatidylcholine (POVPC) (Cayman Chemicals), 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (PAPC, Hycult Biotechnology) and oleic acid were dissolved at 16.4-16.84 mM, and sphingosine-1-phosphate (S1P) at 10 mM in ethanol. Oxidized PAPC (OxPAPC, Hycult Biotechnology) had unknown molecular mass but was assumed to have the same mass as PAPC and was similarly dissolved in ethanol. 9-Nitro oleate (Cayman Chemicals) was dissolved at 1.5267 mM in ethanol. N-Oleoylethanolamide and oleamide were dissolved at 50 mM in DMSO. Stocks of prostaglandin-E_2 (PGE_2) and (±)-15-deoxy-16R-hydroxy-17-cyclobutyl PGE_1 ((R)-butaprost) at 10 mM and 3-(4-[2-chlorophenyl]-9-methyl-6*H*-thieno[3,2-*f*][1,2,4]triazolo[4,3-α]diazepine-2-yl)-1-propanone (WEB2086, Tocris Bioscience) at 100 mM were prepared in ethanol. Vascular endothelial growth factor (VEGF) was dissolved in

water. The stock of pertussis toxin (PTX) was 0.2 mg.ml^{-1} in glycerol; denatured PTX was generated by boiling by PTX for 10 min. 2-Aminoethoxydiphenylborate (2-APB) was dissolved at 75 mM in DMSO. Pluronic acid F-127 (Invitrogen) was stored at 10 % w/v in DMSO at room temperature. Probenecid was freshly prepared in SBS. Final working concentrations of solvents were: 0.01 % methanol, 0.05 % ethanol, and 0.06 % DMSO. Functional anti-TRPC5 (T5E3) and anti-TRPC1 (T1E3) antibodies have been described[12],[14],[20]. For control experiments, pre-adsorption with antigenic peptide (10 μM) occurred overnight at 4 °C prior to use. T1E3 was used at 1:500 dilution and T5E3 at 1:100. Cells were exposed to antibody during loading with Ca^{2+} indicator and for the 0.5 hr wash period. Subsequently cells were washed with fresh SBS to remove excess antibody. For the DN-TRPC5 construct and vector, see the Supplemental Material.

Data analysis and presentation

Mean data are presented as mean ± standard error of the mean (SEM), where the n represents the number of independent experiments and the N represents the number of wells of a 96-well plate used in a single experiment. For patch-clamp experiments, n was the number of recordings from individual cells. Student *t*-tests were used for comparisons between pairs of data. Data from linear wound assays were analyzed by ANOVA followed by a Newman Keuls multiple comparison test. *P* values <0.05 were taken as indicating significant difference. Data were analyzed and presented using Origin software (Microcal Inc.).

RESULTS

Ca^{2+} signals in cells over-expressing TRPC5

Stimulation of Ca^{2+}-entry in HEK 293 cells conditionally expressing human TRPC5 was investigated in 96-well Ca^{2+}-indicator assays. In half of each plate, cells were induced to express TRPC5 by tetracycline (Tet+), while in the other half cells were not exposed to tetracycline (Tet−) to provide controls for TRPC5-independent effects. Cells were exposed to the test substance while maintaining the vehicle constant. Small effects occurred on solution application (Fig 1a, b) but much larger rises in Ca^{2+} were observed on exposure of TRPC5 (Tet+) cells to 10 μM oxidized PAPC, 3 μM PGPC or 3 μM POVPC (Fig 1a-d). There were no stimulatory effects of PAPC or the vehicle (Fig 1a, b). There were also no effects of PGPC or POVPC in control (Tet−) cells (Fig 1a-e). Therefore, PGPC and POVPC did not have TRPC5-independent effects, such as evoking Ca^{2+}-release. In TRPC5 Tet+ cells, effects of PGPC occurred at >0.1 μM (Fig 1e). Effects of POVPC mostly occurred at similar concentrations to those of PGPC except some experiments showed additional effects at 10 nM (Fig 1f). The data suggest that PGPC and POVPC are relatively potent stimulators of TRPC5.

Ionic current in cells over-expressing TRPC5

An alternative method for measuring TRPC channel activity is whole-cell patch-clamp recording. Large ionic currents were evoked in TRPC5 (Tet+) cells by PGPC (Fig 2a, b). Currents were blocked by the non-specific TRPC5 inhibitor 2-APB (Fig 2a) and they reversed polarity near 0 mV, consistent with non-selective cationic permeability of the underlying channels (Fig 2c). Importantly, the evoked current-voltage relationships (I-Vs) had double-rectifying characteristics expected of TRPC5 (Fig 2c)[14],[15],[21]. POVPC evoked similar responses (Fig 2d). Effects of PGPC and POVPC appeared to occur in two phases – early and late (e.g. Fig 2a, c, d), as has previously been observed for receptor-activation of TRPC5[21]. The data are consistent with PGPC and POVPC stimulating TRPC5 and suggest activation via a receptor-coupling mechanism.

Lack of effect on other TRP channels in the same expression system

As further controls for the expression system and to investigate specificity, PGPC and POVPC were tested against other TRP channels expressed via the same conditional system as TRPC5. No stimulation of Ca^{2+}-entry was observed in response to PGPC or POVPC in TRPM2- or TRPM3-expressing cells (Supplemental Fig I).

Ca^{2+} signals in VSMCs endogenously expressing TRPC5

Endogenous expression of TRPC5 usually occurs concomitant with expression of other TRP channels, leading to heteromultimeric channels. To investigate TRPC5 in such a context we used human saphenous vein VSMCs that express TRPC5 along with other TRP proteins including TRPC1[12,20]. PGPC and POVPC elicited Ca^{2+} responses in cells from about half of the donors (Fig 3a, Supplemental Fig II). Effects occurred at concentrations >0.1 μM (Fig 3b) but, as in HEK 293 cells over-expressing TRPC5, there were anomalous responses to 10 nM POVPC (Fig 3b).

TRPC-dependent Ca^{2+}-entry

Responses to PGPC and POVPC in VSMCs were often transient, resembling the time-course of Ca^{2+} events caused by Ca^{2+}-release (Fig 3a). Similar data were obtained using two different Ca^{2+} indicator dyes, fura2 (Fig 3a) and fluo4 (data not shown), or when recording signals from individuals cells using microscope-based imaging (Fig 3c). Unlike Ca^{2+}-release events, however, responses to PGPC and POVPC were absent when Ca^{2+} was omitted from the extracellular medium (Fig 3d, e). ATP-evoked Ca^{2+}-release was unaffected by the absence of extracelullar Ca^{2+} (Fig 3e). The data suggest that PGPC and POVPC evoke predominantly Ca^{2+} entry in VSMCs.

To investigate if TRPC5-containing channels were involved in PGPC- and POVPC-evoked Ca^{2+} entry in VSMCs we first used anti-TRPC5 and anti-TRPC1 antibodies that target extracellular loops and acutely inhibit channel function[12,14,20]. Either antibody inhibited responses to PGPC (Fig 4a-c) and POVPC (Fig 4d, e) in paired experiments using antibodies preadsorbed to antigenic peptides as controls. As an independent test of the contribution of TRPC5-containing channels we used an ion-pore mutant of TRPC5 (DN-TRPC5) that fails to pass current and acts as a dominant negative, presumably because it damages ion permeation by entering in the heteromultimeric complex[12]. Transfection of this mutant into VSMCs inhibited PGPC-evoked Ca^{2+}-entry (Fig 4f; $P<0.05$, $n=8$). The data suggest that endogenous channels containing TRPC5 and TRPC1 contributed to Ca^{2+} entry evoked by PGPC and POVPC.

Positive impact on cell migration

TRPC channel activity has been linked to cell migration[12]. Therefore, the effect of PGPC on VSMC migration was investigated in linear wound assays with and without the presence of DN-TRPC5 (Fig 5a). PGPC was found to enhance cell movement into the wounded area (Fig 5b; Supplemental Fig III). Transfection of VSMCs from 8 patients with DN-TRPC5 did not affect baseline cell movement but prevented the stimulatory effect of PGPC (Fig 5b). The data suggest that PGPC promotes VSMC migration via a mechanism that depends on ion permeation through TRPC channels.

Dependence on $G_{i/o}$ signaling

Lipid factors may act directly on TRPC channels or through intermediate signaling pathways. Sphingosine-1-phosphate, for example, acts via a G protein-coupled receptor where as lysophosphatidylcholine acts relatively directly[12,15]. In the case of sphingosine-1-

phosphate, receptor coupling occurs via a G_i or G_o protein as indicated by sensitivity to pertussis toxin[12] which ADP-ribosylates and inactivates $G_{i/o}$ proteins.

Pertussis toxin strongly inhibited effects of PGPC and POVPC on TRPC5 (Fig 6a, b). Inhibition of the PGPC effect tended to be less than inhibition of the POVPC effect, as shown by comparison of the summary data (Fig 6a, b). In confirmation of previous results[15], pertussis toxin lacked effect on TRPC5 stimulation by lysophosphatidylcholine (Fig 6c). The data suggest that PGPC and POVPC acted on TRPC5 through a $G_{i/o}$ protein signaling pathway and that lysophosphatidylcholine acted via a different mechanism.

In VSMCs, pertussis toxin partially inhibited responses to PGPC (Fig 6d, e) and almost abolished responses to POVPC (Fig 6e). The data suggest that PGPC and POVPC couple to endogenous TRPC channels substantially through a $G_{i/o}$ signaling pathway and presumably because they bind and activate a membrane receptor.

Investigation of the receptor type

Previous studies have suggested that oxidized phospholipids are ligands at prostaglandin E (PGE) and platelet-activating factor (PAF) G protein-coupled receptors[3,8,22,23]. However, neither PGE_2 (n=3, data not shown) nor the synthetic PGE_2 receptor agonist (R)-butaprost stimulated TRPC5 (Supplementary Figure IV) and a high concentration of the PAF receptor antagonist WEB2086 failed to inhibit PGPC-evoked responses in VSMCs (Fig 6f). VEGF receptors are suggested to be involved in oxidized phospholipid effects[24], but we confirmed that the VSMCs and Tet+ HEK 293 cells show no Ca^{2+} responses to 100 ng.mL^{-1} VEGF; contrasting with endothelial cells from the saphenous vein (data not shown). The data indicate that previously suggested oxidized phosholipid receptors are not involved in mediating TRPC channel stimulation.

To obtain more information on the character of the receptor we tested 3 µM 9-nitro-oleate, 3 µM oleic acid, 10 µM oleoylethanolamide and 10 µM oleamide in Ca^{2+} measurements assays of TRPC5 Tet+ cells, using 3 µM PGPC as a positive control. None of these additional lipid factors evoked TRPC5 activity (3 independent experiments for each lipid, data not shown).

DISCUSSION

The study reveals that bioactive oxidation products of a common membrane phospholipid have acute stimulatory effects on Ca^{2+}-entry via TRPC1/5 channels and a G protein signaling mechanism, without causing Ca^{2+}-release. The mechanism was shown to be relevant to proliferating VSMCs.

Dominant negative TRPC5 mutant had no effect on basal migration of VSMCs but prevented migration evoked by PGPC. The result adds to an expanding picture of positive roles of TRPC1/5 in remodeling[12,23,25,26]. The effect is consistent with a pro-atherogenic effect of the phospholipids and may be important in physiological events of formation, remodeling and response-to-injury of blood vessels. PGPC and POVPC were found to be potent stimulators of the channels, acting at concentrations considerably lower than those used in many studies or detected in animals fed atherogenic diets[2-5,27].

PGPC and POVPC generated relatively sustained Ca^{2+} signals in the TRPC5 Tet+ cells but mostly transient signals in VSMCs. The difference may have arisen because of the more complex channel composition in the VSMCs. TRPC1 was involved in the VSMC signal and is susceptible to Ca^{2+}-induced inactivation[28]; it may, therefore confer the decay in the PGPC and POVPC responses. A previous study described opposite effects of PGPC and POVPC

on endothelial cells[6]. Our data show equivalent effects of the oxidized phospholipids on VSMCs except for two features: greater inhibition of POVPC responses by pertussis toxin, and anomalous responses to 10 nM POVPC. The 10 nM POVPC effect occurred in HEK 293 cells induced to express TRPC5 but not non-induced cells, suggesting dependence on TRPC5. A separate intriguing observation was made with lysophosphatidylcholine, which is chemically closely related to PGPC and POVPC and functionally active in similar contexts[29]. Unlike PGPC and POVPC, lysophosphatidylcholine stimulated TRPC5 independently of G protein signaling (Fig 6)[15]. Chemical features in PGPC and POVPC presumably prevented these lipids from mimicking the effect of lysophosphatidylcholine.

The receptors mediating the TRPC effects of PGPC and POVPC were not identified. PGE_2 and PAF receptors are unlikely to be involved. PGE_2 and (R)-butaprost were ineffective and POVPC was previously found to lack effect at PGE_2 receptors[22]. The PAF antagonist WEB2086 failed to block the action of PGPC and a previous study found that WEB2086 inhibited effects of POVPC but not PGPC[23]. A candidate receptor for the PGPC and POVPC effects in our experiments was an S1P receptor because S1P also acts via a $G_{i/o}$ pathway[12]. However, PGPC and POVPC could not have been agonists at S1P receptors because, unlike S1P, they did not cause Ca^{2+}-release. Although oxidized phospholipids bind to scavenger receptors like CD36, these receptors are not thought to couple to G protein signaling.

The data suggest that TRPC channels are components of an initial reception mechanism enabling cellular responses to biologically-active oxidized phospholipids. The mechanism is indicated to involve binding to a previously unrecognized G protein-coupled receptor that couples to the channels but not Ca^{2+}-release. An identified functional consequence is the promotion of VSMC migration, consistent with prior suggestion that oxidized phospholipids play key roles in the proliferating phenotype of VSMCs[16].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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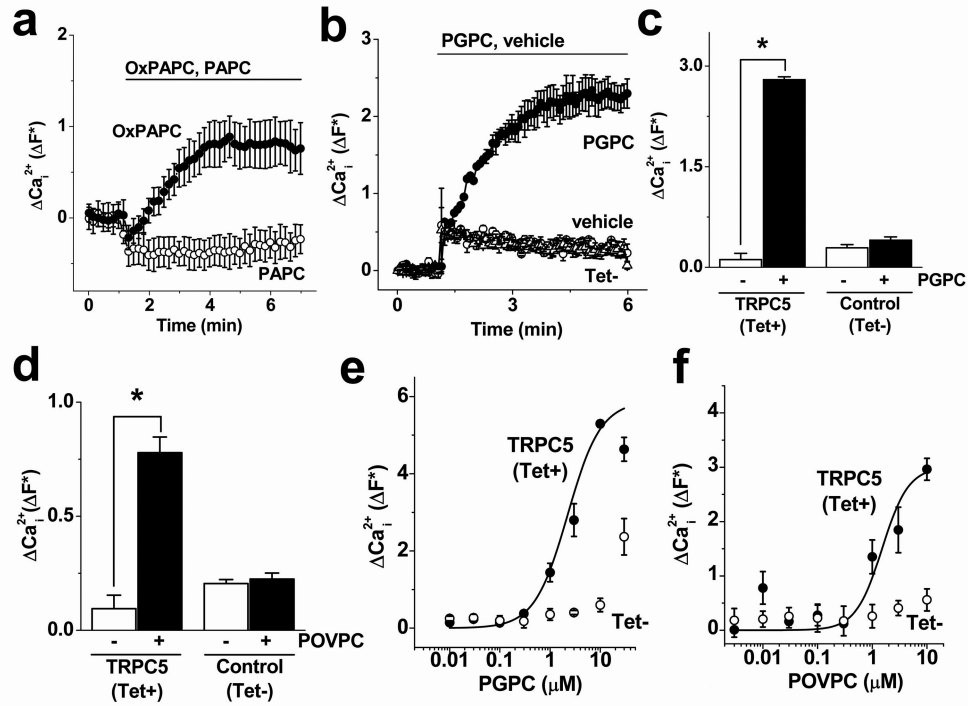
Figure 1. Ca^{2+}-entry stimulated by PGPC and POVPC in TRPC5-expressing HEK 293 cells
Ca^{2+} measurement data, where black symbols indicating responses to OxPAPC, PGPC or
POVPC in TRPC5-expressing (Tet+) cells and white symbols indicating PAPC, vehicle
controls or responses in Tet– cells. The ordinate scale is the change (Δ) in fluo4
fluorescence divided by 10^4 (F*) in arbitrary units. (**a**) Responses to bath-applied 10 μM
OxPAPC or 10 μM PAPC (N=6 each). (**b**) Responses to bath-applied 3 μM PGPC or
vehicle (N=6 each). (**c, d**) Mean data for PGPC, POVPC and vehicle responses in Tet+ and
Tet– cells (n/N=3/18 for each point). (**e, f**) Concentration-response data for PGPC and
POVPC in Tet+ and Tet– cells (n/N=3/9 for each point). Curves are Hill equations fitted to
Tet+ cell data with mid-points at 2.24 μM (**e**) and 1.52 μM (**f**).
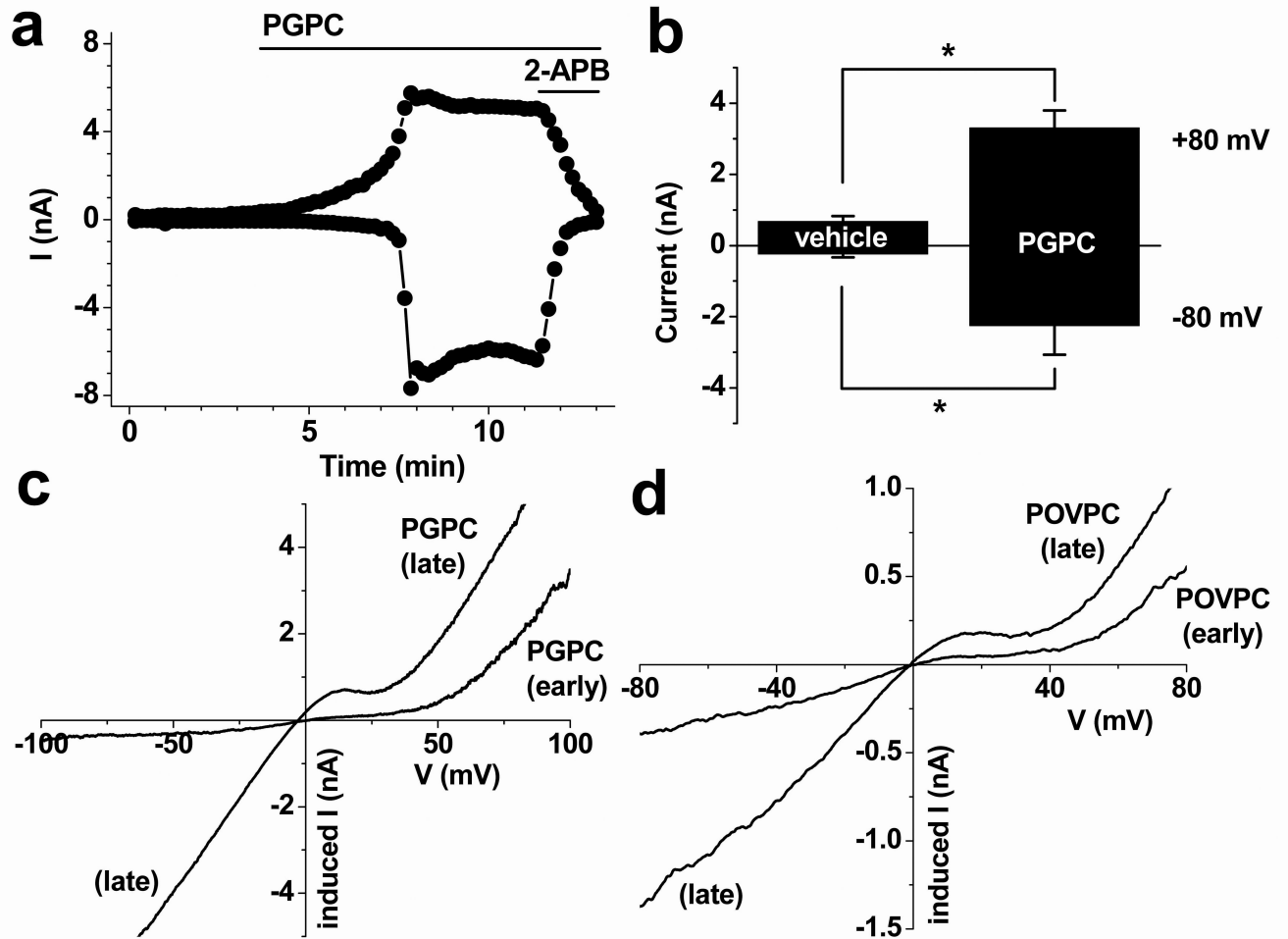
Figure 2. Ionic current stimulated by PGPC or POVPC in TRPC5-expressing HEK 293 cells
Whole-cell patch-clamp recordings from Tet+ cells under voltage-clamp. (**a**) Time-series plot showing the effect of bath-applied 3 µM PGPC in the continuous presence of the ethanol vehicle, and then inhibition by 75 µM 2-APB. (**b**) Mean time-matched data comparing current responses to the vehicle (ethanol) and 3 µM PGPC at −80 mV and +80 mV (n=6 each). (**c**) For the experiment of (**a**), current-voltage relationships (I-Vs) for currents induced by PGPC at the early and late stages of the response. (**d**) Response to bath-applied 3 µM POVPC shown as I-Vs for currents induced by POVPC.
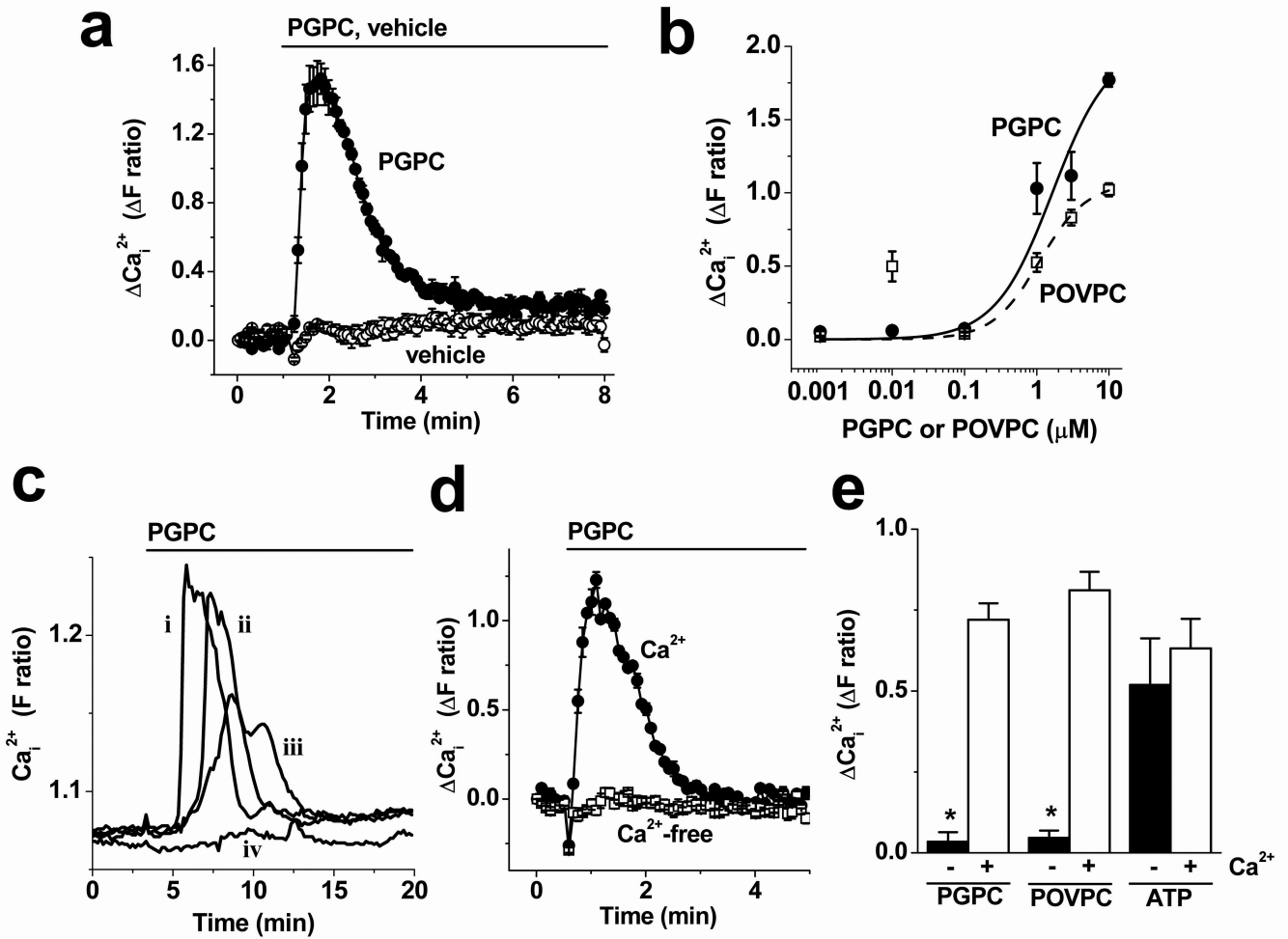
Figure 3. Responses of VSMCs to PGPC and POVPC

Data are from intracellular Ca^{2+} measurements. (**a**) Typical response to bath-applied 3 μM PGPC compared with vehicle control. (**b**) Concentration-response data for PGPC and POVPC (n/N=4/12 for each point). Mid-points of the fitted Hill equations were at 1.5 μM (PGPC) and 1.09 μM (POVPC). (**c**) Example single VSMC responses to 3 μM PGPC observed using the microscope system (4 cells, labeled i-iv). (**d**) Responses to 3 μM PGPC in the presence and absence of extracellular Ca^{2+} (N=6 for each). (**e**) As for (**d**) but mean data from independent experiments for 3 μM PGPC, 3 μM POVPC and 100 μM ATP (n/N=3/18 for each).
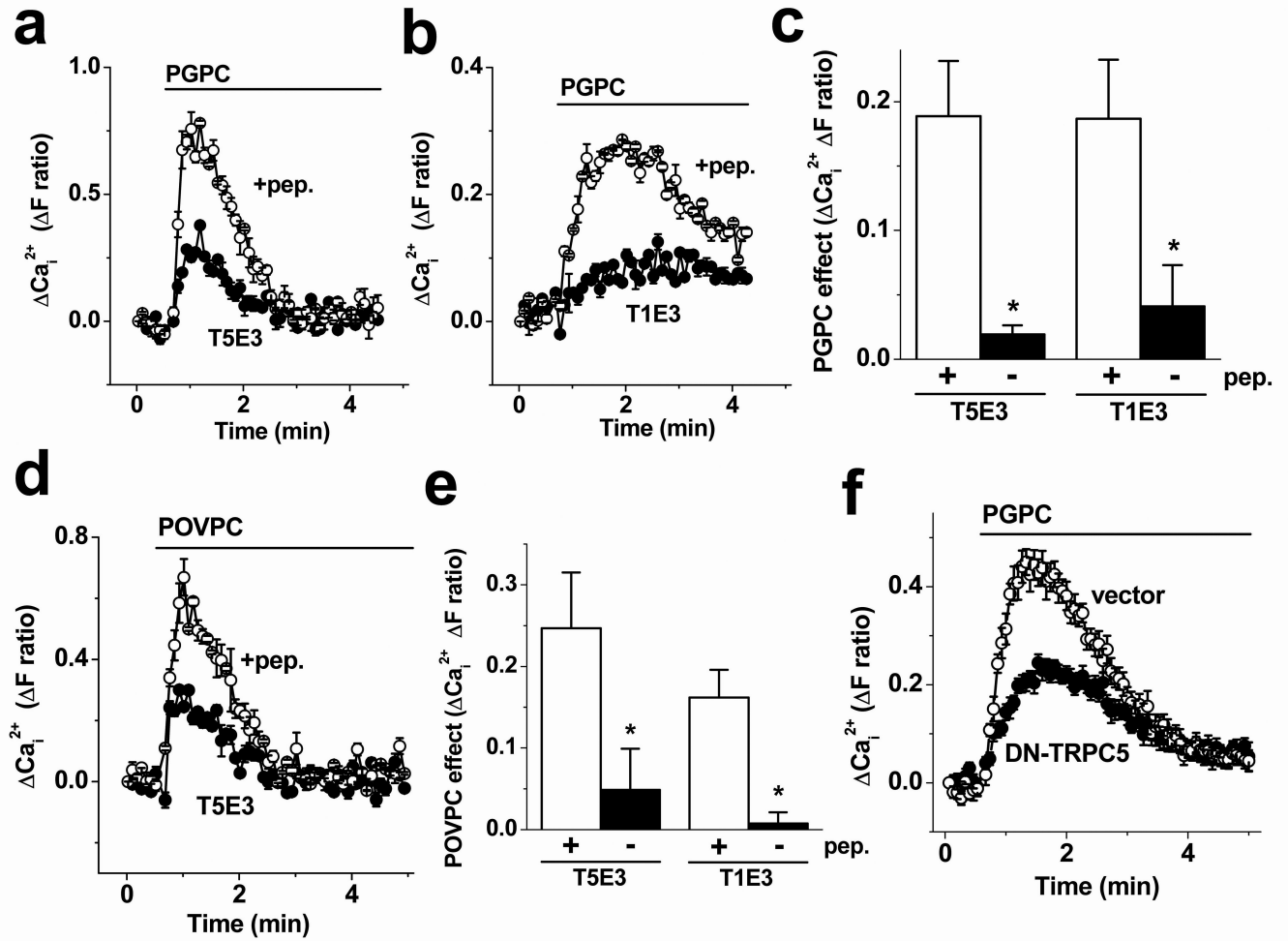
Figure 4. Contribution of endogenous TRPC5 and TRPC1

Data are from intracellular Ca^{2+} measurements from VSMCs. (**a**, **b**) Responses to 3 μM PGPC after pretreatment with anti-TRPC antibody alone or anti-TRPC antibody preadsorbed to its antigenic peptide (+pep.) (N=5 each): T5E3 anti-TRPC5 antibody (**a**); T1E3 anti-TRPC1 antibody (**b**). (**c**) As for (**a**, **b**) but mean data for independent experiments (n/N=3/15). (**d**, **e**) As for (**a, c**) but using POVPC instead of PGPC (n/N=3/15). (**f**) Example from 8 independent paired experiments of the response to 3 μM PGPC after transfection with dominant negative mutant TRPC5 (DN-TRPC5) or vector control (N=16 for each).
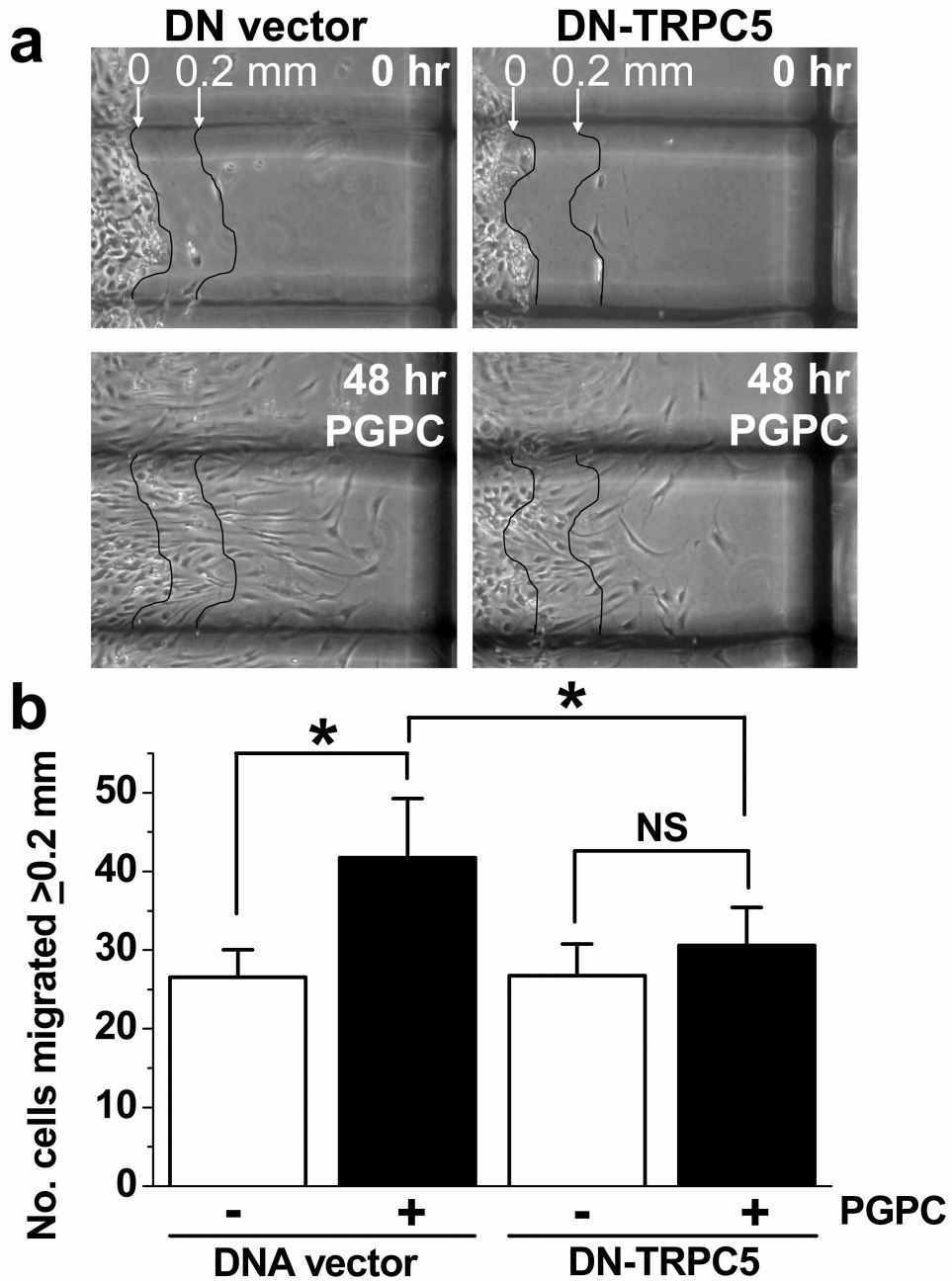
Figure 5. VSMC migration evoked by PGPC and depending on TRPC channel function
(**a**) Representative images showing VSMCs at time zero (0 hr) and 48 hr after scratch wound in the presence of 3 μM PGPC. The images show a paired comparison of cells from the same patient transfected with DNA vector or vector expressing DN-TRPC5. In each image, VSMCs before migration are on the left of the thin line that delineated the origin (0 mm); the parallel thin to the right defines the 0.2 mm distance required for cells to migrate before counting. The thick dark shadow is the 1 mm grid. (**b**) For experiments of the type shown in (**a**), mean data for VSMCs of 8 patients after transfection with DNA vector or

vector containing the dominant negative (DN) TRPC5 insert. VSMCs were treated with vehicle (ethanol) or 3 μM PGPC and the numbers of migrated VSMCs were measured.
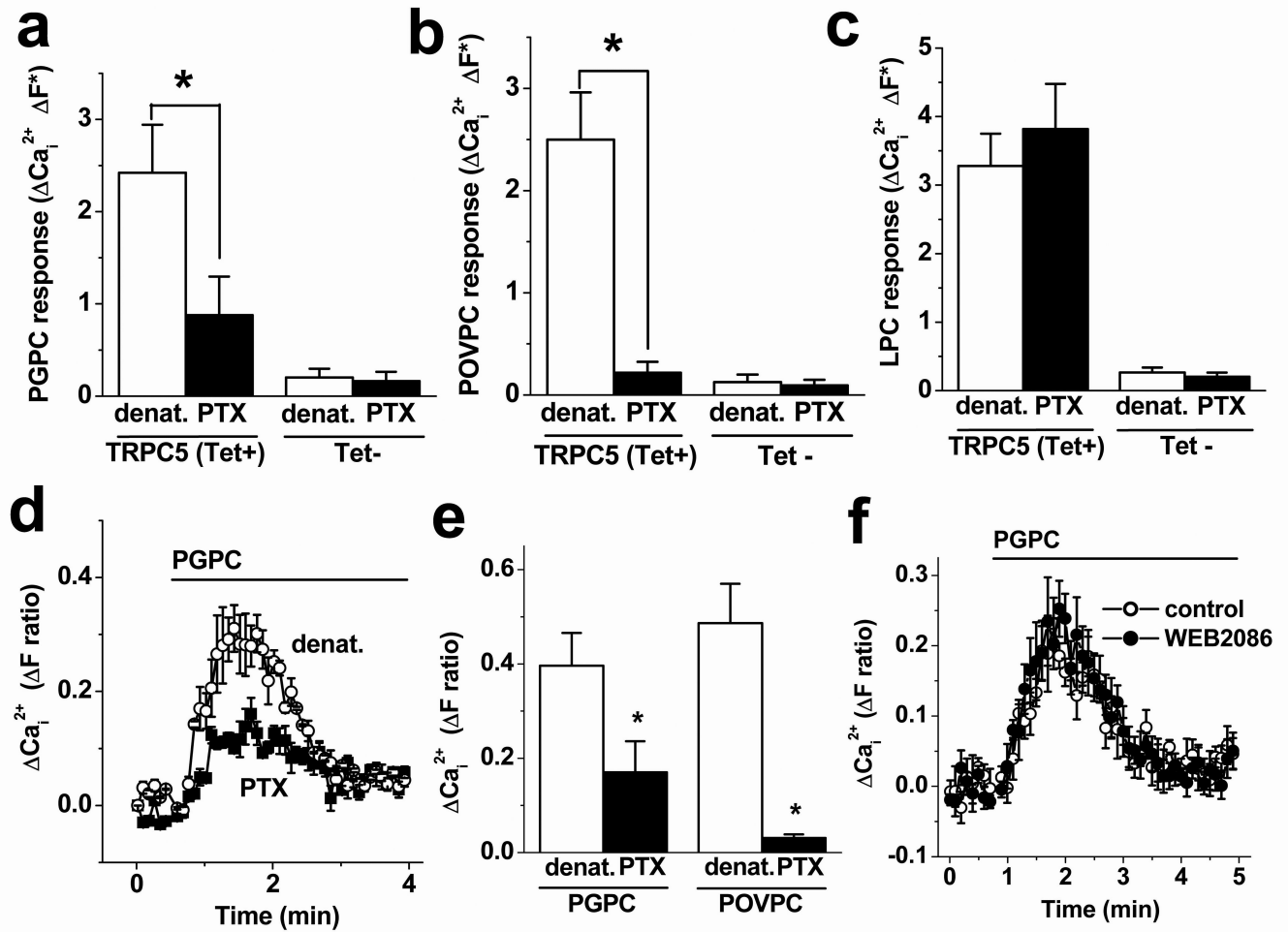
Figure 6. Role of $G_{i/o}$-signaling but not PAF receptors

Data are from intracellular Ca^{2+} measurements from TRPC5-expressing HEK 293 cells (**a-c**) and VSMCs (**d-f**). Cells were pretreated with 1 μg ml^{-1} pertussis toxin (PTX) or denatured (denat.) PTX for 4 hr at 37 °C. (**a-c**) Responses were evoked by 3 μM PGPC (**a**, n/N=3/9), 5 μM POVPC (**b**, n/N=3/9) or 5 μM lysophosphatidylcholine (**c**, n/N=3/18). (**d**) Representative paired experiment showing responses to 3 μM PGPC after treatment with PTX or denatured PTX (N=5 each). (**e**) Responses were evoked by 3 μM PGPC or 5 μM POVPC (n/N=4/20 each). (**f**) Responses to 3 μM PGPC in cells pre-incubated and maintained in the presence of 100 μM WEB2086 or its vehicle (N=6 each and representative of 3 independent experiments).