Membrane translocation of TRPC6 channels and endothelial migration are regulated by calmodulin and PI3 kinase activation

Pinaki Chaudhuri[a], Michael A. Rosenbaum[b], Pritam Sinharoy[c], Derek S. Damron[c], Lutz Birnbaumer[d,1], and Linda M. Graham[a,e,1]

[a]Department of Biomedical Engineering, Cleveland Clinic, Cleveland, OH 44195; [b]Surgical Service, Louis Stokes Cleveland Veterans Affairs Medical Center, Cleveland, OH 44106; [c]Department of Biological Sciences, Kent State University, Kent, OH 44240; [d]Neurobiology Laboratory, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709; and [e]Department of Vascular Surgery, Cleveland Clinic, Cleveland, OH 44195

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Lipid oxidation products, including lysophosphatidylcholine (lysoPC), activate canonical transient receptor potential 6 (TRPC6) channels leading to inhibition of endothelial cell (EC) migration in vitro and delayed EC healing of arterial injuries in vivo. The precise mechanism through which lysoPC activates TRPC6 channels is not known, but calmodulin (CaM) contributes to the regulation of TRPC channels. Using site-directed mutagenesis, cDNAs were generated in which Tyr^{99} or Tyr^{138} of CaM was replaced with Phe, generating mutant CaM, Phe^{99}-CaM, or Phe^{138}-CaM, respectively. In ECs transiently transfected with pcDNA3.1-myc-His-Phe^{99}-CaM, but not in ECs transfected with pcDNA3.1-myc-His-Phe^{138}-CaM, the lysoPC-induced TRPC6-CaM dissociation and TRPC6 externalization was disrupted. Also, the lysoPC-induced increase in intracellular calcium concentration was inhibited in ECs transiently transfected with pcDNA3.1-myc-His-Phe^{99}-CaM. Blocking phosphorylation of CaM at Tyr^{99} also reduced CaM association with the p85 subunit and subsequent activation of phosphatidylinositol 3-kinase (PI3K). This prevented the increase in phosphatidylinositol (3,4,5)-trisphosphate (PIP3) and the translocation of TRPC6 to the cell membrane and reduced the inhibition of EC migration by lysoPC. These findings suggest that lysoPC induces CaM phosphorylation at Tyr^{99} by a Src family kinase and that phosphorylated CaM activates PI3K to produce PIP3, which promotes TRPC6 translocation to the cell membrane.

endothelial | calmodulin | PI3 kinase | TRPC6

Endothelial cell (EC) migration is required for healing after arterial injuries, such as those that occur with angioplasties. Oxidized low-density lipoprotein and lysophosphatidylcholine (lysoPC), the major lysophospholipid of oxidized low-density lipoprotein, are abundant in plasma and atherosclerotic lesions and inhibit EC migration (1). A brief influx of calcium is required to initiate EC migration (2), but lysoPC causes a prolonged influx of Ca^{2+} that disrupts the cytoskeletal dynamics required for normal EC migration (3, 4). Specifically, lysoPC activates canonical transient receptor potential 6 (TRPC6) channels, as shown by patch clamp recording, with Ca^{2+} influx (3). The increased $[Ca^{2+}]_i$ initiates events that result in TRPC5 channel activation (3). The later activation of TRPC5 compared with TRPC6 and the failure of TRPC6 and TRPC5 to coimmunoprecipitate indicates that they do not form a heteromeric complex. The importance of this pathway is found in TRPC6-deficient EC, where lysoPC has little effect on EC migration (3). Furthermore, a high cholesterol diet markedly inhibits endothelial healing in wild-type (WT) mice, but has no effect in TRPC6-deficient ($TRPC6^{-/-}$) mice (5). The mechanism of TRPC6 activation by lysoPC is not fully elucidated, limiting the ability to block this important pathway.

Calmodulin (CaM), a small, highly conserved, intracellular calcium-binding protein (6), binds to TRPC channels and regulates their activation. TRPC proteins, including TRPC6, possess a C-terminal CaM-binding domain that overlaps with a binding site for the inositol trisphosphate receptor, and CaM and the inositol triphosphate receptor compete for binding at this site (7). Removal of CaM from the common binding site results in activation of TRP3 channels (8). TRPC proteins contain additional binding sites for CaM and other Ca^{2+}-binding proteins, indicating a complex regulatory mechanism in response to changes in $[Ca^{2+}]_i$ that includes positive and negative regulation of channels (9). In addition to CaM regulating TRPC proteins by direct binding, CaM-dependent kinases activate TRPC channels (10). CaM activity and peptide binding affinity is altered by its phosphorylation state and bound Ca^{2+}, and Ca^{2+} can regulate the phosphorylation of CaM (11). LysoPC activates tyrosine kinases, including Src family tyrosine kinases (12), and Src family kinases can phosphorylate CaM (13). The role of CaM and CaM phosphorylation in TRPC6 channel activation or in EC migration is incompletely understood.

TRPC6 channel activation generally requires externalization; however, the mechanism of TRPC6 channel translocation to the plasma membrane is not clear. In HEK cells overexpressing TRPC6, stimulation of Gq protein-coupled receptors causes TRPC6 externalization and localization to caveolae or lipid rafts by an exocytotic mechanism (14). In smooth muscle cells, phosphatidylinositol 3-kinase (PI3K) is involved in carbachol-induced TRPC6 externalization (15). The mechanism by which lysoPC induces TRPC6 externalization in EC is unknown.

Significance

Endothelial cell migration is required for vessel repair after damage during angioplasty. Migration is inhibited by lipid oxidation products, including lysophosphatidylcholine (lysoPC), that are abundant in atherosclerotic plaques. Inhibition of migration is dependent on Ca^{2+} entering through canonical transient receptor potential 6 (TRPC6) channels externalized by lysoPC's action. Here we uncover an unappreciated role for activation of PI3K by calmodulin (CaM) requiring phosphorylation of its Tyr^{99}. Phosphatidylinositol (3,4,5)-trisphosphate (PIP3) formed by PI3K facilitates insertion of endocellular TRPC6 channels into the plasma membrane. The CaM-PI3K signaling mechanism may also operate in externalization of additional TRPC channels and for other membrane functions residing primarily on endomembranes prior to stimulation by extracellular signals, including glucose transporter 4 in response to insulin, and aquaporin-2 in response to vasopressin.

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[1]To whom correspondence may be addressed. Email: Birnbau1@gmail.com or grahamL@ccf.org.

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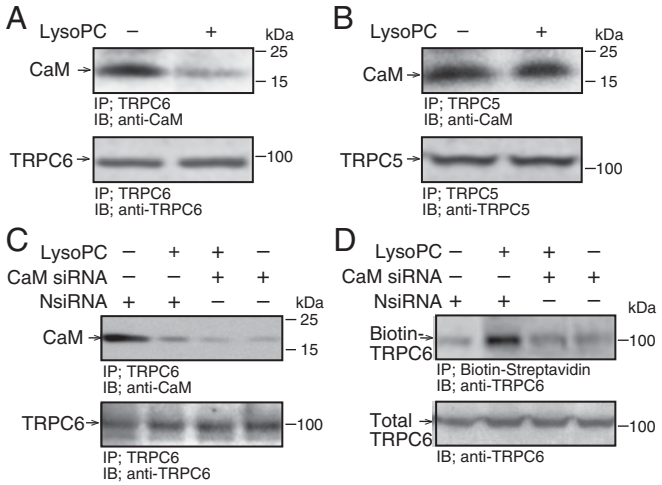

Fig. 1. LysoPC induces TRPC6-CaM dissociation but down-regulation of CaM reduces TRPC6 externalization. (*A* and *B*) BAECs were incubated with lysoPC (12.5 μM) for 15 min. TRPC6 or TRPC5 was immunoprecipitated and associated CaM identified by immunoblot analysis. In aliquots removed after immunoprecipitation, total TRPC6 or TRPC5 was determined ($n = 4$). (*C* and *D*) BAECs were transiently transfected with negative control siRNA (NsiRNA) or CaM siRNA (20 nM) for 24 h, then incubated with lysoPC. TRPC6-CaM association was identified as above ($n = 3$) or TRPC6 externalization was determined by biotinylation assay ($n = 4$).

PI3K produces phosphatidylinositol (3,4,5)-trisphosphate (PIP3) from phosphatidylinositol (4,5)-bisphosphate (PIP2) in the inner leaflet of the plasma membrane and participates in numerous intracellular signaling processes, including TRPC6 activation (16). PI3K is composed of a p85 regulatory subunit (p85α, p85β, or p55γ) and a p110 catalytic subunit (p110α, p110β, p110γ, or p110δ), and activity can be influenced by CaM association (17).

The purpose of the present study is to explore the underlying mechanism of lysoPC-induced TRPC6 activation. We identify a mechanism in which phosphorylation of CaM at Tyr^{99} plays a key role in lysoPC-induced TRPC6 externalization and inhibition of EC migration.

Results

LysoPC Induces Dissociation of CaM from TRPC6 and CaM-Mediated TRPC6 Externalization. LysoPC (1-palmitol-2-hydroxy-*sn*-glycerol-3-phosphocholine; Avanti Polar Lipids) caused significant but not complete dissociation of CaM from TRPC6 ($n = 4$, $P < 0.01$ compared with control, Fig. 1*A*). Interestingly, lysoPC caused no dissociation of CaM from TRPC5 ($n = 4$, Fig. 1*B*), showing that changes were specific for TRPC6 and not due to CaM degradation.

Transient transfection of bovine aortic ECs (BAECs) with CaM small interfering RNA (siRNA) decreased CaM expression to 20 ± 3% of control (Fig. S1) and decreased CaM-TRPC6 association to 20 ± 2% of that in BAECs transfected with negative control siRNA (NsiRNA) under control conditions and to 7 ± 2% of that in BAECs transfected with NsiRNA after incubation with lysoPC ($n = 3$, $P < 0.01$; Fig. 1*C*). Importantly, when CaM was down-regulated, lysoPC-induced TRPC6 externalization was significantly inhibited ($n = 4$, $P < 0.01$ compared with NsiRNA; Fig. 1*D*). These findings suggested that CaM was necessary for lysoPC-induced TRPC6 externalization, and that simply decreasing CaM association with TRPC6 did not promote TRPC6 externalization.

LysoPC Induces Phosphorylation of CaM at Tyr^{99} by a Src Family Kinase That Is Dependent on Ca^{2+} but Independent of TRPC6. The effect of lysoPC on CaM phosphorylation was assessed by immunoblot analysis using antibodies specific for CaM phosphorylated at Tyr^{99}, Tyr^{138}, or Ser^{81} and Thr^{79}. In BAECs incubated with lysoPC, CaM phosphorylation at Tyr^{99} was increased 2.1 ± 0.4-fold compared with control ($n = 5$, $P < 0.01$; Fig. 2*A*), but CaM phosphorylation at Tyr^{138} was unchanged ($n = 3$, Fig. 2*B*). Epidermal growth factor (100 nM), known to induce Tyr^{138} phosphorylation, was the positive control. LysoPC did not increase CaM phosphorylation at Ser^{81} or Thr^{79} ($n = 4$, Fig. 2*C*). Pretreatment of BAECs with a Src family tyrosine kinase inhibitor, PP2 (2 μM), blocked lysoPC-induced CaM phosphorylation at Tyr^{99} (Fig. S2*A*), inhibited lysoPC-induced CaM dissociation from TRPC6 (Fig. S2*B*) and TRPC6 externalization (Fig. S2*C*), and preserved BAEC migration in lysoPC (Fig. S2*D*). These results suggested that lysoPC induced CaM phosphorylation specifically at Tyr^{99} by activation of a Src family kinase.

CaM interactions with its target proteins are regulated by Ca^{2+} loading as well as phosphorylation (13). TRPC6 activation can be regulated by Ca^{2+} (10). To assess the role of Ca^{2+} in the lysoPC-induced phosphorylation of CaM and subsequent TRPC6 activation, BAECs were incubated with BAPTA/AM (25 μM or 300 μM). After 30 min, lysoPC (12.5 μM) was added for 15 min in the presence of Ca^{2+}-containing Krebs-Ringer (KR) buffer. In BAECs preincubated in BAPTA/AM (25 μM), lysoPC induced CaM phosphorylation at Tyr^{99} ($n = 3$, Fig. 2*D*), but in BAECs preincubated in BAPTA/AM (300 μM), lysoPC did not induce CaM phosphorylation at Tyr^{99} ($n = 3$, Fig. 2*E*). Similarly, lysoPC did not induce TRPC6 externalization in BAECs preincubated with 300 μM of BAPTA/AM ($n = 3$, Fig. 2*F*). These results suggested that a local increase of Ca^{2+} is essential for lysoPC-induced CaM phosphorylation and subsequent TRPC6 externalization.

To assess the role of TRPC6 in lysoPC-induced CaM phosphorylation, $TRPC6^{-/-}$ mouse aortic endothelial cells (MAECs) were studied. Incubation with lysoPC (10 μM) induced CaM

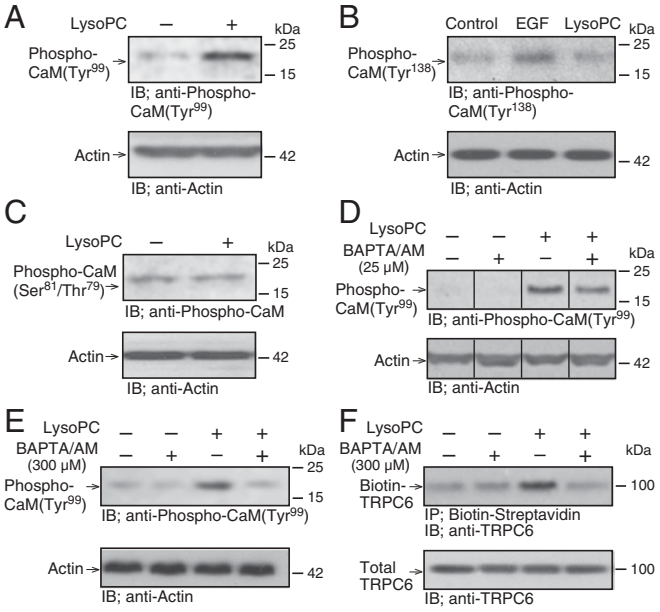

Fig. 2. LysoPC induces CaM phosphorylation at Tyr^{99}, which is Ca^{2+} dependent, but not CaM phosphorylation at Tyr^{138}, Ser^{81}, or Thr^{79}. (*A*–*F*) BAECs were incubated with lysoPC (12.5 μM) for 15 min. (*A*–*C*) Phospho-CaM was identified by immunoblot analysis. Actin served as loading control. (*A*) Phospho-CaM(Tyr^{99}) was identified ($n = 5$). (*B*) Phospho-CaM(Tyr^{138}) was identified ($n = 3$). Epidermal growth factor (EGF, 100 nM) for 30 min served as a positive control. (*C*) Phospho-CaM(Ser^{81}/Thr^{79}) was identified ($n = 4$). (*D*) BAECs preincubated with BAPTA/AM (25 μM) for 30 min before adding lysoPC. Phospho-CaM(Tyr^{99}) was detected by immunoblot analysis ($n = 3$). (Lines indicate lanes rearranged from same gel.) (*E* and *F*) BAECs were incubated with BAPTA/AM (300 μM) for 30 min before adding lysoPC. (*E*) Phospho-CaM was detected by immunoblot analysis ($n = 3$). (*F*) TRPC6 externalization was determined by biotinylation assay ($n = 3$).

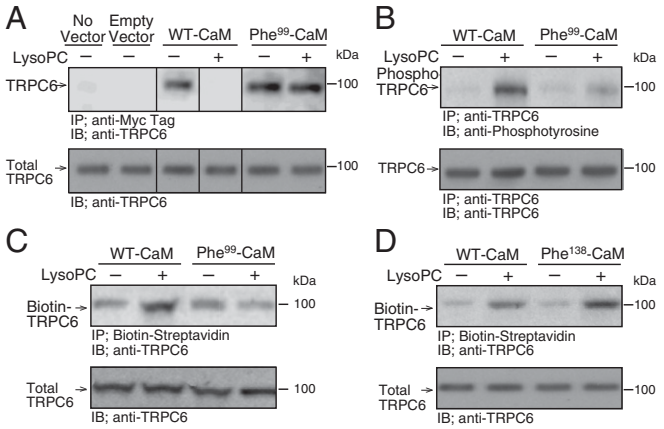

Fig. 3. In BAECs expressing CaM mutated at Tyr^{99}, lysoPC fails to induce TRPC6-CaM dissociation, TRPC6 tyrosine phosphorylation, and TRPC6 externalization. (*A–D*) BAECs were transiently transfected for 24 h with pcDNA3.1-myc-His-WT-CaM, pcDNA3.1-myc-His-Phe^{99}-CaM, or pcDNA3.1-myc-His-Phe^{138}-CaM. At 24 h, BAECs were incubated with lysoPC (12.5 μM) for 15 min. (*A*) Myc-conjugated CaM was immunoprecipitated and associated TRPC6 was detected by immunoblot analysis ($n = 4$). (Lines indicate lanes rearranged from same gel.) (*B*) TRPC6 was immunoprecipitated, then immunoblot analysis using anti-phosphotyrosine or anti-TRPC6 antibody identified tyrosine-phosphorylated TRPC6 or total TRPC6 ($n = 4$). (*C* and *D*) TRPC6 externalization was determined by biotinylation assay ($n = 4$).

phosphorylation at Tyr^{99} in TRPC6$^{-/-}$ MAECs as well as in WT MAECs ($n = 3$, Fig. S3), suggesting that CaM phosphorylation was independent of TRPC6.

CaM Phosphorylation at Tyr^{99} Is Required for LysoPC-Induced TRPC6 Externalization. To evaluate the role of CaM phosphorylation at Tyr^{99} in TRPC6 externalization, mutant CaMs were generated, in which Tyr was replaced with Phe, which cannot be phosphorylated. BAECs were transiently transfected with plasmids containing the vector pcDNA 3.1-myc-His with or without cDNA for WT-CaM, Phe^{99}-CaM, or Phe^{138}-CaM for 24 h, and overexpression was confirmed after 48 h by immunoblot analysis ($n = 3$, Fig. S4 *A* and *B*). LysoPC increased CaM phosphorylation at Tyr^{99} in BAECs overexpressing WT-CaM ($P < 0.01$), but not in BAECs overexpressing Phe^{99}-CaM ($n = 4$, Fig. S4*C*). In another approach, Tyr^{99} was replaced by Asp, which would behave as a constitutively phosphorylated CaM, but BAECs transiently transfected with this mutant were not viable.

In BAECs overexpressing WT-CaM, lysoPC induced: (*i*) CaM dissociation from TRPC6 (7.8 ± 0.3-fold decrease compared with control, $P < 0.01$, $n = 4$; Fig. 3*A*), (*ii*) TRPC6 tyrosine phosphorylation (2.8 ± 0.3-fold increase compared with control, $P < 0.01$, $n = 4$; Fig. 3*B*), and (*iii*) TRPC6 externalization (2.6 ± 0.5-fold increase over control, $P < 0.01$, $n = 4$; Fig. 3*C*). These events did not occur in BAECs overexpressing Phe^{99}-CaM, suggesting that Tyr^{99} phosphorylation was required. The finding was not unique to BAECs. In human ECs (EA.hy 926), phosphorylation of CaM at Tyr^{99} was required for TRPC6 externalization (Fig. S4 *D* and *E*).

To confirm the specificity of CaM phosphorylation at Tyr^{99} for TRPC6 externalization, BAECs overexpressing Phe^{138}-CaM, in which Tyr^{138} was replaced with Phe, were studied. LysoPC induced TRPC6 externalization in BAECs overexpressing WT-CaM or Phe^{138}-CaM with a 2.6 ± 0.5-fold or 2.6 ± 0.3-fold increases, respectively, compared with control ($n = 3$, $P < 0.01$; Fig. 3*D*), suggesting that CaM phosphorylation at Tyr^{138} was not required.

CaM Phosphorylation at Tyr^{99} Is Required for LysoPC-Induced TRPC6-Dependent Increase in $[Ca^{2+}]_i$. BAECs were transiently transfected with a plasmid containing cDNA for the empty vector or the vector with WT-CaM, Phe^{99}-CaM, or TRPC6. Other BAECs were transfected with a combination of two plasmids. Overexpression was confirmed by immunoblot analysis at 24 h (Fig. S5 *A* and *B*). The lysoPC-induced rise in $[Ca^{2+}]_i$ was similar in BAECs transfected with empty vector or WT-CaM ($n = 8$, Fig. 4 *A* and *B*), but Phe^{99}-CaM significantly decreased the lysoPC-induced rise in $[Ca^{2+}]_i$ to 52.3 ± 2.4% of that seen with WT-CaM overexpression ($n = 8$, $P < 0.001$ compared with WT-CaM, Fig. 4 *B* and *C*). The lysoPC-induced rise in $[Ca^{2+}]_i$ was increased by 33.2 ± 4.0% in BAECs overexpressing TRPC6 ($n = 8$, $P < 0.001$, compared with empty vector, Fig. 4 *A* and *D*), supporting a role for TRPC6 in the rise of $[Ca^{2+}]_i$. Similarly, a 33.3 ± 5.2% increase in peak $[Ca^{2+}]_i$ was observed in BAECs overexpressing TRPC6 and WT-CaM ($n = 8$, Fig. 4*E*). The peak in $[Ca^{2+}]_i$ was significantly less in BAECs overexpressing TRPC6 and Phe^{99}-CaM, only 40.6 ± 1.8% of that seen in BAECs overexpressing TRPC6 and WT-CaM ($n = 8$, $P < 0.001$; Fig. 4 *E* and *F*). These results, summarized graphically in Fig. 4*G*, suggest that CaM phosphorylation at Tyr^{99} is critical for lysoPC-induced TRPC6-mediated increase of $[Ca^{2+}]_i$.

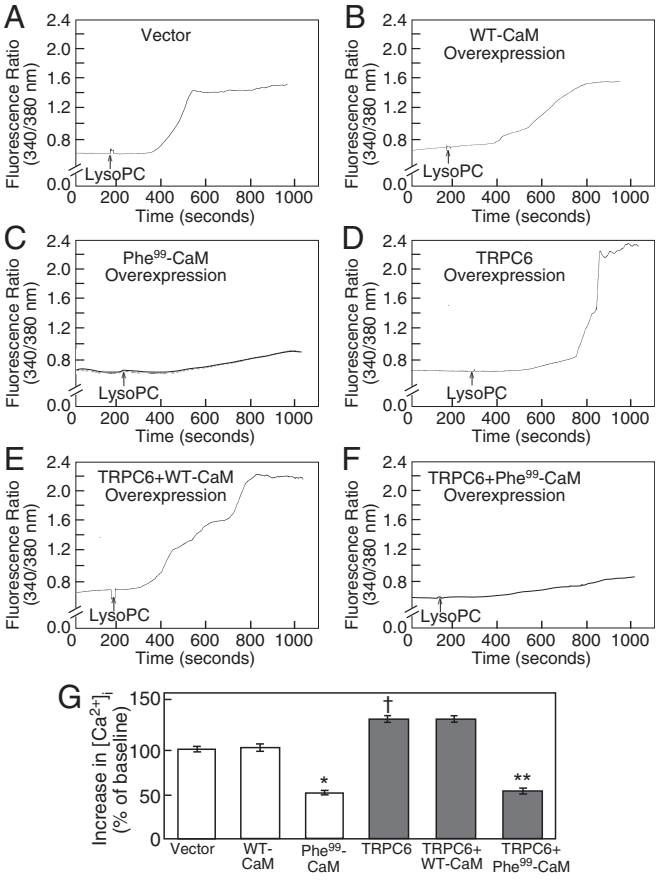

Fig. 4. In BAECs overexpressing Phe^{99}-CaM, lysoPC does not increase $[Ca^{2+}]_i$. (*A–F*) BAECs were transiently transfected with a plasmid containing empty vector or vector with WT-CaM, Phe^{99}-CaM, or TRPC6, or a combination of plasmids as indicated for 16 h, made quiescent for 8 h, then loaded with fura 2-AM. After adjusting the baseline, lysoPC (12.5 μM) was added (arrow) and relative change of $[Ca^{2+}]_i$ measured. A representative tracing of $n = 8$ cells is shown. (*G*) The mean ± SD of $[Ca^{2+}]_i$ changes are depicted in graphic form ($n = 8$ measurements per condition). The change in $[Ca^{2+}]_i$ was calculated as peak fluorescence ratio minus baseline ratio divided by baseline ratio (*$P < 0.001$ compared with overexpression of WT-CaM, †$P < 0.002$ compared with vector, **$P < 0.001$ compared with overexpression of TRPC6 and WT-CaM).

CaM-Dependent PI3K Activation Induces TRPC6 Externalization. The above studies suggested that CaM phosphorylated at Tyr^{99} activated a mechanism responsible for TRPC6 externalization. CaM kinase II was reported to be involved in TRPC6 activation in HEK cells overexpressing TRPC6 (10), and CaM kinase II is expressed in ECs. Pretreatment of BAECs for 1 h with a specific, cell-permeable, CaM kinase II inhibitor, autocamtide-2-related inhibitory peptide (10 μM, Millipore), did not alter lysoPC-induced TRPC6 externalization ($n = 4$, Fig. S6*A*).

The role of proline-rich tyrosine kinase 2 (Pyk2), a tyrosine kinase that is activated by lysoPC (18), interacts with CaM (19), and regulates ion channel function (20), was assessed. In BAECs transiently transfected with Pyk2 siRNA for 24 h, immunoblot analysis at 48 h confirmed down-regulation of Pyk2 ($n = 3$, Fig. S6*B*). LysoPC-induced TRPC6 externalization, however, was not altered by Pyk2 down-regulation ($n = 3$, Fig. S6*C*).

PI3K is involved in carbachol-induced TRPC6 externalization in smooth muscle cells (15), and PI3K activity is enhanced by CaM association with the p85 subunit of PI3K (17). To assess the role of PI3K, BAECs were transiently transfected with p110α siRNA or p85α siRNA for 24 h. Immunoblot analysis after 48 h confirmed down-regulation of p110α or p85α to 18 ± 2% or 12 ± 2% of basal level, respectively ($n = 3$, $P < 0.04$ compared with NsiRNA; Fig. S7 *A* and *B*). LysoPC-induced TRPC6 externalization was blocked by down-regulation of p110α or p85α ($n = 4$, $P < 0.02$, compared with NsiRNA transfected BAECs; Fig. 5 *A* and *B*), but total endogenous TRPC6 level was not altered. These findings supported a role for lysoPC-induced PI3K activation in TRPC6 externalization. Pretreating BAECs with a PI3K inhibitor, LY294002 (20 μM), for 1 h before incubation with lysoPC (12.5 μM) also blocked lysoPC-induced TRPC6 externalization ($n = 4$, $P < 0.02$ compared with lysoPC alone; Fig. 5*C*), but did not alter lysoPC-induced CaM dissociation from TRPC6 ($n = 3$, Fig. 5*D*).

To confirm the ability of lysoPC to activate PI3K, PIP3 production was assessed. Immunofluorescence microscopy studies demonstrated that lysoPC stimulated PIP3 production, but LY294002 blocked this increase ($n = 3$, Fig. 5*E*). Based on overlapping colors, PIP3 and TRPC6 colocalized to discrete areas of the cell membrane ($n = 3$, Fig. 5*E*). LysoPC (12.5 μM) increased PIP3 production measured by ELISA 2.0 ± 0.3-fold ($n = 4$, $P < 0.001$ compared with control), but pretreatment with LY294002 blocked PIP3 production in response to lysoPC ($n = 4$, Fig. 5*F*). These studies were consistent with lysoPC activating PI3K.

To determine if PI3K activation required TRPC6, PIP3 production was assessed in WT and $TRPC6^{-/-}$ MAECs. Basal PIP3 production measured by ELISA was similar in both cell types. LysoPC increased PIP3 production in both WT MAECs and $TRPC6^{-/-}$ MAECs with a 2.3 ± 0.1-fold and 2.2 ± 0.1-fold increase, respectively, compared with control ($n = 3$, Fig. 5*G*). These results were confirmed by confocal immunofluorescence microscopy ($n = 3$, Fig. S8). These findings suggested that lysoPC-induced PI3K activation and PIP3 production did not require TRPC6.

CaM Phosphorylation at Tyr^{99} Regulates PI3K Activation. The role of CaM phosphorylation in CaM binding to the p85 subunit and the interaction of p85 and p110 subunits was assessed. In BAECs incubated with lysoPC, the association of p85α and phospho-CaM as well as CaM was increased ($n = 4$, $P < 0.001$ compared with control; Fig. 6*A*). The similar density of bands for phospho-CaM and total CaM suggested that the majority of the CaM associated with p85α was phosphorylated. Therefore, using BAECs overexpressing WT-CaM or Phe^{99}-CaM, the requirement of CaM phosphorylation at Tyr^{99} was assessed. LysoPC increased the association of p85α and WT-CaM (3.92 ± 0.4-fold increase compared with control, $P < 0.01$) but not p85α and Phe^{99}-CaM ($n = 4$, Fig. 6*B*), suggesting that CaM phosphorylation at Tyr^{99} was required for lysoPC-stimulated CaM-p85α subunit association.

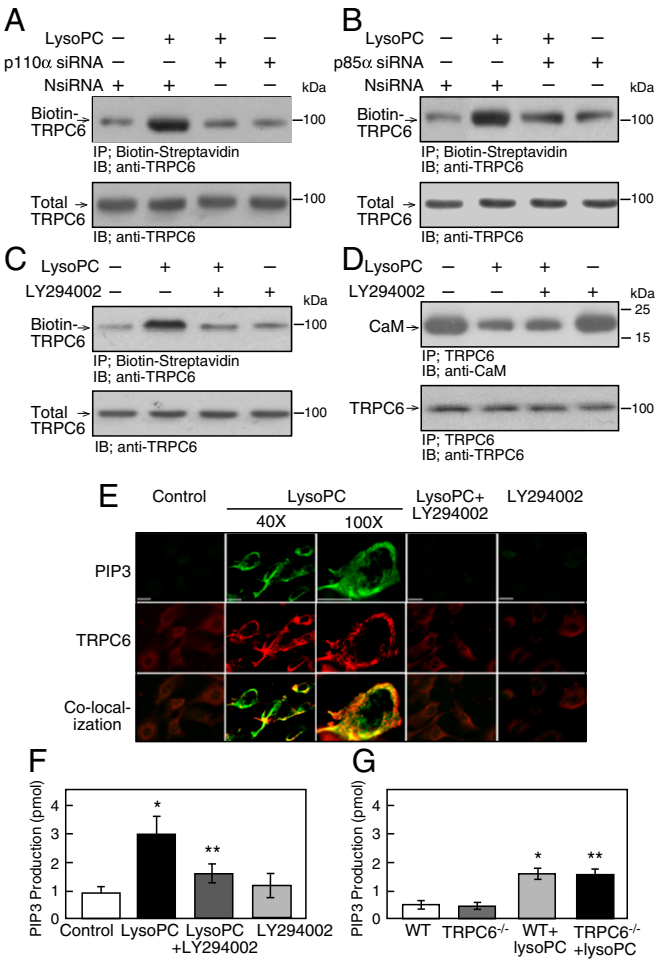

Fig. 5. LysoPC induces PIP3 production and PIP3-TRPC6 colocalization. (*A* and *B*) BAECs were transiently transfected with NsiRNA, p110α siRNA, or p85α siRNA, then incubated with lysoPC (12.5 μM) for 15 min. TRPC6 externalization was detected by biotinylation assay ($n = 4$). (*C* and *D*) BAECs were pretreated with LY294002 (20 μM) for 1 h, then lysoPC. (*C*) TRPC6 externalization was determined by biotinylation assay ($n = 4$). (*D*) TRPC6 was immunoprecipitated, and associated CaM or total TRPC6 was identified by immunoblot analysis ($n = 3$). (*E*) Confocal immunofluorescence microscopy identified PIP3 (green), TRPC6 (red), and PIP3-TRPC6 colocalization (yellow). Representative images of three experiments are shown. Columns 1, 2, 4, 5 show 40× magnification; column 3 shows 100× magnification. (Scale bar, 100 μm.) (*F*) PIP3 production was measured by ELISA and expressed as mean ± SD ($n = 4$, $^*P < 0.001$ compared with control and $^{**}P < 0.02$ compared with lysoPC alone). (*G*) WT or $TRPC6^{-/-}$ MAECs were incubated with lysoPC (10 μM). PIP3 production was measured by ELISA and expressed as mean ± SD ($n = 3$, $^*P < 0.01$ compared with WT and $^{**}P < 0.01$ compared with $TRPC6^{-/-}$).

Under control conditions, PIP3 localization by confocal immunofluorescence microscopy was similar in BAECs overexpressing WT-CaM or Phe^{99}-CaM. After incubation with lysoPC, staining for PIP3 increased in the membrane of BAECs overexpressing WT-CaM but not in BAECs overexpressing Phe^{99}-CaM ($n = 3$, Fig. 6*C*). Discrete areas of colocalization of PIP3 and TRPC6 were noted in BAECs overexpressing WT-CaM but not in BAECs overexpressing Phe^{99}-CaM ($n = 3$, Fig. 6*C*). Basal PIP3 production measured by ELISA was similar in both groups of BAECs (Fig. 6*D*). After incubation with lysoPC, PIP3 production in BAECs overexpressing WT-CaM increased 2.3 ± 0.2-fold compared with baseline ($P < 0.01$), but did not change in BAECs overexpressing Phe^{99}-CaM ($n = 3$, Fig. 6*D*). These results supported the pivotal role of CaM

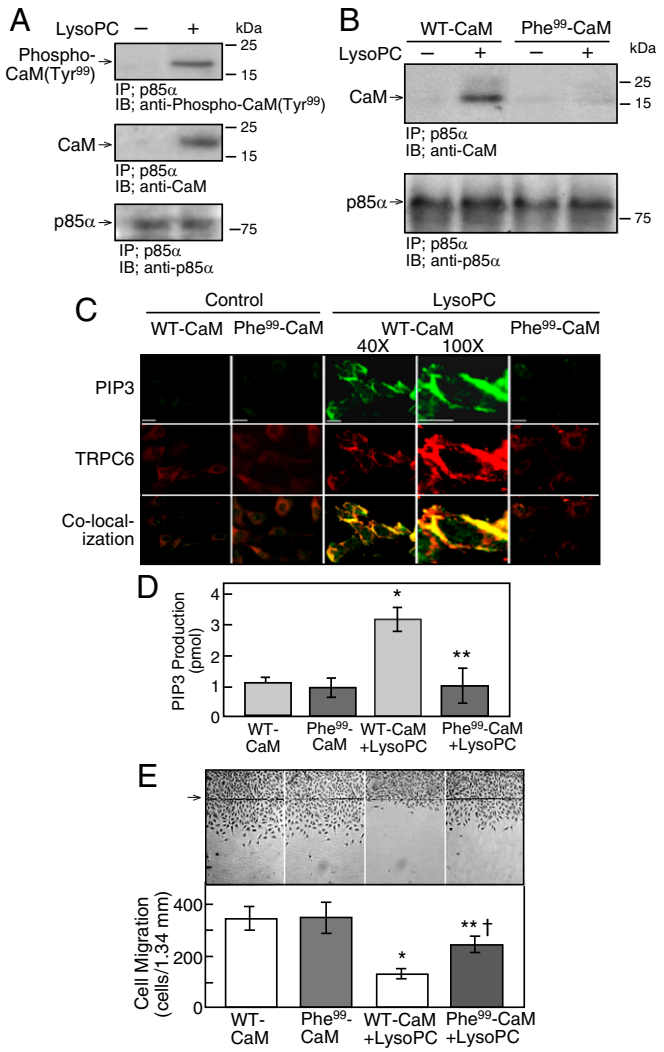

Fig. 6. In BAECs overexpressing Phe^{99}-CaM, lysoPC does not induce CaM-PI3K association, PIP3 production, PIP3-TRPC6 colocalization, or inhibit EC migration. (*A*) BAECs were incubated with lysoPC (12.5 μM) for 15 min. The p85α subunit of PI3K was immunoprecipitated, and associated phospho-CaM(Tyr^{99}), CaM, or total p85α subunit identified by immunoblot analysis (n = 4). (*B–F*) BAECs were transiently transfected to overexpress WT-CaM or Phe^{99}-CaM. (*B–D*) BAECs were incubated with lysoPC. (*B*) The p85α subunit of PI3K was immunoprecipitated, and associated CaM or total p85α subunit was identified by immunoblot analysis (n = 4). (*C*) Confocal immunofluorescence microscopy was used to identify PIP3 (green), TRPC6 (red), or PIP3-TRPC6 colocalization (yellow). Representative images of three experiments are shown. Columns 1, 2, 3, 5 show 40× magnification; column 4 shows 100× magnification. (Scale bar, 100 μm.) (*D*) PIP3 production was measured by ELISA (n = 3). Results are represented as mean ± SD (*P < 0.01 compared with WT-CaM control; **P < 0.001 compared with WT-CaM incubated with lysoPC). (*E*) Migration was assessed after 24 h in the presence or absence of lysoPC (12.5 μM). Arrow identifies the starting line of migration. (*Upper*) Representative images of three experiments are shown 40× magnification. (Scale bar, 100 μm.) (*Lower*) Migration represented as mean ± SD (n = 3, *P < 0.001 compared with WT-CaM control, **P < 0.001 compared with Phe^{99}-CaM control, and $^{\dagger}P$ < 0.001 compared with WT-CaM with lysoPC).

phosphorylation at Tyr^{99} in lysoPC-induced PI3K activation and TRPC6 externalization.

Basal migration of BAECs transiently transfected with empty vector or overexpressing WT-CaM or Phe^{99}-CaM was similar. LysoPC inhibited BAEC migration by 63% in BAECs overexpressing WT-CaM, but only by 30% in BAECs overexpressing Phe^{99}-CaM (n = 3, P < 0.001 compared with WT-CaM with lysoPC; Fig. 6*E*). These results revealed for the first time to our knowledge that CaM phosphorylation at Tyr^{99} contributes to lysoPC's anti-migratory activity by activating PI3K and promoting externalization of TRPC6. Our model for the proposed sequence of events is shown in Fig. 7.

Discussion

CaM contributes to the regulation of TRPC channels, including TRPC6. In HEK cells stably transfected to overexpress TRPC6, CaM inhibitors decrease CaM binding to TRPC6, TRPC6 channel activity, and Ca^{2+} influx in response to receptor activation by carbachol when intracellular stores are depleted (21), suggesting that dissociation of CaM from TRPC6 results in decreased channel activity. CaM inhibitors, however, may decrease TRPC6 channel activity by blocking CaM's activation of PI3K. In fact, we find that CaM dissociation from TRPC6 is necessary but not sufficient for lysoPC-induced TRPC6 externalization (Fig. 1). When CaM is down-regulated with siRNA, decreasing CaM binding to TRPC6, TRPC6 is not externalized.

Phosphorylation of CaM at Tyr^{99} by a Src family kinase is required for lysoPC-induced dissociation of CaM from TRPC6. Activation of Src kinase requires Ca^{2+} and can occur as a result of increased $[Ca^{2+}]_i$ (22, 23). In our previous study, we have shown lysoPC-induced TRPC6 externalization in BAECs was not blocked in Ca^{2+}-free KR buffer or by using BAPTA/AM (25 μM) (3). At this concentration, BAPTA/AM blunts the global increase in $[Ca^{2+}]_i$, but may not chelate all Ca^{2+}. Results of the present study, using a higher concentration of BAPTA/AM, suggest that a small increase in Ca^{2+} is required for kinase activation and CaM phosphorylation with subsequent TRPC6 externalization (Fig. 2).

The source of Ca^{2+} required for kinase activation is unclear. LysoPC could disrupt the membrane lipid bilayer affecting ion channels or acting as a detergent, but the concentration used is below the critical micellar concentration of 40–50 μM (1) and the membrane detergent, saponin, does not increase TRPC6 phosphorylation (Fig. S9*A*) as does lysoPC (3). LysoPC alters cell membrane microviscosity, potentially altering ion channel function, but preincubation with α-tocopherol, which restores microviscosity to normal (24), does not prevent lysoPC-induced TRPC6

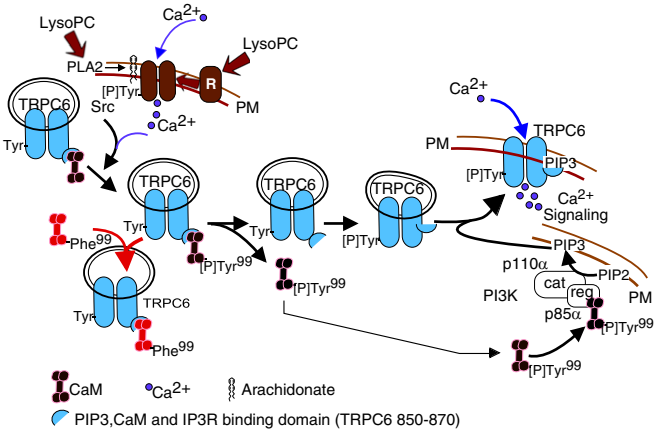

Fig. 7. Model of events following EC exposure to lysoPC. LysoPC activates a Src family kinase that phosphorylates (P) CaM at Tyr^{99}. Phosphorylated CaM dissociates from TRPC6. TRPC6 is phosphorylated. Phosphorylated CaM binds to the p85α subunit of PI3K, activating it. The increased PIP3 in the plasma membrane (PM) serves to anchor TRPC6 there. When CaM is mutated at Tyr^{99} to Phe^{99}, this sequence of events is disrupted. LysoPC might initiate these events through receptor activation or release of arachidonate and activation of arachidonate-regulated Ca^{2+} channels. Neither the identity of the receptor (R) nor the identity of the channel activated to allow entry of the initial local Ca^{2+} trigger are known (brown colored).

externalization (Fig. S9*B*). Pertussis toxin does not alter lysoPC-induced TRPC6 externalization (Fig. S9*C*), suggesting that G_i-proteins are not involved. LysoPC can induce arachadonic acid release (25), causing Ca^{2+} entry through arachidonate-regulated Ca^{2+} channels (26). This could contribute to the Ca^{2+} needed for Src kinase activation and subsequent events.

Phosphoinositides, especially PIP3, bind to TRPC6, and have been reported to disrupt CaM binding and increase TRPC6 current (16). In our studies, lysoPC induces dissociation of TRPC6 and CaM, even when PI3K and PIP3 production are inhibited (Fig. 5*D*), suggesting that PIP3 binding is not required to displace CaM from TRPC6. Dissociation of CaM from TRPC6 by both PIP3 binding and CaM phosphorylation may contribute to TRPC6 externalization. Phospho-CaM, by promoting PI3K activation and increased PIP3 production, may create a feed-forward effect, causing a prolonged elevation of $[Ca^{2+}]_i$ that contributes to pathological effects of lysoPC, including inhibition of EC migration.

Although tethering of Ca^{2+} to CaM alters its protein affinity and the Ca^{2+}-CaM complex regulates many proteins, our studies suggest that CaM phosphorylation at Tyr^{99} is essential in lysoPC-induced PI3K activation. CaM regulates PI3K activation by binding to an SH2 domain of the p85 regulatory subunit of PI3K (17), and this can cause a conformational change that releases the p110 catalytic subunit from the inhibitory effects of the p85 subunit, increasing p110's catalytic activity (27).

PI3K activation results in elevated levels of PIP3 and PIP3-TRPC6 association in the EC plasma membrane. Similarly, vasopressin-induced TRPC6 externalization is mediated by PI3K activation in smooth muscle cells (15). PI3K inhibition with LY294002 blocks lysoPC-induced PIP3 production and externalization of TRPC6 (Fig. 5). LY294002 can also inhibit PI4K, leading to reduced generation of PIP2 and depletion of membrane PIP2, but not at the low concentration (20 μM) used in our studies (15, 28). The role of PI3K in TRPC6 externalization is confirmed by down-regulating PI3K subunits (Fig. 5).

PIP3 formation is critical for lysoPC-induced TRPC6 externalization. PIP3 in the cell membrane can bind to TRPC6, specifically the C terminus, and promote anchoring of TRPC6-containing vesicles in the plasma membrane, increasing TRPC6 activity and $[Ca^{2+}]_i$ (15, 16, 29). Membrane colocalization may be due to direct binding of TRPC6 to PIP3 or binding through a protein partner. PIP3 facilitates docking of proteins with a PH domain, and TRPC6 is reported to have two putative PH-like motifs (30), but their role in PIP3-TRPC6 interaction is unclear. PIP3 binding to a PH domain can induce conformational changes that affect protein function, or PIP3 binding can serve to colocalize proteins and regulate interactions such as oligomerization (31). On the other hand, TRPC6 might bind to PIP3 through a protein partner as has been reported for other TRPC proteins (32). Further studies are needed to determine if an adapter protein is required for TRPC6 binding to PIP3.

Materials and Methods

Expanded methods are available in *SI Materials and Methods*. Animal use was approved by the Institutional Animal Care and Use Committee of the Cleveland Clinic.

The full-length human CaM cDNA (MGC-7) was obtained from ATCC. PCR-based site-directed mutagenesis was used to generate cDNAs for mutant CaMs in which Tyr^{99} or Tyr^{138} was replaced with Phe or Asp (ExonBio), generating mutants Phe^{99}-CaM, Asp^{99}-CaM, or Phe^{138}-CaM. Sequence analysis verified the mutations. BAECs at 60% confluence were transiently transfected with 2 μg of plasmids containing pcDNA3.1-myc-His, pcDNA 3.1-myc-His-WT-CaM, pcDNA3.1-myc-His-Phe^{99}-CaM, pcDNA3.1-myc-His-Asp^{99}-CaM, pcDNA3.1-myc-His-Phe^{138}-CaM, and pcDNA3-TRPC6 using Effectene (Qiagen) according to the manufacturer's protocol.

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