Caveolae Facilitate but Are Not Essential for Platelet-Activating Factor-Mediated Calcium Mobilization and Extracellular Signal-Regulated Kinase Activation

Caroline Poisson, Simon Rollin, Steeve Véronneau, Simon M. Bousquet, Jean-François Larrivée, Christian Le Gouill, Guylain Boulay, Jana Stankova and Marek Rola-Pleszczynski

*J Immunol* 2009; 183:2747-2757; Prepublished online 20 July 2009; doi: 10.4049/jimmunol.0802651
http://www.jimmunol.org/content/183/4/2747

**References**
This article *cites 68 articles*, 39 of which you can access for free at: http://www.jimmunol.org/content/183/4/2747.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Caveolae Facilitate but Are Not Essential for Platelet-Activating Factor-Mediated Calcium Mobilization and Extracellular Signal-Regulated Kinase Activation

Caroline Poisson,* Simon Rollin,* Steeve Véronneau,* Simon M. Bousquet,† Jean-François Larrivée,* Christian Le Gouill,* Guylain Boulay,† Jana Stankova,* and Marek Rola-Pleszczynski2*

Certain proteins, including receptors and signaling molecules, are known to be enriched in caveolae and lipid rafts. Caveolin-1, the major structural protein of caveolae, specifically interacts with many signaling molecules and, thus, caveolae and lipid rafts are often seen as preassembled signaling platforms. A potential binding site for caveolin-1 is present in the platelet-activating factor receptor (PAFR) sequence, and many downstream signaling components of PAFR activation preferentially localize in caveolae. The aim of this study was to investigate whether the PAFR was localized in caveolae/lipid raft domains and, if so, what would be the significance of such localization for PAFR signaling. In this study, we demonstrate that PAFR localizes within membrane microdomains, in close proximity to caveolin-1 in living cells, with potential interaction through a caveolin-1-binding sequence in the PAFR C terminus. Caveolin-1, however, is not essential for PAFR localization in lipid rafts. Disruption of caveolae/lipid rafts with methyl-β-cyclodextrin markedly reduced PAF-triggered inositol phosphate production and cytosolic calcium flux, suggesting that PAFR signaling through the Gq protein was critically dependent on integrity of lipid rafts and/or caveolae. Interestingly, whereas in caveolin-1-expressing cells lipid raft disruption markedly decreased PAFR-mediated activation of the ERK/ MAPK pathway, in cells lacking caveolae, such as leukocytes, lipid raft disruption had either the same inhibitory effect (Ramos B cells) or no effect (monocytes) on PAFR capacity to signal through the ERK/MAPK pathway. In conclusion, PAFR appears to localize within caveolae or lipid rafts in different cell types, and this location may be important for specific signaling events.

*Immunology Division, Department of Pediatrics, and †Department of Pharmacology, Faculty of Medicine, Université de Sherbrooke, Sherbrooke, Canada

Received for publication August 12, 2008. Accepted for publication June 10, 2009.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by grants from the Canadian Institutes of Health Research (to G.B., J.S., and M.R.-P.) and by a Canada Research Chair in Inflammation (to G.B., J.S., and M.R.-P.). G.B., J.S., and M.R.-P. are members of the Fonds de la recherche en santé du Québec-funded Étienne-LeBel Clinical Research Center.

2 Address correspondence and reprint requests to Dr. Marek Rola-Pleszczynski, Immunology Division, Department of Pediatrics, Faculty of Medicine and Health Sciences, Université de Sherbrooke, 3001 North 12th Avenue, Sherbrooke, QC Canada J1H 5N4. E-mail address: marek.rola-pleszczynski@usherbrooke.ca

3 Abbreviations used in this paper: PAF, platelet-activating factor; AEBSF, 4-(2-aminooethyl)benzenesulfonyl fluoride; BRET, bioluminescence resonance energy transfer; [Ca2+]i, intracellular Ca2+ concentration; CD, methyl-β-cyclodextrin; CHO, Chinese hamster ovary; EtOH, ethanol; GPCRs, G protein-coupled receptors; HA, hemagglutinin; IP, inositol phosphate; WT, wild type.

Copyright © 2009 by The American Association of Immunologists, Inc. 0022-1767/09/$2.00

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0802651

Platelet-activating factor (PAF)1 (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) is a very potent inflammatory mediator and was the first bioactive phospholipid to be characterized structurally (1). It mediates its actions upon binding to PAFR, a member of the G protein-coupled receptor superfamily (2). PAF and PAFR are known to play an important role in many pathophysiologic disorders, including anaphylaxis, bronchial asthma, endotoxic shock, atherosclerosis, streptococcal infection, skin diseases, and disorders of the CNS (3). PAFR has been the object of extensive research in the last 10 years, and some of the signaling pathways used by this receptor have been defined, although variations have been reported depending on cell types and methodologies (as reviewed in Ref. 4). Activation of the Gq/11 G protein subunits, phosphorylation of p42/p44 MAPK, and elevation of intracellular calcium levels are, however, quite well-established signaling pathways triggered by this receptor. The structure-function relationship of the receptor has also been studied by mutagenesis and transfection systems. The latter helped reveal, among others, the amino acids implicated in ligand binding, stability, G protein coupling, internalization, Jak kinase activation, and phosphorylation of the PAFR, in addition to proposing mechanisms for desensitization and transcriptional regulation of the receptor (4–9). In addition, analysis of the sequence of PAFR revealed the presence of a putative binding motif for caveolin-1.

Caveolin-1 is the major structural protein of caveolae, which are a subtype of lipid rafts, also described as liquid-ordered domains of the plasma membrane enriched in cholesterol and glycosphingolipids. Certain proteins, including receptors and signaling molecules, are known to be enriched in caveolae and lipid rafts. It has been demonstrated that caveolin-1 specifically interacts with many signaling molecules, such as ERK1/2 (10), Src (11–13), Ras (14), protein kinase C (15), and Gq protein (16), and Gq protein coupling, internalization, Jak kinase activation, and phosphorylation of the PAFR, in addition to proposing mechanisms for desensitization and transcriptional regulation of the receptor (4–9). In addition, analysis of the sequence of PAFR revealed the presence of a putative binding motif for caveolin-1.
Given the presence of a potential binding site for caveolin-1 in the PAFR sequence (aa 293–300: YXFXxxxxF) and the many downstream signal components of the PAFR activation cascade that are preferentially localized in caveolae, the aim of this study was to investigate whether the PAFR was localized in caveolae/lipid raft domains and, if so, what would be the significance of such localization for PAFR signaling.

Materials and Methods

Materials

Reagents were obtained from the following sources: oligonucleotides were synthesized at Sigma Genosys (Sigma-Aldrich). BSA, methyl β-D-cyclodextrin (CD), MES, leupeptin, aprotinin, soybean trypsin inhibitor, 4-(2-amino-ethyl)benzenesulfonyl fluoride (AEBSF), Na₃VO₄, HBSS, and protein A-Sepharose were from Sigma-Aldrich. Calcycin A was from BIOMOL. DMEM high glucose, RPMI 1640, fura 2, and pDNA3 vector were from Invitrogen. [3H]WEB2086 and DeepBlueC were from Promega (Fisher Scientific). Nitrocellulose transfer membrane, AG-X1 resin, and Poly-prep column were from Bio-Rad. Myo-[3H]Insitol was from Amersham Biosciences. Caveolin-1 high glucose without insulin was from United States Biological. FuGENE6 transfection reagent was from Roche Diagnostics. PAF C-16 was from Calbiochem. Pirenlicin/streptomycin solution was from Wisent Biotech Pvt Ltd. DMEM high glucose, RPMI 1640, and DMEM/F-12 containing 100 µg/ml penicillin, 100 µg/ml streptomycin, 100 IU of penicillin G, and 100 µg/ml streptomycin was from Invitrogen. [3H]inositol and 0.2% FBS. Forty-eight hours posttransfection, cells were seeded in 0.5–1 ml of immunoprecipitation buffer (PBS, 1% Triton X-100, 0.5% Nonidet P-40, 5 mM EDTA, 1 µM leupeptin, 10 µg/ml soybean trypsin inhibitor, 2 µg/ml aprotinin, 100 µg/ml AEBSF, 1 mM Na₃VO₄, 10 µM NaF, and 10 mM calyculin A). Lysates were precleared with 50 µl of protein A-Sepharose bead suspension at 4°C with continuous rotation for 1 h. Lysates were then centrifuged at 13,000 rpm for 10 min, and the precleared lysates were incubated with indicated Abs overnight at 4°C. A total of 30 µl of BSA-treated bead suspension was then added and incubated for 2 h at 4°C. After incubation, beads were washed several times with buffer and proteins were eluted from beads by addition of 4× SDS sample buffer, resolved by SDS-PAGE, and subjected to Western blot analysis using goat anti-PAFR and rabbit anti-caveolin-1 Abs (Santa Cruz Biotechnology).

Cell culture, transfections, and stimulations

All cell cultures were originally from American Type Culture Collection. HEK 293, Ramos, and A431 cells were, respectively, cultured in DMEM high glucose, RPMI 1640, and DMEM/F-12 containing 100 µg/ml penicillin and 100 IU of streptomycin and supplemented with 10% FBS (Sigma-Aldrich). Chinese hamster ovary (CHO) cells were cultured in DMEM/F-12 supplemented with 5% FBS. FuGENE6 transfection reagent was used for all transfections, according to the manufacturer’s instructions. Before all stimulations, cells were starved (0.1% FBS) overnight and stimulations were conducted in medium containing 0.1% FBS and 0.1% BSA.

Human peripheral blood monocytes were obtained by venipuncture from medication-free normal volunteers following informed consent, as approved by the institutional Ethics Review Board, as previously described (18).

Cholesterol depletion and repletion

To deplete cholesterol, unless specified otherwise, cells were treated with CD (10 mM in buffer A: 10 mM HEPES, 0.15 M NaCl, 4 mM KCl, 11 mM glucose (pH 7.5)) for 30–45 min at 37°C. Then the cells were washed with buffer A and immediately used for functional activity or binding assays. Cholesterol depletion was performed, as described by Mandal et al. (19). Cholesterol (water-soluble; Sigma-Aldrich) was loaded onto cells by incubating them with cholesterol/CD complexes (1 mM cholesterol:10 mM CD) and immediately used for functional activity or binding assays. Cholesterol (water-soluble; Sigma-Aldrich) was loaded onto cells by incubating them with cholesterol/CD complexes (1 mM cholesterol:10 mM CD) and immediately used for functional activity or binding assays. To deplete cholesterol, unless specified otherwise, cells were treated with CD (10 mM in buffer A: 10 mM HEPES, 0.15 M NaCl, 4 mM KCl, 11 mM glucose (pH 7.5)) for 30–45 min at 37°C. Then the cells were washed with buffer A and immediately used for functional activity or binding assays. Cholesterol depletion was performed, as described by Mandal et al. (19). Cholesterol (water-soluble; Sigma-Aldrich) was loaded onto cells by incubating them with cholesterol/CD complexes (1 mM cholesterol:10 mM CD) and immediately used for functional activity or binding assays. Human peripheral blood monocytes were obtained by venipuncture from medication-free normal volunteers following informed consent, as approved by the institutional Ethics Review Board, as previously described (18).

Caveolin-1 cloning

Caveolin-1 was cloned from HUVEC total RNA extract. RT-PCR was performed to obtain cDNAs, and a final PCR was performed to obtain caveolin-1 using the following primers: 5′-GAGGTTACCCGACGATGGG-3′ and 5′-TCGCTAGCTCATCTTCTTCTGCA ACTTCTGCG-3′. Caveolin-1 was then transferred into pGEM-3Zf vector using EcoRI and Xbal restriction enzymes. Caveolin-1 cDNA was tagged with nuclease resistant HA epitope by tagging with a synthetic oligonucleotide (5′-GGGGTACCATCGTTCGTTGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT
25 nM HEPES (pH 7.3)) containing 0.1% FBS, and incubated with indicated concentrations of CD or PBS for 30 min at 37°C. Cells were centrifuged and resuspended in buffer containing 3 μM fura 2-AM (Invitrogen) for 15 min at 25°C, washed, and resuspended in 1 ml of buffer for fura 2 hydrolysis at 25°C for 1–1.5 h. After incubation, cells were washed twice in buffer and intracellular fluorescence was recorded at 37°C using a Hitachi F-2500 fluorescence spectrophotometer (Chromatoque HPLC) with the following settings: 340 nm EX WL, 510 nm EM WL, 400 V, 5 nM EX Slit, 10 nM EM Slit, and 600 s recording time. Cells were stimulated with 100 nM PAF. Triton X-100 and EGTA solution (100 mM Tris and 125 mM EGTA (pH 9.0)) were used to determine maximal and minimal fluorescence values, respectively. Calcium mobilization (nM) was calculated using FL Solutions software.

**Intracellular calcium mobilization (microscopy)**

Based on previously published methods (20, 21), A431 cells were seeded on coverslips and allowed to adhere. Cells were then washed with HBSS and loaded with 0.2 μM fura 2 in HBSS for 20 min at 25°C. Cells were washed with HBSS and pretreated or not with cycloheximide for 30 min at 25°C. Cells were washed again, inserted into a circular open-bottom chamber, and placed on the stage of Zeiss Axiosvert microscope fitted with an Attofluor Digital Imaging and Photometry System (Attofluor). Approximately 50–60 cells were selected on each coverslip for fluorescence recording. Intracellular calcium release was measured by fluorescence videomicroscopy at 25°C using alternating excitation wavelengths of 334 and 380 nm and monitoring emitted fluorescence at 520 nm. Free intracellular Ca2+ concentration ([Ca2+]) was calculated from 334/380 fluorescence ratios, as previously described (22). PAF was diluted to its final concentration in HBSS/0.1% BSA and applied to cells by surface perfusion.

**Intracellular signaling**

HEK 293 cells were seeded in 6-well plates and transfected with plasmid encoding c-Myc-tagged PAFR 48 h before experiment. One 100-mm petri dish of A431 and 5 × 10⁵ Ramos were used for each time point. For all cell types, stimulations were conducted for 5 min at 37°C with 10 nM PAF or EtOH after pretreatment of cells with 10 mM cycloheximide or 1 × 10⁻⁴ M WEB2086. Cells were then lysed in ice-cold buffer (10 mM Tris (pH 7.4), 10 mM NaCl, 3 mM MgCl₂, 0.5 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.5% deoxycholate, 1 μM leupeptin, 10 μg/mL soybean trypsin inhibitor, 2 μg/mL aprotinin, 100 μg/mL AEBSF, 1 mM Na₃VO₄, 10 μM NaF, and 10 nM calyculin A). Lysates were then centrifuged, and supernatants were stored at −80°C until used. Otherwise, cleared cell lysates were mixed with 4× Laemmli buffer and subjected to SDS-PAGE and Western blot analysis using mouse anti-p-ERK1/2 Ab. Membranes were then stripped and reprobed with rabbit anti-ERK1 Ab (Santa Cruz Biotechnology).

**Radioligand-binding assay**

Competition-binding curves were done, as previously described (23), with modifications. Briefly, experiments were done on HEK 293 cells transiently expressing the c-Myc-tagged PAFR. Cells were harvested and washed twice in PBS, pretreated with 10 mM CD for 30 min at 37°C, and then washed again with PBS. Binding reactions were conducted in 250 μL of HEPES Tyrode’s buffer (140 mM NaCl, 2.7 mM KCl, 1 mM CaCl₂, 12 mM NaHCO₃, 5.6 mM d-glucose, 0.49 mM MgCl₂, 0.37 mM Na₃PO₄, and 25 mM HEPES (pH 7.4)) containing 0.1% BSA. A total of 5 × 10⁴ cells was incubated with 10 nM [3H]WEB2086 (PerkinElmer) and increasing concentrations of nonradioactive PAF for 90 min at room temperature. Reactions were stopped by centrifugation, pellets were washed in Tyrode’s buffer, and cell-associated radioactivity was measured by liquid scintillation.

**GST pull-down assay**

BL21 Escherichia coli transformed with plasmids encoding all the intracellular loops and the C-tail of PAFR were grown to log phase at 37°C in YT medium with ampicillin (100 μg/ml). Expression of the fusion proteins was induced with 1 mM isopropyl-1-thio-D-galactopyranoside for 4 h at 37°C. Bacterial pellets were lysed in lysis buffer (1.2 M d-glucose, 10 mM EDTA, 50 mM Tris-HCl (pH 8.0), 0.1 mg/mL AEBSF, and 4 μg/mL lysozyme) for 30 min on ice. For complete lysis, bacterial lysates were frozen in liquid N₂ and subsequently thawed at 60°C and put back on ice. After a 30-min centrifugation at 4°C (13,000 rpm), supernatants containing GST-fusion proteins were purified using glutathione-agarose beads for 3 h at 4°C. Purified GST-fusion proteins were subsequently quantified by Coomassie staining of a 10% SDS-PAGE. A total of 1 μg of each freshly purified recombinant protein was incubated for 12 h at 4°C with cell lysate of HEK 293 cells transiently transfected with 5 μg of human pcDNA-caveolin-1. Beads were pelleted and washed three times with ice-cold radioimmunoprecipitation assay solution (150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1% Igepal CA-630, 0.5% sodium-deoxycholate, 0.1% SDS, and 5 mM EDTA). Bound proteins were eluted with SDS-PAGE sample buffer for 20 min, then heated at 60°C for 5 min, and stored on ice. Proteins were separated by a 10% SDS-PAGE and subjected to Western blot analysis.

**Statistical analysis**

Data were analyzed using Student’s t test or ANOVA with Neumann-Keuls multiple comparison test, as appropriate, using the Prism 4.0 software (GraphPad). A p value of <0.05 was considered significant.

**Results**

PAFR is localized to low-density microdomains

Caveolae isolation was performed, as previously described (24), on CHO cells stably expressing a c-Myc epitope-tagged human PAFR (23). PAFR (as revealed with anti-c-Myc Ab) was found to colocalize with caveolin-1, an integral membrane protein of caveolae (25), at the 5–35% sucrose interface, corresponding to low-density membrane domains (Fig. 1A). This colocalization was also observed using HEK 293 cells transiently expressing PAFR (Fig. 1B), as revealed with an anti-PAFR Ab. To ensure specificity of this PAFR position in the sucrose gradient, similar experiments were performed on COS-7 cells transiently expressing a PAFR-GFP construct, and fluorescence was measured in each fraction of the sucrose gradient.
These experiments confirmed both PAFR localization in low-density membrane fractions and colocalization of PAFR with caveolin-1. We next looked at a cell line that naturally expresses PAFR to verify the localization of the receptor without overexpression. We used the A431 keratinocyte cell line, which also naturally expresses caveolin-1 to help determine whether caveolin-1 is essential for PAFR localization in low-density membrane domains. As shown in Fig. 1C, PAFR is partially localized in lipid rafts in A431 cells, because only part of the PAFR pool colocalizes with caveolin-1 in fraction 5.

**PAFR and caveolin-1 are colocalized in living cells**

Because our method for isolating low-density membrane microdomains could not discriminate between caveolae and noncaveolar rafts, we therefore performed BRET2 analysis to verify whether PAFR was localized within 10 angstroms from caveolin-1. We cotransfected different plasmid combinations (500 ng of each plasmid) into HEK 293 cells and compared BRET2 ratios among combinations (Fig. 2D). Luciferase-coupled CD4 and GFP-coupled PAFR combination gave a ratio of 0.046, which was significantly different from luciferase-coupled PAFR and GFP-coupled caveolin-1 combination (0.097, \( p < 0.01 \)) and LUC-coupled caveolin-1 and GFP-coupled PAFR combination (0.077, \( p < 0.05 \)), using one-way ANOVA with Newman-Keuls multiple comparison test. We had previously shown that PAFR can form oligomers at the membrane in resting cells (26). We thus used a combination of luciferase-coupled PAFR and GFP-coupled PAFR as a positive control for BRET2 comparison. The resulting BRET2 signal (0.121, \( p < 0.01 \)) was significantly higher than that of luciferase-coupled CD4 and GFP-coupled PAFR combination and similar to BRET2 ratios obtained by guest on October 3, 2017 http://www.jimmunol.org/Downloaded from

**FIGURE 2.** BRET2 assay for interaction between caveolin-1 and PAFR on HEK 293 cells. A, A total of 300 ng of luciferase-coupled caveolin-1 (Cav-1-LUC) or 300 ng of luciferase-coupled CD4 cDNA (CD4-LUC) was cotransfected in HEK 293 cells with increasing amounts of PAFR-GFP cDNAs, and BRET2 signal was analyzed. B, Same experiment was done using 400 ng of PAFR-LUC with increasing amounts of Cav-1-GFP. Graph represents means of two to four independent experiments, each done in triplicate. C, Cells were transfected with 500 ng of each construct, and BRET2 signal was analyzed. Graph represents means of three to five independent experiments, each done in triplicate (\(*, p < 0.05; **, p < 0.01\)). D, Colocalization of caveolin-GFP (green) with PAFR-Myc (red) in transiently transfected HEK 293 cells, as revealed by confocal microscopy following a 10-min stimulation with either ethanol (PAF vehicle: Vh) or \( 10^{-7} \) M PAF. Lower right panel in each block shows colocalization mask (white). Scale bar = 10 \( \mu \text{m} \).
between caveolin-1 and PAFR. Caveolin-1 is also well known to form homodimers as well as oligomers (12, 13, 27–30), these complexes being the structural assembly units of caveolae. We therefore used a combination of luciferase-coupled caveolin-1 and GFP-coupled caveolin-1 constructs to assess the maximal BRET2 ratio possible because the diameter of caveole (50–100 nm) is 5–10 times greater than the maximal distance for a positive BRET2 signal. As expected, the BRET2 ratio was markedly greater (0.433, p < 0.01) than the negative control. Concentration-response curves using 300 ng of luciferase-coupled caveolin-1 and increasing amounts of GFP-coupled caveolin-1 ranging from 20 to 400 ng showed a similar pattern to those obtained in Fig. 2, A and C, ratios varying from 0.057 to 0.521 (data not shown). Taken together, these results strongly suggest that at least part of the PAFR pool in live, resting cells is localized in close proximity to caveolin-1. When caveolin-1 is present, it could therefore play a role in positioning PAFR within lipid rafts, or caveolae.

Additional evidence for PAFR and caveolin colocalization was provided by confocal microscopy, using GFP-labeled caveolin and Myc-tagged PAFR, as illustrated in Fig. 2D. Colocalization persisted following stimulation with 10⁻⁷ M PAF.

**PAFR coimmunoprecipitates with caveolin-1** BRET2 experiments showed PAFR and caveolin-1 to be closely localized in the cell membrane of living cells. Because of the putative binding motif for caveolin-1 in the C-terminal portion of the PAFR, we performed coimmunoprecipitation assays to see whether both proteins could be physically associated. HEK 293 cells were transfected with c-Myc epitope-tagged human PAFR or HA epitope-tagged human caveolin-1, or both, and immunoprecipitation of PAFR using anti-Myc mAb was conducted. Western blot analysis using anti-PAFR Ab confirmed PAFR precipitation and anti-caveolin-1 Ab revealed a 24-kDa band when both constructs were cotransfected (Fig. 3A). The same experiments were performed using anti-HA mAb to immunoprecipitate caveolin-1 (Fig. 3B). Identity of caveolin-1 was verified using anti-caveolin-1 Ab. Using anti-PAFR Ab, we found again that PAFR associated with caveolin-1, when both proteins were coexpressed in cells. Similar results were obtained in COS-7 cells (data not shown). This strongly suggested that PAFR and caveolin-1 were physically associated and, thus, that a portion of the PAFR pool could be complexed with caveolin-1 and localized in caveolar rafts when both proteins were expressed in the same cell.

**Determination of the potential binding site for caveolin-1 on PAFR**

To determine which part of PAFR is responsible for caveolin-1 binding, we used a GST pull-down assay to pull down HA-tagged caveolin-1 with GST-fused intracellular loops and C-tail of PAFR, as described in Materials and Methods (Fig. 3, C and D). Following separation of proteins from the glutathione-agarose beads, SDS-PAGE and Western blot analysis were performed and a 24-kDa band interacting with the GST C-tail of PAFR was revealed using anti-caveolin-1 Ab. This result suggested that the site of PAFR interaction with caveolin-1 was probably situated in the C-terminus domain of the receptor, which corresponds to the position of the putative binding motif for caveolin-1(17) found in PAFR. We next used a PAFR mutant in which the three aromatic residues (Y293, F295, and F300) in the putative caveolin binding site had been mutated to alamines (3ΔA) to assess whether this mutation prevented PAFR association with caveolin. As shown in Fig. 3, E and F, coimmunoprecipitation of caveolin-1 with the mutated PAFR3ΔA was markedly reduced compared with that with WT PAFR, suggesting that this putative binding site was important for PAFR-caveolin-1 association.

**Functional consequences of PAFR3ΔA mutation or caveolin overexpression**

As illustrated in Fig. 4, the mutated PAFR3ΔA receptor failed to respond to PAF in terms of IP production (Fig. 4A) and showed a greatly diminished response in terms of ERK1/2 phosphorylation (Fig. 4B), suggesting that caveolin association with PAFR via its C-terminus domain was important for receptor-mediated signaling. In contrast, overexpression of caveolin-1 did not result in enhanced PAFR signaling, but rather in a relative inhibition of IP production (Fig. 4C) and ERK1/2 phosphorylation (Fig. 4D).

**Integrity of caveolae rafts is important for PAF-induced IP production**

Because PAFR is localized in low-density microdomains, we determined the efficiency of PAFR response, measured by IP production, following disruption of rafts with CD, a cholesterol-sequestering agent. HEK 293 cells transiently transfected with...
human PAFR were treated or not with 10 mM CD for 30 min, washed, and stimulated with graded concentrations of PAF for 15 min to determine IP production (Fig. 5A). When cells were pre-treated with CD, PAF-induced IP production was significantly reduced for PAF concentrations ranging from 1 to 1000 nM, and the EC₅₀ was markedly shifted to the right. CHO cells stably expressing human PAFR were also used to determine the effect of lipid raft disruption on PAF-induced IP production (Fig. 5B), and results

FIGURE 4. Functional effects of PAFR 3αA mutation and caveolin overexpression on IP production and ERK phosphorylation. HEK 293 were transiently transfected with either WT or a mutated (3αA) form of PAFR (A and B), or with WT PAFR ± caveolin-1 (C and D). IP formation was determined following stimulation with either vehicle or 10⁻⁷ M PAF (A and C). ERK1/2 phosphorylation was determined following stimulation with either vehicle or 10⁻⁷ M PAF for 2–60 min (B and D). Membranes were probed with anti-p-ERK1/2 Ab, stripped, and rebotted with anti-tubulin Ab. Graphs illustrate mean (±SEM) relative densitometry values (n = 3).

FIGURE 5. IP determination and binding analysis after lipid raft disruption. To disrupt lipid rafts, cells were treated with 10 mM CD for 30 min at 37°C. Concentration response of PAF-induced IP formation was determined on HEK 293 cells transiently expressing PAFR (A) and CHO cells stably expressing PAFR (B). Graphs are representative of three independent experiments, each done in triplicates (*, p < 0.05; **, p < 0.01; ***, p < 0.001). C, PAF-induced IP formation was determined on CHO stably expressing PAFR after treatment of PBS, CD, or CD/cholesterol mix for 30 min at 37°C. D, [³H]WEB2086-binding experiments were performed on HEK 293 cells transiently expressing PAFR with or without CD treatment. Graph represents means of three independent experiments, each done in triplicate.
showed a similar marked decrease in IP production for PAF concentrations ranging from 1 to 1000 nM, as well as a shift to the right for the EC_{50}. Similar results were also obtained when pretreating PAFR-transfected COS-7 cells with 2 μg/ml filipin, an agent that selectively binds to cholesterol and induces structural disorders within the cell membrane (data not shown). Finally, as shown in Fig. 5C, repletion with cholesterol reversed the inhibitory effect of CD on PAF-induced IP production. These results suggest that PAFR coupling to the Goq protein, which is also localized in lipid rafts, is strongly dependent on the integrity of lipid rafts/caveolae.

**Cyclodextrin treatment does not change ligand affinity for PAFR**

To ensure that cholesterol extraction using CD treatment at a concentration of 10 mM for 30 min did not by itself affect the ability of PAFR to bind to its ligand, we performed radioligand-binding experiments, as previously described (23), on HEK 293 cells (Fig. 5D). Cells were transfected with human PAFR and, 48 h post-transfection, half the cells were treated with CD, and the other half with PBS. Cells were then washed and subjected to PAFR-specific, saturable binding using the radiolabeled PAF antagonist WEB2086 (31, 32), in the presence of increasing concentrations of PAF. No significant difference in binding curves was observed between CD-treated cells and control cells. This was also true for COS-7 cells and CHO cells (data not shown).

**Effect of lipid raft disruption on PAF-induced intracellular calcium release**

Because functional PAFR coupling to Goq was significantly reduced after lipid raft disruption by CD in transfected CHO and HEK 293 cells, we tested the impact of CD treatment on intracellular calcium release in another cell line that naturally expresses PAFR. Fig. 6A shows one representative graph of [Ca^{2+}]/_i_ flux (nM) after PAF stimulation (1 × 10^{-8} M) of A431 cells plated on coverslips for microscopy. Cells treated with 10 mM CD were slower to start calcium release after stimulation than were control cells; however, this was not observed with 1 mM CD treatment. Total calcium release (Fig. 6B) from 10 mM CD-treated cells was significantly reduced compared with control cells (51.22 ± 8.9% loss, p < 0.05). A similar tendency, although not statistically significant, was observed at 1 mM CD (17.28 ± 6.3% loss). These data suggested that PAFR coupling to Goq was dependent on low-density microdomain integrity in caveolae-expressing cells, which corroborates the IP results obtained with transfected cells (Fig. 5).

**Effect of lipid rafts disruption on MAPK signaling pathway**

HEK 293 cells expressing human PAFR were stimulated with 1 × 10^{-8} M PAF for different time periods and lysed. ERK (ERK1/2) activation was assessed by Western blot analysis using Ab against the phosphorylated form of ERK1/2, and loading was verified using Ab directed against total ERK1 (Fig. 7A). Data show a marked increase in ERK1/2 phosphorylation after 5-min stimulation as compared with controls with decreased phosphorylation thereafter. We thus used the 5-min time point for all additional experiments.

HEK 293 cells, transfected with human PAFR cDNA, were left untreated or were incubated with 1 × 10^{-4} M PAFR antagonist WEB2086. For lipid raft disruption, cells were treated with 10, 1, or 0.1 mM CD. Cells were then stimulated with PAF or vehicle (EtOH) and lysed for determination of ERK1/2 phosphorylation. A representative experiment is shown in Fig. 7B. Data in the lower panel are expressed as fold induction of ERK1/2 phosphorylation as compared with vehicle and represent means of four independent experiments. PAF induced a 12.4-fold increase (p < 0.01) in ERK phosphorylation, which was reduced to 3.75 in the presence of WEB2086 and to 4.63 when cells were treated with 10 mM CD (p < 0.05). ERK activation was reduced to a 8.63- and a 7.08-fold increase using 1 and 0.1 mM CD, respectively. The CD effect could be reversed by cholesterol repletion, as shown in Fig. 7C. Stimulation of A431 cells with PAF resulted in a 6.77-fold induction (p < 0.001) of ERK activation, with a complete inhibition (p < 0.001) with WEB2086 and a significant reduction (p < 0.001) with 10 mM CD (Fig. 7D).

**Differential dependence on lipid rafts of PAF-induced Ca^{2+} flux and ERK activation in leukocytes**

Because leukocytes are devoid of caveolin, but readily respond to PAF stimulation, we used the Ramos B cell line and peripheral blood monocytes to assess the dependence on lipid raft integrity of PAF-induced responses in these cells.

Following isolation of lipid rafts from Ramos cells based on a method of Petrie et al. (33), it appeared that most, if not all of the PAFR pool was localized in lipid rafts/caveolae. Graph is representative of three independent experiments, each done on three coverslips of 50 – 60 cells per condition.

We thus used the 5-min time point for all additional experiments.
These results suggest that PAFR signaling in leukocytes is dependent on lipid raft integrity in some, but not all cell types.

**Discussion**

Caveolae and lipid rafts are two types of membrane microdomains that have been the object of extensive research in the past few years. Several proteins have been found to be associated almost exclusively within these domains, as reviewed previously (12, 13, 37, 38). Isolation of these domains can be done in many different ways (37), but there is no existing method that can specifically discriminate between the two types of microdomains. Using standard methods widely used for isolation of caveolae and lipid rafts, we demonstrated that PAFR is localized within these domains in HEK 293, A431, CHO, and Ramos cell lines. Because PAFR localizes in fractions containing caveolin-1 and flotillin-1, which are proteins found to be enriched in caveolae and lipid rafts, we could not clearly determine whether PAFR was localized within caveolae or noncaveolar lipid rafts in cells expressing caveolae. We thus attempted to define whether PAFR could physically interact with a specific component of a subtype of microdomains, namely caveolin-1.

Caveolin-1 is the major structural protein of caveolae. It is thus seen as a very good marker for caveolae. BRET2 is a sensitive method for determining whether two proteins are present in close proximity (within 1–10 nm) from each other in living cells (39). This method was used for determining arrestin binding to GPCR (40), as well as for defining receptor dimerization (41–43). We therefore measured the association of PAFR and caveolin-1 in live resting cells using BRET2. Our data indicate that PAFR is effectively localized within 1–10 nm from caveolin-1 in living cells. Additional results presented in this study suggest that PAFR is not only localized close to caveolin-1 within living cells, but that it can physically bind to caveolin-1. This binding site is found in the C-terminal part of PAFR, which also contains a potential binding motif for caveolin-1, a motif shared by many caveolin-binding proteins, such as G proteins and endothelial NOS. This motif was also found to be present in the AT1 receptor, but apparently was not important for AT1 location in caveolae (44). Our data,
however, suggest that this PAFR motif is important not only for PAFR association with caveolin-1, but also for PAFR-mediated signaling, leading to IP production and MAPK activation.

Whereas a major proportion of the PAFR pool appears to be associated with caveolae in cells expressing caveolin-1, PAFR is also associated with lipid raft microdomains in cells lacking caveolin-1, such as Ramos cells. This suggests that caveolin-1 may not be essential for PAFR localization in low-density microdomains. Palmitoylation is a modification often found in proteins associated with lipid rafts, as reviewed previously (45), and it seems likely that this modification could play a role in lipid raft localization of proteins such as flotillins (46, 47) and G proteins (48). Because PAFR possesses a putative palmitoylation site (conserved cysteine 317) in its C terminus (49), it is conceivable that the palmitate residue could constitute a potential anchoring domain for PAFR within lipid rafts, in the absence of caveolin expression. This could also be true for caveolin-1-expressing cells because the caveolin-1-binding motif may not be responsible for PAFR localization within caveolae.

PAFR has also been shown to be expressed at intracellular sites, such as the nuclear membrane (50), where it can efficiently transmit signals. It is unknown at this time whether intracellular PAFR associates with caveolae or lipid rafts, as it does at the plasma membrane.

Both caveolar and noncaveolar lipid rafts are often seen as signaling platforms because these domains are enriched in many signaling proteins, as reviewed previously (12, 13, 37, 38). As mentioned above, slight differences exist in the signaling components found in these subtypes of microdomains. These differences are partly due to caveolin-1, which has been found to specifically interact with many signaling proteins (17, 51). G proteins are among these signaling components enriched in microdomains (16, 48, 52–54). Several studies indicate that Goq11 (16, 54) is mainly found in caveolae and rafts, and that caveolin-1 can modulate GPCR signaling by facilitating their interaction with Goq11 (55). Because PAFR has been shown to couple to Goq11, Gz1, and Gz0 subunits (4) and because PAFR is localized within microdomains, we investigated the consequences of a disruption of these domains for PAFR coupling to Goq11. We found that IP production, as well as intracellular calcium release, which presumably result mainly from Goq11 activation of phospholipase Cβ (56–58), are both affected by cholesterol extraction with CD in most cell types. This is observed both in cells expressing caveolin-1 (HEK 293, A431) and in cells lacking the latter (Ramos), suggesting that PAFR coupling to Goq11 is critically dependent on the integrity of both types of microdomains. Interestingly, however, at least one cell type lacking caveolae, namely peripheral blood monocytes, readily responds to PAF, but their response is unaffected by microdomain disruption.

PAFR activation of the MAPK family of proteins is well established. In a wide range of cell types, the MAPKs ERK1/2, p38, and JNKs are activated upon PAF stimulation (59–66). ERK (ERK1/2) phosphorylation has been found to be independent of Ras (67), G proteins, and receptor internalization (68). Because ERK

FIGURE 9. Calcium mobilization in Ramos cells and peripheral blood monocytes. A, PAF-induced (1 × 10⁻⁷ M) calcium mobilization was determined after treating 5 × 10⁶ Ramos cells with 1 or 10 mM cyclodextrin. Graph is representative of two to four independent experiments. Calcium mobilization on Ramos cells (B) or freshly isolated monocytes (C) after treatment with either vehicle, 10 mM CD, or CD/cholesterol complexes. Maximum fluorescence peaks (means ± SEM) after PAF stimulation are shown on bottom panels.

FIGURE 10. ERK1/2 phosphorylation on Ramos cell line and monocytes isolated from peripheral blood. A, 1 × 10⁶ Ramos cells were pretreated with vehicle, 100 μM WEB2086, 10 mM CD, or CD/cholesterol complexes, and stimulated with 1 × 10⁻⁸ M PAF for 5 min. Cells were lysed, and proteins were subjected to SDS-PAGE and Western blot analysis using anti-p-ERK1/2 Ab, stripped, and blotted with anti-ERK1 Ab. Blots are representative of three separate experiments.
has been found to physically associate with caveolin-1 (10), we proceeded to determine the importance of lipid rafts and caveolae in PAFR signaling through ERK1/2. In cells expressing caveolin-1, caveola disruption markedly reduced the capacity for PAFR to induce phosphorylation of ERK1/2. This seemed logical, because both PAFR and ERK were found within caveolae, in association with caveolin-1. In this case, caveola disruption would limit PAFR accessibility to ERK proteins, thus reducing its activation. Interestingly, in cells lacking caveolae, lipid raft disruption resulted in very different effects depending on the cell type: in human B lymphocytic Ramos cells, CD treatment resulted in invagination. Interestingly, in cells lacking caveolae, lipid raft disruption resulted in very different effects depending on the cell type: in human B lymphocytic Ramos cells, CD treatment resulted in invagination. Interestingly, in cells lacking caveolae, lipid raft disruption resulted in very different effects depending on the cell type: in human B lymphocytic Ramos cells, CD treatment resulted in invagination. Interestingly, in cells lacking caveolae, lipid raft disruption resulted in very different effects depending on the cell type: in human B lymphocytic Ramos cells, CD treatment resulted in invagination. Interestingly, in cells lacking caveolae, lipid raft disruption resulted in very different effects depending on the cell type: in human B lymphocytic Ramos cells, CD treatment resulted in invagination. Interestingly, in cells lacking caveolae, lipid raft disruption resulted in very different effects depending on the cell type: in human B lymphocytic Ramos cells, CD treatment resulted in invagination. Interestingly, in cells lacking caveolae, lipid raft disruption resulted in very different effects depending on the cell type: in human B lymphocytic Ramos cells, CD treatment resulted in invagination. Interestingly, in cells lacking caveolae, lipid raft disruption resulted in very different effects depending on the cell type: in human B lymphocytic Ramos cells, CD treatment resulted in invagination. Interestingly, in cells lacking caveolae, lipid raft disruption resulted in very different effects depending on the cell type: in human B lymphocytic Ramos cells, CD treatment resulted in invagination. Interestingly, in cells lacking caveolae, lipid raft disruption resulted in very different effects depending on the cell type: in human B lymphocytic Ramos cells, CD treatment resulted in invagination. Interestingly, in cells lacking caveolae, lipid raft disruption resulted in very different effects depending on the cell type: in human B lymphocytic Ramos cells, CD treatment resulted in invagination. Interestingly, in cells lacking caveolae, lipid raft disruption resulted in very different effects depending on the cell type: in human B lymphocytic Ramos cells, CD treatment resulted in invagination. Interestingly, in cells lacking caveolae, lipid raft disruption resulted in very different effects depending on the cell type: in human B lymphocytic Ramos cells, CD treatment resulted in invagination. Interestingly, in cells lacking caveolae, lipid raft disruption resulted in very different effects depending on the cell type: in human B lymphocytic Ramos cells, CD treatment resulted in invagination. Interestingly, in cells lacking caveolae, lipid raft disruption resulted in very different effects depending on the cell type: in human B lymphocytic Ramos cells, CD treatment resulted in invagination. Interestingly, in cells lacking caveolae, lipid raft disruption resulted in very different effects depending on the cell type: in human B lymphocytic Ramos cells, CD treatment resulted in invagination. Interestingly, in cells lacking caveolae, lipid raft disruption resulted in very different effects depending on the cell type: in human B lymphocytic Ramos cells, CD treatment resulted in invagination. Interestingly, in cells lacking caveolae, lipid raft disruption resulted in very different effects depending on the cell type: in human B lymphocytic Ramos cells, CD treatment resulted in


