α₁-Adrenergic Receptors Function Within Hetero-Oligomeric Complexes With Atypical Chemokine Receptor 3 and Chemokine (C-X-C motif) Receptor 4 in Vascular Smooth Muscle Cells

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Background—Recently, we provided evidence that α₁-adrenergic receptors (ARs) in vascular smooth muscle are regulated by chemokine (C-X-C motif) receptor (CXCR) 4 and atypical chemokine receptor 3 (ACKR3). While we showed that CXCR4 controls α₁-ARs through formation of heteromeric receptor complexes in human vascular smooth muscle cells (hVSMCs), the molecular basis underlying cross-talk between ACKR3 and α₁-ARs is unknown.

Methods and Results—We show that ACKR3 agonists inhibit inositol trisphosphate production in hVSMCs on stimulation with phenylephrine. In proximity ligation assays and co-immunoprecipitation experiments, we observed that recombinant and endogenous ACKR3 form heteromeric complexes with α₁B/D-AR. While small interfering RNA knockdown of ACKR3 in hVSMCs reduced α₁B/D-AR:ACKR3, CXCR4:ACKR3, and α₁B/D-AR:CXCR4 complexes, small interfering RNA knockdown of CXCR4 reduced α₁B/D-AR:ACKR3 heteromers. Phenylephrine-induced inositol trisphosphate production from hVSMCs was abolished after ACKR3 and CXCR4 small interfering RNA knockdown. Peptide analogs of transmembrane domains 2/4/7 of ACKR3 showed differential effects on heteromerization between ACKR3, α₁B/D-AR, and CXCR4. While the transmembrane domain 2 peptide interfered with α₁B/D-AR:ACKR3 and CXCR4:ACKR3 heteromerization, it increased heteromerization between CXCR4 and α₁A-B-AR. The transmembrane domain 2 peptide inhibited ACKR3 but did not affect α₁B-AR in β-arrestin recruitment assays. Furthermore, the transmembrane domain 2 peptide inhibited phenylephrine-induced inositol trisphosphate production in hVSMCs and attenuated phenylephrine-induced constriction of mesenteric arteries.

Conclusions—α₁-ARs form hetero-oligomeric complexes with the ACKR3:CXCR4 heteromer, which is required for α₁B/D-AR function, and activation of ACKR3 negatively regulates α₁-ARs. G protein–coupled receptor hetero-oligomerization is a dynamic process, which depends on the relative abundance of available receptor partners. Endogenous α₁-AR function within a network of hetero-oligomeric receptor complexes. (J Am Heart Assoc. 2017;6:e006575. DOI: 10.1161/JAHA.117.006575.)

Key Words: alpha • adrenergic receptor • blood pressure • chemokine • C-X-C motif chemokine ligand 11 • C-X-C motif chemokine ligand 12 • phenylephrine • vasoconstriction

Multiple lines of evidence suggest that the 7-transmembrane domain receptors chemokine (C-X-C motif) receptor (CXCR) 4 and atypical chemokine receptor (ACKR) 3 (formerly known as CXCR7, RDC1, and GPR159) contribute to the regulation of vascular function and blood pressure in health and disease. 1–8 Recently, we reported that CXCR4 activation sensitizes α₁-adrenergic receptor (AR) function in vascular smooth muscle and stabilizes blood pressure during the cardiovascular stress response to hemorrhagic shock, whereas activation of ACKR3 inhibits vascular α₁-AR function and impairs blood pressure. 3 Subsequently, we provided evidence that heteromeric complexes between endogenous CXCR4 and α₁A/B-AR are constitutively expressed in human vascular smooth muscle cells (hVSMCs), through which CXCR4 modulates α₁-AR function. 4,8 The molecular mechanisms underlying cross-talk between ACKR3 and α₁-AR, however, remain elusive.

The concept of GPCR (G protein–coupled receptor) hetero-oligomerization is rapidly evolving and challenges the classical paradigm that a mono- or homodimeric receptor is the

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Received May 22, 2017; accepted June 28, 2017.

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DOI: 10.1161/JAHA.117.006575
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Clinical Perspective

What Is New?

- Heteromeric complexes between atypical chemokine receptor 3 and α1A/B/D-adrenergic receptors are constitutively expressed in human vascular smooth muscle cells.
- Hetero-oligomerization of α1B/D-adrenergic receptor with the chemokine (C-X-C motif) receptor 4: atypical chemokine receptor 3 heteromeric complex is required for α1B/D-adrenergic receptor function.
- Formation of hetero-oligomeric G protein–coupled receptor complexes within the plasma membrane is a dynamic process that depends on the relative abundances of and affinities between available receptor partners.

What Are the Clinical Implications?

- Drugs targeting heteromeric complexes between α1-adrenergic receptors, chemokine (C-X-C motif) receptor 4, and atypical chemokine receptor 3 could provide new pharmacological approaches to regulate vascular function in disease conditions.
- Dynamic alterations in the formation of hetero-oligomeric receptor complexes could explain biological variability of G protein–coupled receptor–mediated functions.

Methods

Proteins, Peptides, and Reagents

Phenylephrine (PE), Phentolamine, 5’-Methylurapidil, Cyclazosin and BMY 7378 were purchased from Sigma Aldrich, CXCL11 and CXCL12 were from Protein Foundry and TC14012 was from Tocris Biosciences. The peptide analogs of transmembrane (TM) 2 (TM2; YILNALADLWVLTVTAVVDDD), TM4 (VVCILWLLACVSLPDTYYLDD), and TM7 (DDDLHVTQCLSLVHCNVNPYLFSN) of ACKR3 were generated using the Fmoc protected amino acids in a solid-phase synthesis on a 433A Applied Biosciences Peptide Synthesizer and Liberty Blue Microwave peptide synthesizer (CEM Corporation) using Fmoc chemistry. The peptides were cleaved from the resin and deprotected with a mixture of 90.0% (v/v) trifluoroacetic acid (TFA) with 2.5% water, 2.5% triisopropyl-silane, 2.5% 2,2'-dicyclohexylquinolinium trifluoroacetate, and 5% thioanisol. Peptides were purified on a preparative (25×250 mm) Atlantis C3 reverse phase column (Agilent Technologies) in a 90 minutes gradient of 0.1% (v/v) trifluoroacetic acid in water and 0.1% trifluoroacetic acid in acetonitrile, with a 10 mL/min flow rate. The fractions containing peptides were analyzed on Agilent 6100 LC/MS spectrometer (Agilent Technologies) with the use of a Zorbax 300SB-C3 PoroShell column and a gradient of 5% acetic acid in water and acetonitrile. Fractions that were more than 95% pure were combined and freeze dried.

Cells

hVSMCs, primary aortic smooth muscle cells (ATCC-PCS-100-012), and human embryonic kidney (HEK) 293T cells (ATCC-CRL-11268) were purchased from American Type Culture Collection. hVSMCs were cultured in vascular basal media (ATCC PCS-100-030) supplemented with the vascular smooth muscle growth kit (ATCC PCS-100-042), 100 U/mL penicillin, and 100 μg/mL streptomycin. hVSMCs were used between passages 2 and 5. HEK293T cells were cultured in high-glucose Dulbecco’s modified Eagle medium containing 10 mg/mL sodium pyruvate, 2 mmol/L L-glutamine, 10% (vol/vol) FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. The HTLA cell line, a HEK293 cell line stably expressing a tTA-dependent luciferase reporter and a β-arrestin2-TEV receptor 4, angiotensin II type I receptor or CXCR4, we studied whether heteromerization of ACKR3 with α1-ARs could constitute a molecular mechanism underlying their cross-talk in vascular smooth muscle. Here, we provide evidence that hetero-oligomeric complexes between endogenous ACKR3 and α1A-AR and between the ACKR3:CXCR4 heteromer and α1B-AR are constitutively expressed in hVSMC, through which ACKR3 regulates α1-AR function.
fusion gene,\textsuperscript{21} was generously provided by the laboratory of Dr Bryan Roth and maintained in high glucose Dulbecco’s modified Eagle medium supplemented with 10% (vol/vol) heat-inactivated FBS, 1x nonessential amino acids, 100 U/mL penicillin, 100 μg/mL streptomycin, 50 μg/mL hygromycin B, and 2 μg/mL puromycin. All cells were cultured in a humidified environment at 37°C, 5% CO\textsubscript{2}.

**Proximity Ligation Assays**

Proximity ligation assays (PLAs) were performed as previously described in detail.\textsuperscript{4,8,22} In brief, cells were grown and fixed on 8-well chamber slides (Nunc). Cells were fixed with 4% (wt/vol) paraformaldehyde for 15 minutes at room temperature and then blocked overnight at 4°C with 3% (wt/vol) BSA in PBS. To visualize individual receptors, slides were incubated with rabbit anti-HA (Abcam 9110), mouse anti-FLAG (Sigma F1804), mouse anti-ACKR3 (R&D MAB42273), rabbit anti-\(\alpha_1\)\textsubscript{A}-AR (Abcam Ab137123), rabbit anti-\(\alpha_1\)\textsubscript{B}-AR (Abcam Ab169523), goat anti-\(\alpha_2\)\textsubscript{D}-AR (Santa Cruz SC27099), or goat anti-CXCR4 (Abcam Ab1670) at 37°C for 105 minutes in a humidifying chamber. To visualize receptor-receptor interactions, slides were incubated with a combination of rabbit anti-HA (Abcam 9110) and mouse anti-FLAG (Sigma F1804), mouse anti-ACKR3 (R&D MAB42273), and goat anti-CXCR4 (Abcam 1670) or rabbit anti-\(\alpha_1\)\textsubscript{A}-AR (Abcam Ab137123) or rabbit anti-\(\alpha_1\)\textsubscript{B}-AR (Abcam Ab169523) or goat anti-\(\alpha_2\)\textsubscript{D}-AR (Santa Cruz SC27099) or with a combination of goat anti-CXCR4 (Abcam 1670) and rabbit anti-\(\alpha_1\)\textsubscript{A}-AR (Abcam Ab137123) or rabbit anti-\(\alpha_1\)\textsubscript{B}-AR (Abcam Ab169523) or rabbit anti-\(\alpha_2\)\textsubscript{D}-AR (Santa Cruz SC27099) at 37°C for 105 minutes in a humidifying chamber. All antibodies were used in dilutions of 1:500. Slides were then washed with PBS and incubated (60 minutes at 37°C in a humidifying chamber) with secondary species-specific antibodies conjugated with plus and minus Duolink II PLA probes (1:5), as appropriate. Negative control slides were incubated with omission of one primary antibody. Slides were washed again with PLA wash buffer A (Duolink II) and then incubated with ligation-ligase solution (30 minutes at 37°C in a humidifying chamber), washed with PLA wash buffer A and then incubated with amplification-polymerase solution (100 minutes at 37°C in a humidifying chamber). Slides were then washed twice with PLA wash buffer B (Duolink II), once with 0.01x PLA wash buffer B and allowed to dry. Slides were then mounted with a minimal volume of Duolink II mounting medium with 4’,6-diamidino-2-phenylindole overnight, and PLA signals (Duolink In Situ Detection Reagents Green \(\lambda_{\text{excitation/emission}}\) 495/527 nm or Red \(\lambda_{\text{excitation/emission}}\) 598/634 nm) were identified as fluorescent spots under a fluorescence microscope (Carl Zeiss Axiosvert 200M with EC Plan-Neofluor objective lenses [40x /1.30 oil] equipped with Axio CamMRc5 and AxioVision Rel. 4.9.1 acquisition software) at room temperature. PLA signals were quantified using the Duolink Image Tool software (Sigma Aldrich). Images were imported in merged.tiff formats containing both signal and nuclei channels. Merged images were visually verified for analytical quality. Comparisons and statistical analyses were performed only when PLA assays were performed on the same day in parallel experiments and fluorescence microscopy was performed with the identical settings. For each experiment and condition, 10 randomly selected nonoverlapping vision fields were analyzed. The anti-GPCR antibodies that were used for PLA, except goat anti-CXCR4 (Abcam Ab1670) and rabbit anti-CXCR4 (Alomone Labs ACR-014), have been validated for sufficient selectivity for their GPCR target previously.\textsuperscript{4,8,23,24} The validation of goat anti-CXCR4 (Abcam Ab1670) and rabbit anti-CXCR4 (Alomone Labs ACR-014) is shown in Figure 1. We detected that incubation of hVSMC with CXCR4 small interfering RNA (siRNA) reduced PLA signals with goat anti-CXCR4 (Abcam Ab1670) by 79±4% and PLA signals with rabbit anti-CXCR4 (Alomone Labs ACR-014) by 75±4%, as compared with cells incubated with nontargeting (NT) siRNA.

**Co-Immunoprecipitation Analyses of Receptor Interactions**

Co-immunoprecipitation experiments with hVSMCs were performed using the Thermo Scientific Pierce Co-immunoprecipitation Kit (catalog No. 26149), as previously described.\textsuperscript{8} Fifty micrograms of anti-ACKR3 (R&D, MAB42273) or anti-mouse IgG\textsubscript{1} (Abcam Ab81032) were incubated with 50 μL AminoLink Plus Coupling Resin for 180 minutes at room temperature. Five hundred micrograms of cell lysate was precleared with 50 μL of the control agarose resin slurry (60 minutes at 4°C). Immobilized anti-ACKR3 resin and anti-IgG\textsubscript{1} resin were incubated with precleared lysate for 24 to 48 hours at 4°C. After incubation, the resins were washed 3 times with 200 μL IP lysis/wash buffer, once with conditioning buffer, and protein was eluted using 50 μL of elution buffer. Samples were analyzed by Western blotting.

**Western Blotting**

Western blotting with rabbit anti-ACKR3 (Abcam Ab38089), rabbit anti-\(\alpha_1\)\textsubscript{A}-AR (Abcam Ab137123), rabbit anti-\(\alpha_1\)\textsubscript{B}-AR (Abcam Ab169523), goat anti-\(\alpha_2\)\textsubscript{D}-AR (Santa Cruz SC27099), and mouse anti-\(\alpha_2\)\textsubscript{C}-AR (Abcam Ab167433) in combination with anti-rabbit, goat, or mouse IgG horseradish peroxidase–linked whole antibody (GE Healthcare and Sigma Aldrich) was performed as previously described.\textsuperscript{8}
Gene Silencing via RNA Interference

ACKR3 and CXCR4 siRNA gene silencing was performed as previously described. In brief, hVSMCs were grown in 2 mL of Accell siRNA delivery media per well (Dharmacon) in 6-well plates (Nunc). Commercially available Accell ACKR3 and CXCR4 siRNA were reconstituted with 1× siRNA buffer to a stock concentration of 100 μmol/L. Cells were then transfected with 1 μmol/L of ACKR3/CXCR4 siRNA and incubated for 72 hours at 37°C, 5% CO2. Accell NT siRNA pool was used as negative control. After 72 hours, cells were assayed for receptor cell-surface expression and used for signaling experiments.

GPCR Gene Transfections

HEK293T cells were transiently transfected with 1.5 μg of DNA encoding either human influenza hemagglutinin (HA)-tagged ACKR3 (HA-ACKR3), FLAG-ACKR3, HA-CXCR4, FLAG-α1a-AR, FLAG-α1b-AR, or FLAG-α1d-AR with a combination of 2 GPCR encoding DNAs, as indicated (all plasmids from Addgene), using Lipofectamine 3000 as per manufacturer’s protocol. Empty vector, pcDNA3, was used as a control. Twenty-four hours later, cells were fixed on chamber slides for PLA.

IP3 ELISA

Inositol trisphosphate (IP3) ELISAs were purchased from LS Bio and performed according to the manufacturer’s protocol (LS BIO F10644). In brief, hVSMCs were grown to confluence in 6-well dishes (Nunc) and then treated as described in the Results section. Cells were then washed once with cold PBS, 225 μL of cold PBS was added to each well, and cells were lysed by ultrasonication. The cell lysate was centrifuged for 10 minutes at 4°C at 1500g to remove cellular debris. The total protein concentration in the supernatant was determined with the Bio-Rad DC Protein Assay according to the manufacturer’s protocol. Equivalent amounts of total protein were added to the ELISA strips diluted in the provided sample diluent (1:5 and 1:10). The assay was then completed as per the manufacturer’s protocol. Optical densities were read on a Biotek Synergy II microplate reader.

Figure 1. Validation of anti–chemokine (C-X-C motif) receptor 4 (CXCR4) antibodies. Human vascular smooth muscle cells were incubated with nontargeting (NT) or CXCR4 small interfering RNA (siRNA) and then used in proximity ligation assays (PLAs) for the detection of CXCR4 with goat anti-CXCR4 (Abcam Ab1670) and rabbit anti-CXCR4 (Alomone Labs ACR-014). A, Representative PLA images showing merged PLA/4,6-diamidino-2-phenylindole dihydrochloride signals. Ctrl: Omission of primary antibody. Scale bars=10 μm. B, Quantification of PLA signals per cell, as in (A). n=10 images per condition. *: p<0.05 vs. cells incubated with NT siRNA.

DOI: 10.1161/JAHA.117.006575
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Page dimensions: 612.0x792.0

[45x763]Hetero-Oligomerization of \( \alpha_1 \)-Adrenergic Receptors was continuously aerated with 95% O2, system (DMT-USA). The intraluminal solution and the vessel pressurized to 80 mm Hg in a DMT 110P pressure myograph cannulae with US Pharmacopeia scale 11-0 sutures, and sectored free from the mesentery, mounted onto 2 glass lungs. Third- or fourth-order mesenteric arteries were dissected in detail with slight modifications.3,4 Male Sprague-Dawley rats (Harlan) were anesthetized with 3.5% iso-flurane on a Biotek Synergy II plate reader.

Pressure Myography

All procedures involving animals were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, 8th Edition, and were approved by the Institutional Animal Care and Use Committee of Loyola University Chicago. Pressure myography was performed as previously described in detail with slight modifications.3,4 Male Sprague-Dawley rats (Harlan) were anesthetized with 3.5% iso-flurane. The mesentery was immediately removed and placed in 130 mmol/L NaCl, 4.7 mmol/L KCl, 1.18 mmol/L KH\(_2\)PO\(_4\), 1.17 mmol/L MgSO\(_4\), 14.9 mmol/L NaHCO\(_3\), 5.5 mmol/L D-glucose, 0.026 mmol/L EDTA, and 1.16 mmol/L CaCl\(_2\) aerated with 95% O\(_2\), 5% CO\(_2\) at 37°C. The animal was then euthanized by cardiacotomy and bilateral decompression of the lungs. Third- or fourth-order mesenteric arteries were dissected free from the mesentery, mounted onto 2 glass cannulae with US Pharmacopeia scale 11-0 sutures, and pressurized to 80 mm Hg in a DMT 110P pressure myograph system (DMT-USA). The intraluminal solution and the vessel bath solution were the same as previously described. The vessel bath solution was continuously aerated with 95% O\(_2\), 5% CO\(_2\) throughout the experiment. The outer diameter of the pressurized vessel was then continuously measured and recorded via digital video-edge detection on addition of increasing doses of PE to the vessel bath.

Data Analyses

Data are expressed as mean±SEM from independent experiments that were performed on different days. Data were analyzed using GraphPad Prism 7 software. Data that passed the Shapiro–Wilk normality test were analyzed with unpaired Student t test or 1-way ANOVA with Dunnett’s multiple comparison post hoc test for multiple comparisons, as appropriate. Data that did not pass the Shapiro–Wilk normality test were analyzed with the Mann–Whitney U test or Kruskal–Wallis H test with Dunn’s multiple comparisons test, as appropriate. Molecular masses corresponding to the migration positions of bands in Western blot analyses were calculated by nonlinear regression analyses based on the migration position of standard proteins. Dose response curves were analyzed using nonlinear regression analyses. A 2-tailed P<0.05 was considered significant.

Results

ACKR3 Agonists Inhibit G\(_\rm{q}\)-Mediated Signaling on \( \alpha_1 \)-AR Activation

We have previously shown that ACKR3 activation antagonizes vasoconstriction of isolated mesenteric resistance arteries upon stimulation with the selective \( \alpha_1 \)-AR agonist PE.3 Because the effects of ACKR3 activation on PE-induced intracellular signaling events in hVSMCs are unknown, we evaluated the effects of the natural ACKR3 agonists CXCL11 and CXCL12 and of the synthetic peptide agonist TC14012, which is also a CXCR4 antagonist, using IP\(_3\) production in hVSMCs upon stimulation with PE as a read-out for \( G_q \)-mediated signaling on \( \alpha_1 \)-AR activation. We first confirmed ACKR3 agonist activity of the ligands in a \( \beta \)-arrestin 2 recruitment assay (PRESTO-Tango). As shown in Figure 2A, the EC\(_{50}\) for \( \beta \)-arrestin 2 recruitment to ACKR3 was 16.5±4.9 nmol/L for CXCL11, 2.4±0.3 nmol/L for CXCL12, and 87.5±19.0 nmol/L for TC14012. The efficacy of TC14012 to recruit \( \beta \)-arrestin 2 to ACKR3 was lower than the efficacy of the natural chemokine agonists. PE stimulation of hVSMCs increased cellular IP\(_3\) concentrations 5.3-fold. Pretreatment of cells with all ACKR3 agonists abolished this response, indicating that ACKR3 activation inhibits \( \alpha_1 \)-AR-mediated signaling (Figure 2B).

ACKR3 Forms Heteromeric Complexes With \( \alpha_{1\rm{A}}/\beta_{D/\rm{AR}}\)

Next, we studied whether recombinant ACKR3 would be able to form heteromeric complexes with recombinant \( \alpha_{1\rm{A}} \)-AR subtypes in HEK293T cells. We used proximity ligation assays (PLAs) to visualize and quantify individual receptors and receptor-receptor interactions at single molecule resolution.26

DOI: 10.1161/JAHA.117.006575
Heteromerization between ACKR3 and CXCR4 and between CXCR4 and \( \alpha_{1A/-AR} \) was used as a positive control in PLA experiments because both receptor pairs have previously been shown to interact in expression systems.8,17 When N-terminal HA-tagged ACKR3 was coexpressed with N-terminal FLAG-tagged \( \alpha_{1A/b/d/-AR} \) subtypes, we observed positive signals for receptor-receptor interactions in PLA experiments with anti-FLAG and anti-HA antibodies for each receptor combination (Figure 3A). The number of PLA signals for interactions between HA-ACKR3 and FLAG-\( \alpha_{1A/b/d/-AR} \) were comparable with the number of PLA signals corresponding to FLAG-ACKR3:HA-CXCR4 and HA-CXCR4:FLAG-\( \alpha_{1A/-AR} \) interactions (Figure 3B).

Because these observations suggested that recombinant ACKR3 and \( \alpha_{1A/b/d/-AR} \) subtypes form heteromeric complexes when coexpressed, we then evaluated receptor-receptor interactions in native hVSMCs by PLA. For PLA, we used anti-ACKR3 in combination with anti-\( \alpha_{1A/B/D/-AR} \) or anti-\( \alpha_{2A/C/-AR} \) antibodies directed against extracellular receptor domains. These antibodies have been validated to show sufficient selectivity for their GPCR target23 (Figure 1). Figure 4A shows typical PLA images corresponding to receptor-receptor interactions and Figure 4B the quantification of the PLA signals per cell from 3 independent experiments. We detected positive PLA signals for interactions between ACKR3 and \( \alpha_{1A/B/D/-AR} \) and between ACKR3 and \( \alpha_{2B/-AR} \) in hVSMCs. The number of PLA signals for interactions between ACKR3 and \( \alpha_{2A/C/-AR} \), however, were not distinguishable from the number of signals obtained in negative control experiments.

To confirm that the positive PLA signals for interactions between ACKR3 and \( \alpha/-ARs \) correspond to direct physical receptor-receptor interactions, we performed co-immunoprecipitation experiments with hVSMCs (Figure 4C through 4H). ACKR3 could be immunoprecipitated with anti-ACKR3 (Figure 4C). \( \alpha_{1A/B/D/-AR} \) and \( \alpha_{2B/-AR} \) were detectable in ACKR3 immunoprecipitates (Figure 4D through 4G), whereas \( \alpha_{2C/-AR} \) could not be detected (Figure 4H). It should be noted that we observed a band of 50 kDa in the ACKR3 immunoprecipitate in Western blot experiments with anti-\( \alpha_{1D/-AR} \), but we were unable to detect \( \alpha_{1D/-AR} \) in the hVSMC lysate (input). We interpret this observation to reflect low abundance of \( \alpha_{1D/-AR} \) on hVSMC and high enrichment of \( \alpha_{1D/-AR} \) in the ACKR3 immunoprecipitate. Furthermore, the band corresponding to \( \alpha_{2B/-AR} \) in the cell lysate, only the band migrating slightly below 50 kDa was detectable in the ACKR3 immunoprecipitate. This band matches well with the predicted molecular mass of 49.9 kDa (UniProtKB—P18089). Collectively, our observations suggest that heteromeric complexes between ACKR3 and \( \alpha_{1A/B/D/-AR} \) are constitutively expressed in hVSMCs.

ACKR3 Silencing Inhibits \( \alpha_{1/-AR} \)

To assess the functional role of heteromeric complexes between ACKR3 and \( \alpha_{1A/B/D/-AR} \) on \( \alpha_{1/-AR} \) function, we then aimed to reduce heteromeric complexes in hVSMCs by...
Figure 3. Atypical chemokine receptor 3 (ACKR3) forms heteromeric complexes with α1a/1b/1d-adrenergic receptors (ARs) in human embryonic kidney 293T (HEK293T) cells. A, Typical proximity ligation assay (PLA) images for the detection of individual receptors and receptor-receptor interaction in HEK293T cells transfected with DNA encoding influenza hemagglutinin (HA)– or FLAG-tagged receptors. Ctrl: Cells transfected with pcDNA. Images show merged PLA/4′,6-diamidino-2-phenylindole dihydrochloride signals. Scale bars=10 μm. B, Quantification of PLA signals per cell, as in (A). n=3 independent experiments with n=10 images per condition and experiment.
ACKR3 knockdown with siRNA. Typical PLA images for the detection of receptor-receptor interactions in hVSMCs are shown in Figure 5A and the quantifications of the PLA signals from 4 independent experiments are shown in Figure 5B and 5C. Incubation of hVSMCs with siRNA for ACKR3 reduced PLA signals for ACKR3 by more than 70%, as compared with cells incubated with NT siRNA. PLA signals for $\alpha_{1A/1B/1D}$-AR or CXCR4 were indistinguishable between hVSMCs incubated with NT or ACKR3 siRNA (Figure 5B).

Quantification of the PLA signals for receptor-receptor interactions showed that siRNA knockdown of ACKR3 significantly reduced ACKR3: $\alpha_{1A/1B/1D}$-AR and ACKR3:CXCR4 interactions but did not affect ACKR3: $\alpha_{1A/1D}$-AR or CXCR4: $\alpha_{1A/1D}$-AR interactions (Figure 5C). When hVSMCs were tested for PE-induced IP$_3$ production after incubation with NT siRNA or ACKR3 siRNA, we observed that PE increased cellular IP$_3$ concentrations 4.8-fold after incubation with NT siRNA but failed to increase IP$_3$ concentrations in cells after incubation with ACKR3 siRNA (Figure 5D). Because ACKR3 siRNA
knockdown did not significantly reduce ACKR3:α1A-AR but abolished PE-induced IP$_3$ production in hVSMCs, we then evaluated the relative contribution of the α1-AR subtypes to this signaling response. As shown in Figure 5E, PE-induced IP$_3$ production could be inhibited with the nonselective α1-AR antagonist phentolamine and with the selective α1B/D-AR antagonists cyclazosin and BMY 7378, respectively. In contrast, the selective α1A-AR antagonist 5'-methylurapidil did not inhibit PE-induced IP$_3$ production in hVSMCs.

We previously showed that CXCR4 knockdown reduces heteromeric complexes between CXCR4 and α1B/D-AR and between CXCR4 and ACKR3, leading to loss of α1-AR signaling. Because CXCR4 likely exists as a homodimer and also within CXCR4:ACKR3 heteromeric complexes, we then evaluated the relative contribution of the α1-AR subtypes to this signaling response.

Figure 5. Atypical chemokine receptor 3 (ACKR3) gene silencing reduces ACKR3:α1B/D-AR signaling in human vascular smooth muscle cells (hVSMCs). A, Representative proximity ligation assay (PLA) images for the detection of individual receptors (left) and receptor-receptor interactions (right) in hVSMCs incubated with nontargeting (NT) or ACKR3 small interfering RNA (siRNA). Images show merged PLA/4',6-diamidino-2-phenylindole dihydrochloride signals. Ctrl: Omission of one primary antibody. Scale bars=10 μm. B, Quantification of PLA signals per cell for the detection of individual receptors, as in (A). n=4 independent experiments with n=10 images per condition and experiment. *P<0.05 vs cells incubated with NT siRNA. C, Quantification of PLA signals per cell for receptor-receptor interactions, as in (A). n=4 independent experiments with n=10 images per condition and experiment. *P<0.05 vs cells incubated with NT siRNA. D, Inositol trisphosphate (IP$_3$) production of hVSMCs incubated with NT or ACKR3 siRNA upon stimulation with vehicle or 1 μmol/L phenylephrine (PE). n=4 independent experiments. *P<0.05 vs vehicle. E, IP$_3$ production of hVSMCs upon stimulation with vehicle or 1 μmol/L phenylephrine for 5 minutes. Cells were pretreated (30 minutes, 37°C) with 10 μmol/L of vehicle or α1-AR antagonists. n=3 independent experiments. *P<0.05 vs vehicle.
complexes, we then tested whether knockdown of ACKR3 also affects heteromerization between CXCR4 and $\alpha_{1A/B/D}$-AR and vice versa (Figure 6). Representative PLA images for the detection of ACKR3 and CXCR4: $\alpha_{1A/B/D}$-AR heteromers after incubation of cells with nontargeting (NT) or ACKR3 small interfering RNA (siRNA). Images show merged PLA/4',6-diamidino-2-phenylindole dihydrochloride (DAPI) signals. Ctrl: Omission of one primary antibody. Scale bars=10 μm. B, Typical PLA images for the detection of CXCR4 and ACKR3: $\alpha_{1A/B/D}$-AR heteromers in hVSMCs incubated with NT or CXCR4 siRNA. Images show merged PLA/DAPI signals. Ctrl: Omission of one primary antibody. Scale bars=10 μm. C, Quantification of PLA signals per cell for the detection of ACKR3 and CXCR4: $\alpha_{1A/B/D}$-AR heteromers, as in (A). n=3 independent experiments with n=10 images per condition and experiment. *P<0.05 vs cells incubated with NT siRNA. D, Quantification of PLA signals per cell for the detection of CXCR4 and ACKR3: $\alpha_{1A/B/D}$-AR heteromers, as in (B). n=3 independent experiments with n=10 images per condition and experiment. *P<0.05 vs cells incubated with NT siRNA. E, Inositol trisphosphate (IP$_3$) production of hVSMCs incubated with NT, CXCR4, or ACKR3 siRNA upon stimulation with vehicle or 1 μmol/L phenylephrine (PE). n=3 independent experiments. *P<0.05 vs vehicle. DOI: 10.1161/JAHA.117.006575
were reduced to 30±3% and 36±5% of the signals for cells incubated with NT siRNA, respectively. Consistent with our previous findings,8 we observed that PE-induced IP₃ production was abolished after incubation of hVSMCs with CXCR4 siRNA or ACKR3 siRNA, as compared with hVSMCs incubated with NT siRNA (Figure 6E).

**TM Domain–Derived Peptide Analogs of ACKR3 Interfere With Receptor Heteromerization**

TM-derived peptide analogs of GPCRs, including TM peptide analogs of CXCR4 and β₂-AR, have previously been shown to interfere with receptor heteromerization and function.4,27,28 Thus, we tested whether peptide analogs of TM2, TM4, and TM7 of ACKR3 would also be able to affect formation of heteromeric ACKR3 complexes in PLA experiments. Representative PLA images for the visualization of individual receptors and receptor-receptor interactions after incubation of hVSMCs with vehicle or ACKR3 TM peptide analogs are shown in Figure 7A and 7B. The quantifications of the corresponding PLA signals for individual receptors and receptor-receptor interactions from 3 independent experiments are shown in Figure 8A and 8B. The TM peptide analogs did not significantly affect the number of PLA signals for ACKR3, α₁A/B/D-AR, or CXCR4 (Figure 8A). When PLA was performed to detect receptor-receptor interactions, however, we observed differential effects of the TM peptide analogs on the number of PLA signals in hVSMCs (Figure 8B). The TM2 peptide analog significantly reduced PLA signals corresponding to ACKR3:α₁B/D-AR complexes but did not affect signals for ACKR3:α₁A-AR complexes. The TM4 peptide analog reduced PLA signals for ACKR3:α₁B-AR complexes without affecting ACKR3:α₁A/D-AR interactions. In contrast, the TM7 peptide analog significantly increased the number of PLA signals for ACKR3:α₁A-AR complexes but did not alter signals corresponding to ACKR3:α₁B/D-AR heteromers. While PLA

**Figure 7.** Peptides derived from transmembrane domains of atypical chemokine receptor 3 (ACKR3) alter receptor heteromerization in human vascular smooth muscle cells (hVSMCs). hVSMCs were treated with vehicle or transmembrane (TM) 2/4/7 peptide analogs (10 μmol/L, 30 minutes at 37°C), washed and used for proximity ligation assay (PLA). A, Typical PLA images for the detection of individual receptors. Images show merged PLA/4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) signals. Ctrl: Omission of primary antibody. Scale bars=10 μm. B, Typical PLA images for the detection of receptor-receptor interactions. Images show merged PLA/DAPI signals. Ctrl: Omission of one primary antibody. Scale bars=10 μm. AR indicates adrenergic receptor; CXCR4, chemokine (C-X-C motif) receptor 4.
signals for ACKR3:CXCR4 heteromeric complexes were reduced after incubation with all TM peptide analogs (TM2>TM4>TM7), all peptide analogs significantly increased signals for CXCR4:α1A-AR. Furthermore, the TM2 and TM4 peptides also increased signals for CXCR4:α1B-AR complexes but did not affect signals for CXCR4:α1D-AR complexes. The TM7 peptide did not significantly affect signals for CXCR4:α1B-AR or CXCR4:α1D-AR complexes.

Figure 8. Peptides derived from transmembrane domains of atypical chemokine receptor 3 (ACKR3) alter receptor heteromerization in human vascular smooth muscle cell (hVSMC)—proximity ligation assay (PLA) signal quantification. A, Quantification of PLA signals per cell for the detection of individual receptors, as in Figure 7A. n=3 independent experiments with n=10 images per condition and experiment. B, Quantification of PLA signals per cell for the detection of receptor-receptor interactions, as in Figure 7B. n=3 independent experiments with n=10 images per condition and experiment. *P<0.05 vs vehicle. AR indicates adrenergic receptor; CXCR4, chemokine (C-X-C motif) receptor 4.

TM Domain–Derived Peptide Analogs of ACKR3 Interfere With Receptor Function

We then studied whether the TM peptide analogs of ACKR3 modulate signaling of recombinant ACKR3 and α1B-AR. None of the TM peptide analogs activated ACKR3 in β-arrestin recruitment assays (Figure 9A). The TM2 peptide analog, however, significantly inhibited CXCL12-induced β-arrestin 2 recruitment.
to ACKR3 (EC$_{50}$ for CXCL12, 2.1±0.45 nmol/L; CXCL12 plus TM2, 37.7±14.1 nmol/L [P<0.05 versus CXCL12]; CXCL12 plus TM4, 14.3±5.4 nmol/L [P<0.05 versus CXCL12]; and CXCL12 plus TM7, 3.9±2.0 nmol/L [P>0.05 versus CXCL12]) (Figure 9B). None of the TM peptide analogs affected PE-induced β-arrestin 2 recruitment to $\alpha_{1a}$-AR (Figure 9C).

Next, we tested whether the TM peptide analogs interfere with signaling of endogenous $\alpha_{1a}$-AR in hVSMCs. While the TM7 peptide analog did not influence PE-induced IP$_3$ production in hVSMCs, the TM2 and TM4 peptide analogs inhibited this response (Figure 9D). Next, we reevaluated the effects of CXCL12 on PE-induced IP$_3$ production in hVSMCs (Figure 9E).
Figure 10. Hetero-oligomerization of $\alpha_1$-adrenergic receptor (AR) with atypical chemokine receptor 3 (ACKR3) and chemokine (C-X-C motif) receptor 4 (CXCR4)—working model. A, Hetero-oligomeric complexes between $\alpha_{1B/D}$-AR and the ACKR3:CXCR4 heteromer are responsible for $\alpha_{1B/D}$-AR signaling on catecholamine stimulation in human vascular smooth muscle cells (hVSMCs). Activation of ACKR3 inhibits, whereas disruption of the ACKR3:CXCR4 hetero-oligomer complex inactivates $\alpha_{1B/D}$-AR signaling and shifts the patterns of receptor heteromerization within the entire receptor network towards a new equilibrium, which leads to the assembly of newly formed heteromeric complexes between $\alpha_{1A/B}$-AR and the ACKR3 and/or CXCR4 protomers/homodimers. B, Disruption of ACKR3:CXCR4 heteromers with peptide analogs of transmembrane (TM) domains of ACKR3 leads to the assembly of newly formed heteromeric receptor complexes. The TM2 peptide analog interferes with ACKR3:CXCR4 and $\alpha_{1B/D}$-AR:ACKR3:CXCR4 hetero-oligomerization, which inhibits $\alpha_{1B/D}$-AR signaling/function and leads to the formation of increased numbers of $\alpha_{1A/B}$-AR:CXCR4 complexes, and presumably to increased numbers of ACKR3 protomers/homodimers (not shown). The TM7 peptide disrupts ACKR3:CXCR4 heteromers without affecting $\alpha_{1B/D}$-AR:ACKR3:CXCR4 hetero-oligomerization, leading to increases in $\alpha_{1A}$:ACKR3 and $\alpha_{1A}$-AR:CXCR4 heteromers. Because $\alpha_{1B/D}$-AR:ACKR3:CXCR4 hetero-oligomers are not affected by the TM7 peptide, $\alpha_{1B/D}$-AR signaling/function is maintained.

Discussion

In the present study, we provide evidence that heteromeric complexes between ACKR3 and $\alpha_{1A/B/D}$-AR are constitutively expressed in hVSMCs. Our findings suggest that hetero-oligomerization of $\alpha_{1B/D}$-AR with the CXCR4:ACKR3 heteromeric complex is required for $\alpha_{1B/D}$-AR function (Figure 10A). Furthermore, our observations indicate that the formation of hetero-oligomeric GPCR complexes within the plasma membrane is a dynamic process that depends on the relative abundances of and affinities between available receptor partners (Figure 10B).

The finding that ACKR3 agonists inhibited $\alpha_1$-AR-mediated IP$_3$ production is consistent with the effects of the ACKR3 agonists on $\alpha_1$-AR-mediated constriction of isolated resistance arteries that we previously described and confirms functional cross-talk between ACKR3 and $\alpha_1$-ARs in hVSMCs.$^3$ Furthermore, the observed effects of CXCL12 in the present study are consistent with our previous findings that CXCL12 functions predominantly as an ACKR3 agonist in isolated mesenteric rat arteries, which attenuates PE-induced vasoconstriction, and during the cardiovascular stress response to hemorrhagic shock in rats, where CXCL12 impairs hemodynamic stability and blood pressure.$^3$ This behavior could be explained by the higher affinity of CXCL12 for ACKR3 than for CXCR4.$^{16}$ CXCL12, however, also enhanced the potency of PE to increase blood pressure in normal animals via CXCR4,
which indicates that its functions depend on the relative contribution of CXCR4 and ACKR3 to the specific experimental or pathophysiological environment.8

We detected that recombinant and endogenous ACKR3 and $\alpha_{1A/B/D}$-AR are in close proximity in cells. Because endogenous $\alpha_{1A/B/D}$-AR could also be co-immunoprecipitated with ACKR3 in hVSMCs, these data suggest that ACKR3 physically interacts with $\alpha_{1A/B/D}$-AR and that the close proximity between the receptor partners that we detected by PLA corresponds to direct receptor-receptor interactions. In contrast to interaction patterns between CXCR4 and $\alpha_{12}$-AR that we previously observed, our current findings indicate that ACKR3 also heteromerizes with $\alpha_{2B}$-AR in hVSMCs, suggesting selectivity of the observed receptor-receptor interactions.8

We have previously shown that siRNA knockdown of CXCR4 in hVSMCs reduces heterogenic CXCR4:ACKR3 and CXCR4: $\alpha_{1A/B}$-AR complexes and inhibits $\alpha_{1}$-AR function upon PE stimulation.8 The findings of the present study that siRNA knockdown of either ACKR3 or CXCR4 reduces heteromeric complexes between ACKR3: $\alpha_{1D}$-AR and between CXCR4: $\alpha_{1B/D}$-AR suggest that $\alpha_{1B/D}$-AR form hetero-oligomeric complexes with the CXCR4:ACKR3 heteromer and that such hetero-oligomerization is required for $\alpha_{1B/D}$-AR function. This assumption is supported by our observations on the effects of the TM2 peptide analog of ACKR3. While this peptide reduced $\alpha_{1B/D}$-AR:ACKR3 and ACKR3:CXCR4 heteromerization, abolished PE-induced IP$_3$ production in hVSMCs, and inhibited PE-induced constriction of isolated mesenteric arteries, it also increased the number of CXCR4: $\alpha_{1A/B}$-AR complexes (Figure 10B). As the TM2 peptide analog did not inhibit $\beta$-arrestin 2 recruitment to recombinant $\alpha_{2A}$-AR on PE stimulation, these findings further indicate that it inhibits $\alpha_{1B/D}$-AR function through interference with receptor hetero-oligomerization. In combination with the observation that CXCL12 did not enhance PE-induced signaling in hVSMCs in the presence of the TM2 peptide, these data support the notion that the hetero-oligomeric complex between the CXCR4:ACKR3 heteromer and $\alpha_{1B/D}$-AR, but not the heteromer between $\alpha_{1B/D}$-AR and the CXCR4 protomer or homodimer, regulates $\alpha_{1B/D}$-AR function in hVSMCs. The observation that the TM7 peptide did not affect PE-induced signaling is consistent with this concept as it did not reduce ACKR3: $\alpha_{1B/D}$-AR and CXCR4: $\alpha_{1B/D}$-AR, despite partial interference with ACKR3:CXCR4 heteromerization (Figure 10B).

Research on the biological roles of ACKR3 has been hampered by the lack of pharmacological ACKR3 inhibitors. The findings of the present study identify the TM2 peptide analog as an ACKR3 inhibitor without intrinsic agonist activity within the tested range of concentrations. Thus, we cannot exclude that inhibition of ACKR3 also contributes to the inhibitory effects of the TM2 peptide analog on $\alpha_{1}$-AR signaling in hVSMCs. Nevertheless, such a mechanism would be consistent with the assumption that conformational changes of ACKR3 on agonist or antagonist binding inhibit $\alpha_{1B/D}$-AR via allosteric interactions within the hetero-oligomeric receptor complex. We observed that ACKR3 knockdown did not affect CXCR4: $\alpha_{1A}$-AR heteromerization and that CXCR4 knockdown did not affect ACKR3: $\alpha_{1D}$-AR heteromerization. Furthermore, all TM peptide analogs interfered with ACKR3:CXCR4 heteromerization but increased CXCR4: $\alpha_{1A}$-AR heteromers. As we have previously shown that CXCR4 knockdown in hVSMCs reduces CXCR4: $\alpha_{1A}$-AR heteromers,8 these observations suggest that $\alpha_{1A}$-AR predominantly forms heteromeric complexes with CXCR4 and ACKR3 protomers or homodimers, whereas $\alpha_{1B/D}$-AR preferentially form hetero-oligomeric complexes with the CXCR4:ACKR3 heteromer. We have previously shown that partial depletion of vascular smooth muscle CXCR4 in a Cre-lox mouse model did not reduce CXCR4: $\alpha_{1A/B}$-AR heteromerization.4 Similarly, partial depletion of ACKR3 in hVSMCs did not reduce ACKR3: $\alpha_{1A}$-AR heteromerization in the present study. These observations imply that the formation of heteromeric receptor complexes is not a direct function of total expression levels of a single receptor and depends on relative affinities between interacting receptor partners and their relative abundances on the cell surface. In such a system, ACKR3: $\alpha_{1A}$-AR heteromerization may be favored over the formation of other ACKR3 heteromers and ACKR3 protomers or homodimers. Reduction of ACKR3: $\alpha_{1A}$-AR heteromerization would then only occur when the relative abundance of ACKR3 falls below the number of $\alpha_{1A}$-ARs that are available for heteromerization.

We observed that the ACKR3 TM peptide analogs selectively interfered with ACKR3 heteromerization but also increased the number of other ACKR3 and CXCR4 heteromeric complexes. These findings suggest that binding of the peptides to their corresponding TM receptor domains interferes with distinct receptor heteromerization interfaces, either by direct blockade of interactions sites or via allosteric effects on the target receptor conformation. Disruption of hetero-oligomeric receptor complexes by the TM peptides then leads to the assembly of newly formed hetero-oligomeric receptor complexes, depending on the relative affinities for and abundances of available receptor partners and receptor interaction sites (Figure 10). Thus, these observations suggest that the formation of hetero-oligomeric receptor complexes within the plasma membrane is a dynamic process, in which interference with heteromerization between receptor partners shifts the patterns of receptor heteromerization within the entire receptor network towards a new equilibrium. This assumption would be consistent with previous observations that recombinant GPCRs exist on the cell surface in a dynamic equilibrium between monomeric receptors and
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dimeric/oligomeric receptor complexes.\(^{29-31}\) Provided that receptor heteromerization modulates the pharmacological properties of each receptor partner, such regulation of heteromeric receptor complex formation implies that receptor function is rapidly adapted to the specific environment and explains biological variability of GPCR-mediated functions.

We did not employ an unrelated control peptide, e.g., a scrambled peptide of similar amino acid composition, in our experiments. Nevertheless, the differential effects of the TM peptide analogs on receptor heteromerization and function serve as rigorous controls for each other.

Although depletion of ACKR3 in hVSMCs and incubation of hVSMCs with the TM2 and TM4 peptide analogs did not affect expression levels of ACKR3:\( \alpha_1A \)-AR complexes, \( \alpha_1 \)-AR-mediated signaling on PE stimulation was abolished under both conditions. Furthermore, the TM7 peptide increased the number of ACKR3:\( \alpha_1A \)-AR heteromers on hVSMCs but did not affect PE-induced \( \alpha_1 \)-AR function. PE-induced IP\(_3\) production, however, was not sensitive to the selective \( \alpha_1A \)-AR antagonist 5'-methylurapidil but could be inhibited with a pan \( \alpha_1 \)-AR antagonist and with selective \( \alpha_1B/D \)-AR antagonists. This suggests that the relative contribution of \( \alpha_1A \)-AR to the combined \( \alpha_1 \)-AR response is limited under our experimental conditions, which is consistent with the relative functional relevance of \( \alpha_1 \)-AR subtypes previously reported.\(^{32-34}\) Thus, we cannot currently comment on the functional role of \( \alpha_1A \)-AR:ACKR3 heteromers. Similarly, possible functions of ACKR3:\( \alpha_2D \)-AR heteromeric complexes remain to be determined in future studies.

Conclusions

We show that recombinant and endogenous ACKR3 form heteromeric complexes with \( \alpha_1 \)-AR, that endogenous heteromeric ACKR3:\( \alpha_1B/D \)-AR complexes can be disrupted with TM-derived peptide analogs of ACKR3 without altering expression levels of individual receptors, and that disruption of heteromeric ACKR3:\( \alpha_1B/D \)-AR complexes results in the loss of \( \alpha_1B/D \)-AR function. Thus, our findings fulfill recently proposed criteria for GPCR heteromers in native tissues.\(^{11}\) Our observations suggest that \( \alpha_1B/D \)-AR function depends on direct physical interactions with the ACKR3:CXCR4 heteromer, which may serve as a molecular scaffold to maintain \( \alpha_1B/D \)-AR in a functional configuration within the plasma membrane. In combination with our previous findings,\(^{3,4,8}\) these data further suggest that ACKR3 on agonist binding negatively regulates the activity of \( \alpha_1B/D \)-AR, whereas CXCR4 on agonist binding sensitizes \( \alpha_1B/D \)-AR function via allosteric interactions within the hetero-oligomeric receptor complex (Figure 10A). Our findings provide new insights into the regulation of \( \alpha_1 \)-AR in hVSMCs and highlight the functional relevance of GPCR hetero-oligomerization for GPCR function. Furthermore, we provide initial evidence that hetero-oligomerization of endogenous GPCRs is a dynamic process, which could explain biological variability of GPCR-mediated signaling events and functions. We believe that drugs targeting heteromeric complexes between \( \alpha_1 \)-AR, CXCR4, and ACKR3 could provide new pharmacological approaches to regulate vascular function in disease conditions.

Sources of Funding

This work was supported by the National Institute of General Medical Sciences (award R01GM107495) and by the National Cancer Institute (award R01CA188427). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Disclosures

None.

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DOI: 10.1161/JAHA.117.006575
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