

α_1 -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 α_1 -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 α_1 -ARs through formation of heteromeric receptor complexes in human vascular smooth muscle cells (hVSMCs), the molecular basis underlying cross-talk between ACKR3 and α_1 -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 $\alpha_{1A/B/D}$ -AR. While small interfering RNA knockdown of ACKR3 in hVSMCs reduced $\alpha_{1B/D}$ -AR:ACKR3, CXCR4:ACKR3, and $\alpha_{1B/D}$ -AR:CXCR4 complexes, small interfering RNA knockdown of CXCR4 reduced $\alpha_{1B/D}$ -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, $\alpha_{1A/B/D}$ -AR, and CXCR4. While the transmembrane domain 2 peptide interfered with $\alpha_{1B/D}$ -AR:ACKR3 and CXCR4:ACKR3 heteromerization, it increased heteromerization between CXCR4 and $\alpha_{1A/B}$ -AR. The transmembrane domain 2 peptide inhibited ACKR3 but did not affect α_{1B} -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— α_1 -ARs form hetero-oligomeric complexes with the ACKR3:CXCR4 heteromer, which is required for $\alpha_{1B/D}$ -AR function, and activation of ACKR3 negatively regulates α_1 -ARs. G protein-coupled receptor hetero-oligomerization is a dynamic process, which depends on the relative abundance of available receptor partners. Endogenous α_1 -ARs 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 α_1 -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 α_1 -AR function and impairs blood pressure.³ Subsequently, we provided evidence that heteromeric complexes between endogenous CXCR4 and $\alpha_{1A/B}$ -AR are constitutively expressed in human vascular smooth muscle cells (hVSMCs), through which CXCR4 modulates α_1 -AR function.^{4,8} The molecular mechanisms underlying cross-talk between ACKR3 and α_1 -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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Clinical Perspective

What Is New?

- Heteromeric complexes between atypical chemokine receptor 3 and $\alpha_{1A/B/D}$ -adrenergic receptors are constitutively expressed in human vascular smooth muscle cells.
- Hetero-oligomerization of $\alpha_{1B/D}$ -adrenergic receptor with the chemokine (C-X-C motif) receptor 4:atypical chemokine receptor 3 heteromeric complex is required for $\alpha_{1B/D}$ -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 α_1 -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.

fundamental signaling unit.^{9,10} In contrast to the traditional view, the concept of GPCR heteromerization assumes that GPCRs function within heteromeric receptor complexes, in which direct physical associations between the receptor partners modulate receptor signaling properties and function. While the existence of numerous GPCR heteromers has been implied based on studies in expression systems, only few of such heteromeric receptor complexes have been validated in native tissues.¹¹

CXCR4 is a typical GPCR that couples to $G\alpha_i$ and recruits β -arrestin to the receptor.¹² ACKR3 has initially been thought to function as a scavenger receptor, which sequesters chemokine ligands from the cell surface, but does not induce cellular signaling events.^{13,14} Subsequent studies provided evidence that ACKR3 activation does induce β -arrestin-mediated and G protein-independent signaling events.^{12,15,16} Furthermore, ACKR3 has been described to form heteromeric complexes with CXCR4, leading to preferential activation of β -arrestin-mediated signaling pathways over canonical G protein pathways on activation of the receptor complex with C-X-C motif chemokine ligand (CXCL)12.^{17,18} While CXCL12 is the cognate ligand of CXCR4 and ACKR3,¹² macrophage migration inhibitory factor (MIF) and ubiquitin are other endogenous ligands of CXCR4, and MIF and CXCL11 are other endogenous ligands of ACKR3.^{12,19} As recent reports suggest that α -ARs and β -ARs can form heteromeric complexes with other GPCRs, such as other ARs, adenosine A1 receptor, dopamine

receptor 4, angiotensin II type I receptor or CXCR4,^{11,20} we studied whether heteromerization of ACKR3 with α_1 -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 α_{1A} -AR and between the ACKR3:CXCR4 heteromer and $\alpha_{1B/D}$ -AR are constitutively expressed in hVSMC, through which ACKR3 regulates α_1 -AR function.

Methods

Proteins, Peptides, and Reagents

Phenylephrine (PE), Phentolamine, 5'-Methylurapidil, Cyclozolin 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; YILNLAIDLWVLTIPVWVDDDD), TM4 (VVCILWLLAFVSLPDTYYLDD), and TM7 (DDDLHVTCQLSLVHCCVNPVLYSFIN) 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'-(ethylenedioxy) diethanethiol and 5% thioanisole. 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 μ g/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 μ g/mL streptomycin. The HTLA cell line, a HEK293 cell line stably expressing a tTA-dependent luciferase reporter and a β -arrestin2-TEV

fusion gene,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, 1× nonessential amino acids, 100 U/mL penicillin, 100 μ g/mL streptomycin, 50 μ g/mL hygromycin B, and 2 μ g/mL puromycin. All cells were cultured in a humidified environment at 37°C, 5% CO₂.

Proximity Ligation Assays

Proximity ligation assays (PLAs) were performed as previously described in detail.^{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- α_{1A} -AR (Abcam Ab137123), rabbit anti- α_{1B} -AR (Abcam Ab169523), goat anti- α_{1D} -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- α_{1A} -AR (Abcam Ab137123) or rabbit anti- α_{1B} -AR (Abcam Ab169523) or goat anti- α_{1D} -AR (Santa Cruz SC27099) or with a combination of goat anti-CXCR4 (Abcam 1670) and rabbit anti- α_{1A} -AR (Abcam Ab137123) or rabbit anti- α_{1B} -AR (Abcam Ab169523) or rabbit anti-CXCR4 (Alomone Labs ACR-014) and goat anti- α_{1D} -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 0.01× 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

Axiovert 200M with EC Plan-Neofluor objective lenses [40×/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.^{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.⁸ Fifty micrograms of anti-ACKR3 (R&D, MAB42273) or anti-mouse IgG₁ (Abcam Ab81032) were incubated with 50 μ L AminoLink Plus Coupling Resin for 180 minutes at room temperature. Five hundred micrograms of cell lysate was precleared with 50 μ L of the control agarose resin slurry (60 minutes at 4°C). Immobilized anti-ACKR3 resin and anti-IgG₁ resin were incubated with precleared lysate for 24 to 48 hours at 4°C. After incubation, the resins were washed 3 times with 200 μ L IP lysis/wash buffer, once with conditioning buffer, and protein was eluted using 50 μ L of elution buffer. Samples were analyzed by Western blotting.

Western Blotting

Western blotting with rabbit anti-ACKR3 (Abcam Ab38089), rabbit anti- α_{1A} -AR (Abcam Ab137123), rabbit anti- α_{1B} -AR (Abcam Ab169523), goat anti- α_{1D} -AR (Santa Cruz SC27099), and mouse anti- α_{2C} -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.⁸

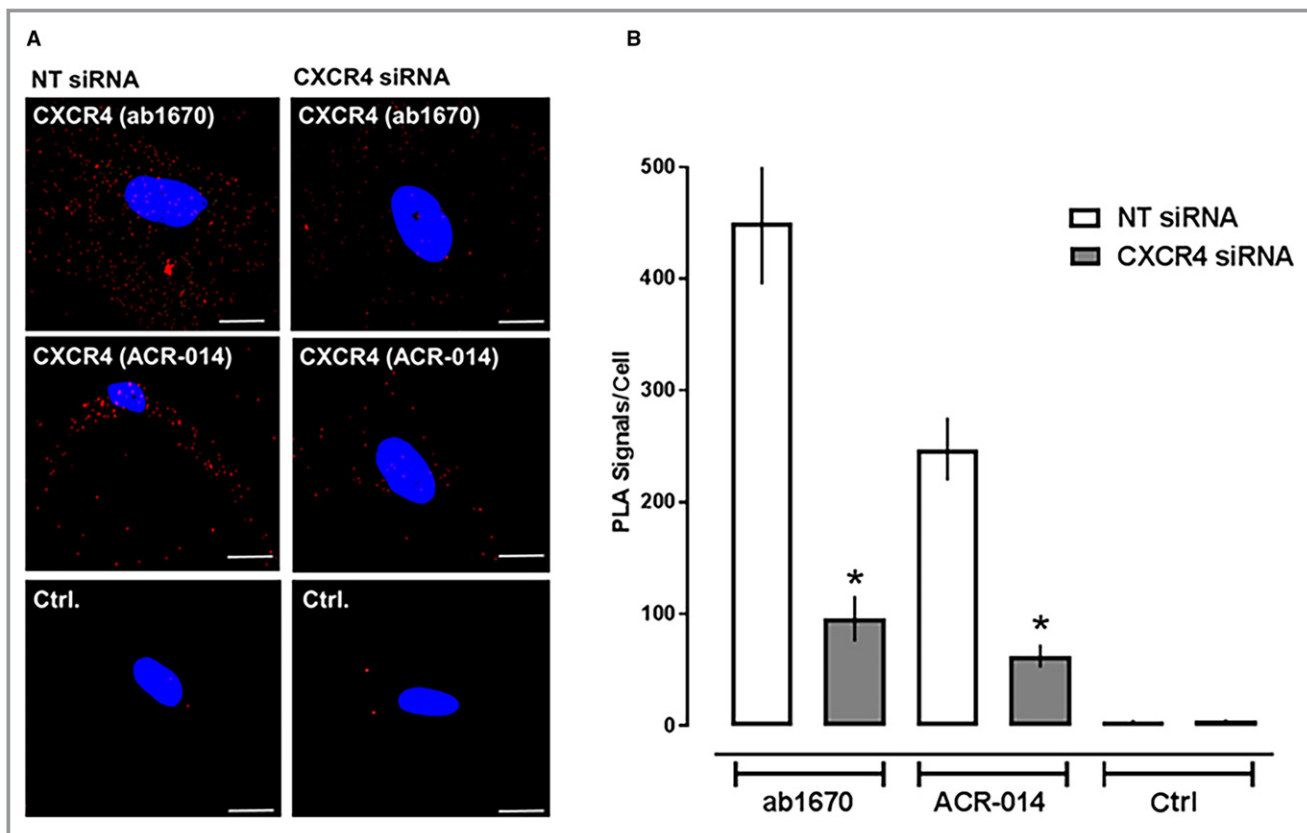


Figure 1. Validation of anti-chemokine (C-X-C motif) receptor 4 (CXCR4) antibodies. Human vascular smooth muscle cells were incubated with nontargeting (NT) or CXCR4 small interfering RNA (siRNA) and then used in proximity ligation assays (PLAs) for the detection of CXCR4 with goat anti-CXCR4 (Abcam 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 μ m. B, Quantification of PLA signals per cell, as in (A). n=10 images per condition. *: p<0.05 vs. cells incubated with NT siRNA.

Gene Silencing via RNA Interference

ACKR3 and CXCR4 siRNA gene silencing was performed as previously described.²⁵ 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 \times siRNA buffer to a stock concentration of 100 μ mol/L. Cells were then transfected with 1 μ mol/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 μ g of DNA encoding either human influenza hemagglutinin (HA)-tagged ACKR3 (HA-ACKR3), FLAG-ACKR3, HA-CXCR4, FLAG- α_{1a} -AR, FLAG- α_{1b} -AR, or FLAG- α_{1d} -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 confluency in 6-well dishes (Nunc) and then treated as described in the Results section. Cells were then washed once with cold PBS, 225 μ L 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 (Bio-Rad 500-0116). 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

(absorbance at 450 nm) and IP_3 concentrations were extrapolated from the standard curve.

PRESTO-Tango β -Arrestin Recruitment Assay

The PRESTO-Tango (parallel receptorome expression and screening via transcriptional output, with transcriptional activation following arrestin translocation) assay was performed as recently described.²¹ The ACKR3 and α_{1B} -AR-Tango plasmids were a gift from Dr Bryan Roth (Addgene plasmids #66265 and #66214). HTLA cells (2.5×10^5 /well) were seeded in a 6-well plate and transfected with 1.5 μ g of the Tango plasmids using Lipofectamine 3000. The following day, transfected HTLA cells (1×10^5 cells/well) were plated onto Poly-L-Lysine precoated 96-well microplates and allowed to attach to the plate surface for at least 4 hours before treatment. Proteins used for treatment were prepared in twice the final concentration in culture media, added at a 1:1 (vol/vol) ratio and incubated overnight at 37°C, 5% CO₂ in a humidified environment. The following morning, media were removed from cell culture plates and replaced with a 100- μ L 1:5 mixture of Bright-Glo (Promega) and 1 \times Hanks' balanced salt solution, 20 mmol/L HEPES solution. Plates were then incubated at room temperature before measuring luminescence 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% isoflurane. The mesentery was immediately removed and placed in 130 mmol/L NaCl, 4.7 mmol/L KCl, 1.18 mmol/L KH₂PO₄, 1.17 mmol/L MgSO₄, 14.9 mmol/L, NaHCO₃, 5.5 mmol/L D-Glucose, 0.026 mmol/L EDTA, and 1.16 mmol/L CaCl₂ aerated with 95% O₂, 5% CO₂ at 37°C. The animal was then euthanized by cardiectomy 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% O₂, 5% CO₂ 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 \pm 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_q-Mediated Signaling on α_1 -AR Activation

We have previously shown that ACKR3 activation antagonizes vasoconstriction of isolated mesenteric resistance arteries upon stimulation with the selective α_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 α_1 -AR activation. We first confirmed ACKR3 agonist activity of the ligands in a β -arrestin 2 recruitment assay (PRESTO-Tango²¹). As shown in Figure 2A, the EC₅₀ for β -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 β -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 α_1 -AR-mediated signaling (Figure 2B).

ACKR3 Forms Heteromeric Complexes With $\alpha_{1A/B/D}$ -AR

Next, we studied whether recombinant ACKR3 would be able to form heteromeric complexes with recombinant α_1 -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

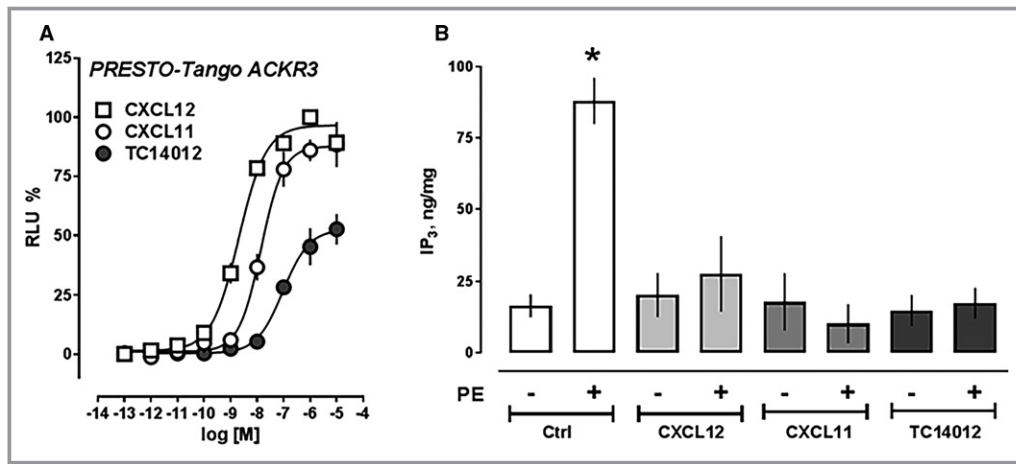


Figure 2. Atypical chemokine receptor 3 (ACKR3) inhibits α_1 -adrenergic receptor (AR) signaling in human vascular smooth muscle cells (hVSMCs). A, β -arrestin 2 recruitment assay (PRESTO-Tango) for ACKR3. Cells were treated with chemokine (C-X-C motif) ligand (CXCL) 12 (open squares), CXCL 11 (open circles), and TC14012 (grey circles). RLU (%): relative luminescence units in percentage of the RLU after treatment with 1 μ mol/L CXCL12 (=100%). n=3 independent experiments. B, hVSMCs were treated with vehicle (control, Ctrl) or ACKR3 ligands (1 μ mol/L, 15 minutes) and then stimulated with 1 μ mol/L phenylephrine (PE) for 5 minutes. Inositol trisphosphate (IP₃) production was measured by ELISA. n=3 independent experiments. * P <0.05 vs. unstimulated cells.

Heteromerization between ACKR3 and CXCR4 and between CXCR4 and α_{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- α_{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- $\alpha_{2A/B/C}$ -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 α_{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 α -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 α_{2B} -AR were detectable in ACKR3 immunoprecipitates (Figure 4D through 4G), whereas α_{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- α_{1D} -AR, but we were unable to detect α_{1D} -AR in the hVSMC lysate (input). We interpret this observation to reflect low abundance of α_{1D} -AR on hVSMC and high enrichment of α_{1D} -AR in the ACKR3 immunoprecipitate. Furthermore, the band corresponding to α_{1A} -AR in the ACKR3 immunoprecipitate migrated with a slightly lower molecular mass (46.1 kDa; 95% confidence interval, 42.2–50.5) than in the hVSMC lysate (49 kDa; 95% confidence interval, 44.8–53.8), which could be explained by differences in posttranslational receptor modifications or partial proteolytic processing, which may have occurred during the experimental procedure. While we observed 3 bands for α_{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 for human α_{2B} -AR (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 α_1 -AR

To assess the functional role of heteromeric complexes between ACKR3 and $\alpha_{1A/B/D}$ -AR on α_1 -AR function, we then aimed to reduce heteromeric complexes in hVSMCs by

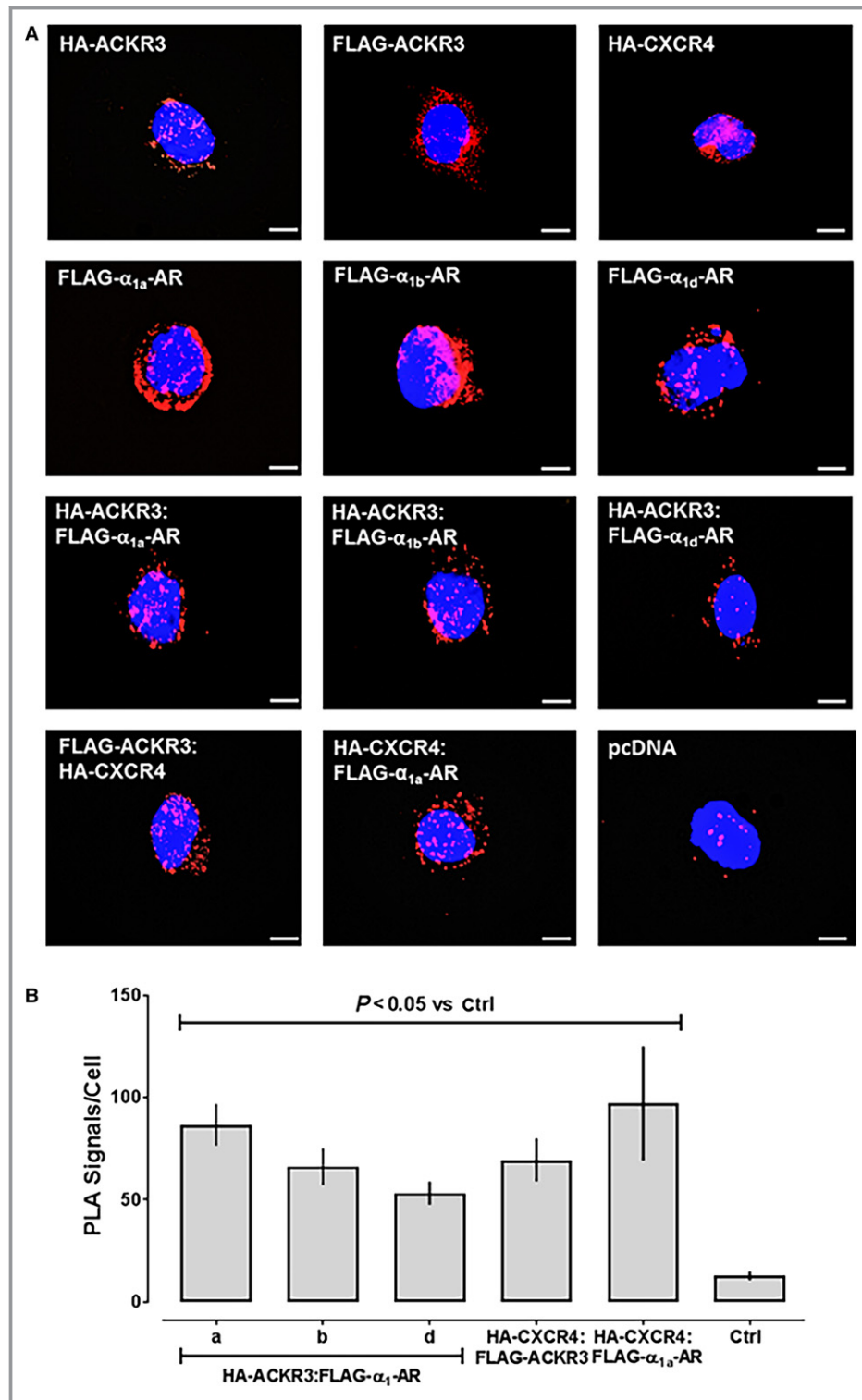


Figure 3. Atypical chemokine receptor 3 (ACKR3) forms heteromeric complexes with $\alpha_{1a/b/d}$ -adrenergic receptors (ARs) in human embryonic kidney 293T (HEK293T) cells. A, Typical proximity ligation assay (PLA) images for the detection of individual receptors and receptor-receptor interaction in 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 μ m. B, Quantification of PLA signals per cell, as in (A). n=3 independent experiments with n=10 images per condition and experiment.

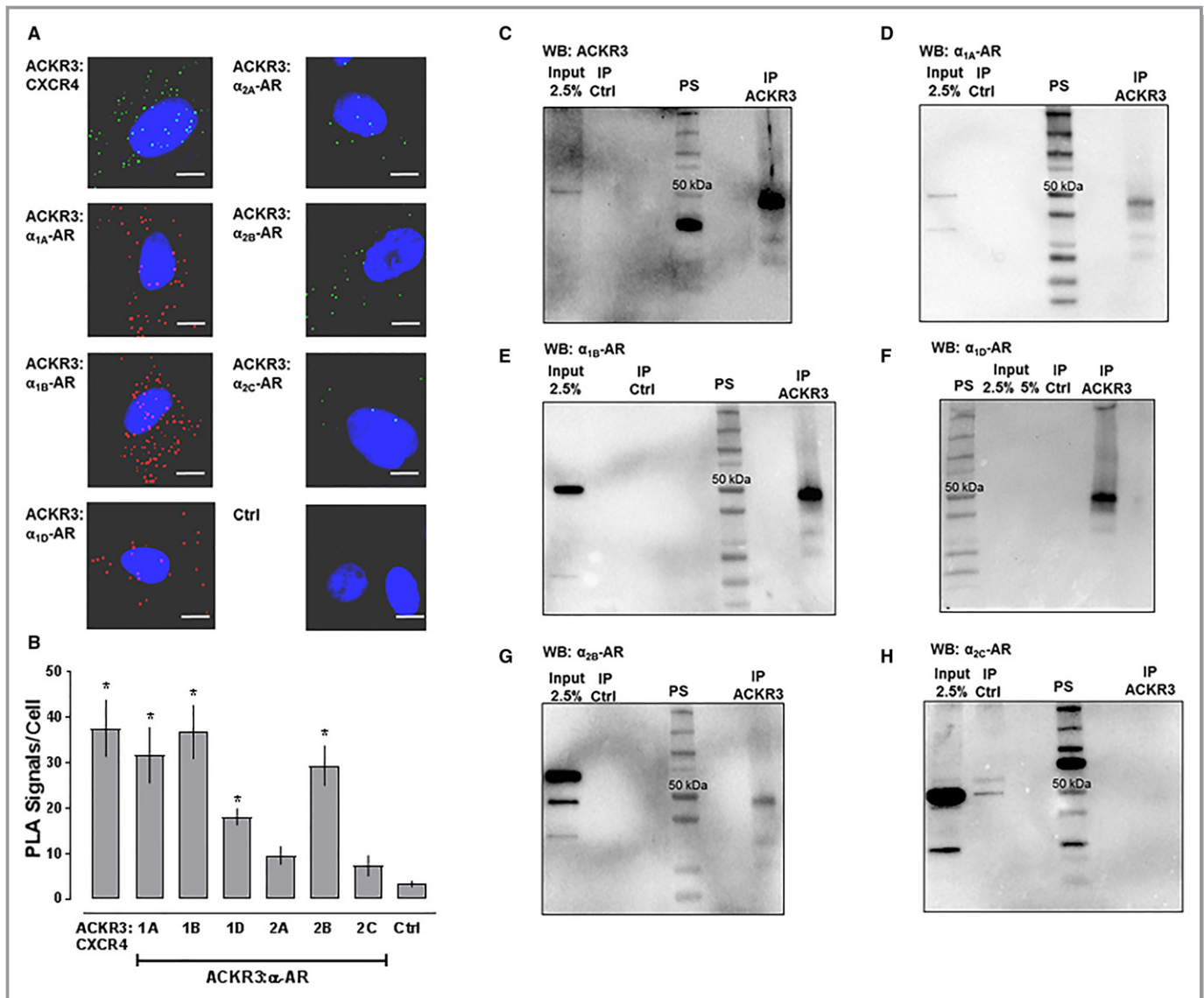


Figure 4. Atypical chemokine receptor 3 (ACKR3) forms heteromeric complexes with $\alpha_{1A/B/D}$ -adrenergic receptor (AR) and α_{2B} -AR in human vascular smooth muscle cells (hVSMCs). A, Representative proximity ligation assay (PLA) images for the detection of receptor-receptor interactions in hVSMCs. Ctrl: Omission of one primary antibody. Images show merged PLA (green or red)/4',6-diamidino-2-phenylindole dihydrochloride signals. Scale bars=10 μ m. B, Quantification of PLA signals for receptor-receptor interactions per cell, as in (A). $n=3$ independent experiments with $n=10$ images per condition and experiment. $*P<0.05$ vs control. C through H, hVSMCs were lysed and ACKR3 was immunoprecipitated (IP) followed by Western blotting (WB) to detect ACKR3 (C), α_{1A} -AR (D), α_{1B} -AR (E), α_{1D} -AR (F), α_{2B} -AR (G), and α_{2C} -AR (H) in the IP samples. IP control: precipitate after incubation of cell lysates with nonreactive or IgG₁-coupled resin. The white light images are overlaid at the corresponding position of the standard proteins (PS). Images are representative of $n=3$ independent experiments.

ACKR3 knockdown with siRNA. Typical PLA images for the detection of individual receptors and receptor-receptor interactions in hVSMCs after incubation with NT siRNA and ACKR3 siRNA 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 $\alpha_{1A/B/D}$ -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: $\alpha_{1B/D}$ -AR and ACKR3:CXCR4 interactions but did not affect ACKR3: α_{1A} -AR or CXCR4: α_{1A} -AR interactions (Figure 5C). When hVSMCs were tested for PE-induced IP₃ production after incubation with NT siRNA or ACKR3 siRNA, we observed that PE increased cellular IP₃ concentrations 4.8-fold after incubation with NT siRNA but failed to increase IP₃ concentrations in cells after incubation with ACKR3 siRNA (Figure 5D). Because ACKR3 siRNA

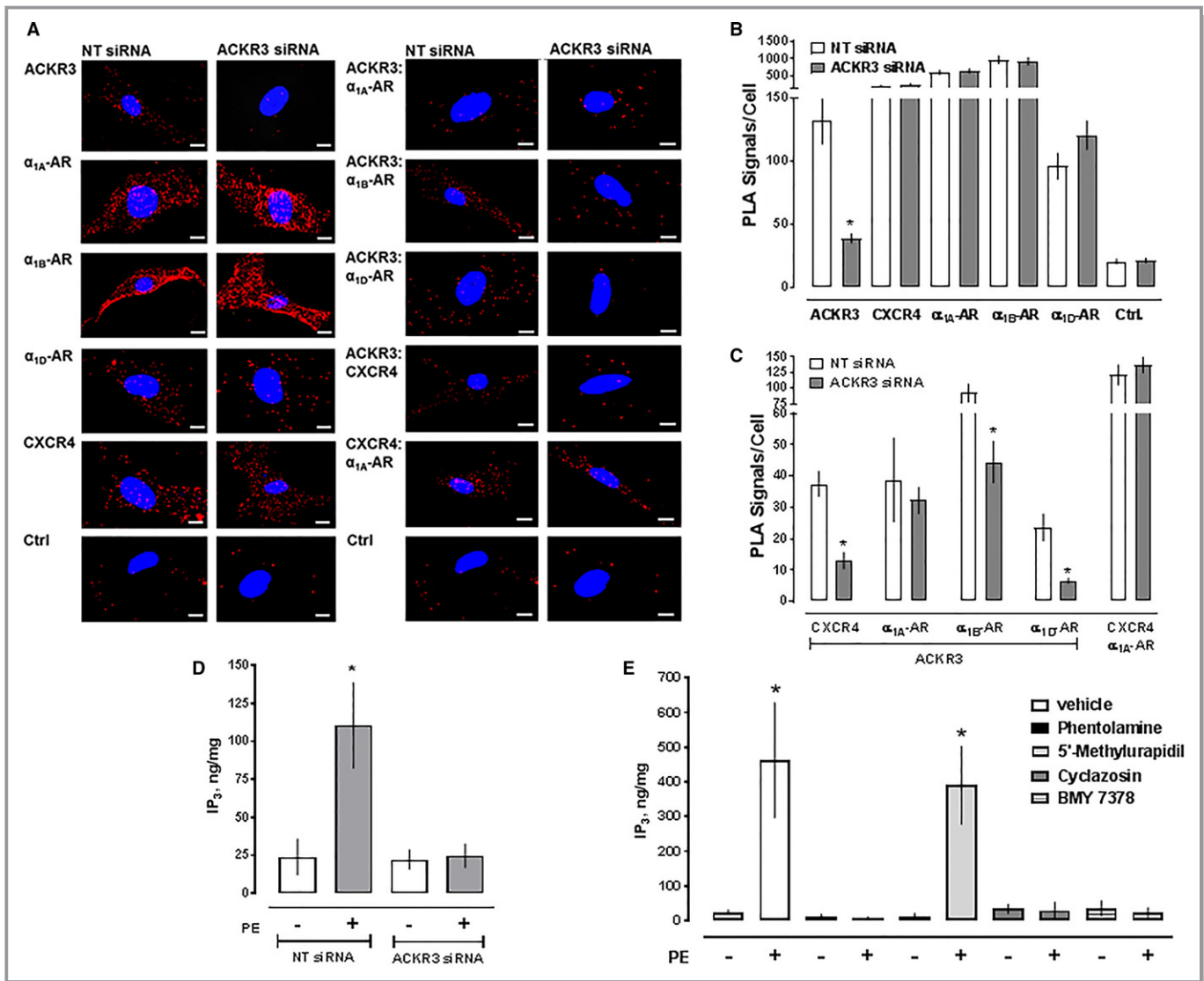


Figure 5. Atypical chemokine receptor 3 (ACKR3) gene silencing reduces ACKR3: $\alpha_{1B/D}$ -adrenergic receptor (AR) and ACKR3: chemokine (C-X-C motif) receptor 4 (CXCR4) heteromerization and inhibits $\alpha_{1B/D}$ -AR signaling in human vascular smooth muscle cells (hVSMCs). A, Representative proximity 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 μ m. 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 (IP₃) production of hVSMCs incubated with NT or ACKR3 siRNA upon stimulation with vehicle or 1 μ mol/L phenylephrine (PE). n=4 independent experiments. * P <0.05 vs vehicle. E, IP₃ production of hVSMCs upon stimulation with vehicle or 1 μ mol/L phenylephrine for 5 minutes. Cells were pretreated (30 minutes, 37°C) with 10 μ mol/L of vehicle or α_1 -AR antagonists. n=3 independent experiments. * P <0.05 vs vehicle.

knockdown did not significantly reduce ACKR3: α_{1A} -AR but abolished PE-induced IP₃ production in hVSMCs, we then evaluated the relative contribution of the α_1 -AR subtypes to this signaling response. As shown in Figure 5E, PE-induced IP₃ production could be inhibited with the nonselective α_1 -AR antagonist phentolamine and with the selective $\alpha_{1B/D}$ -AR antagonists cyclazosin and BMY 7378, respectively. In

contrast, the selective α_{1A} -AR antagonist 5'-methylurapidil did not inhibit PE-induced IP₃ production in hVSMCs.

We previously showed that CXCR4 knockdown reduces heteromeric complexes between CXCR4 and $\alpha_{1A/B}$ -AR and between CXCR4 and ACKR3, leading to loss of α_1 -AR signaling.⁸ Because CXCR4 likely exists as a homodimer and also within CXCR4:ACKR3 heteromeric

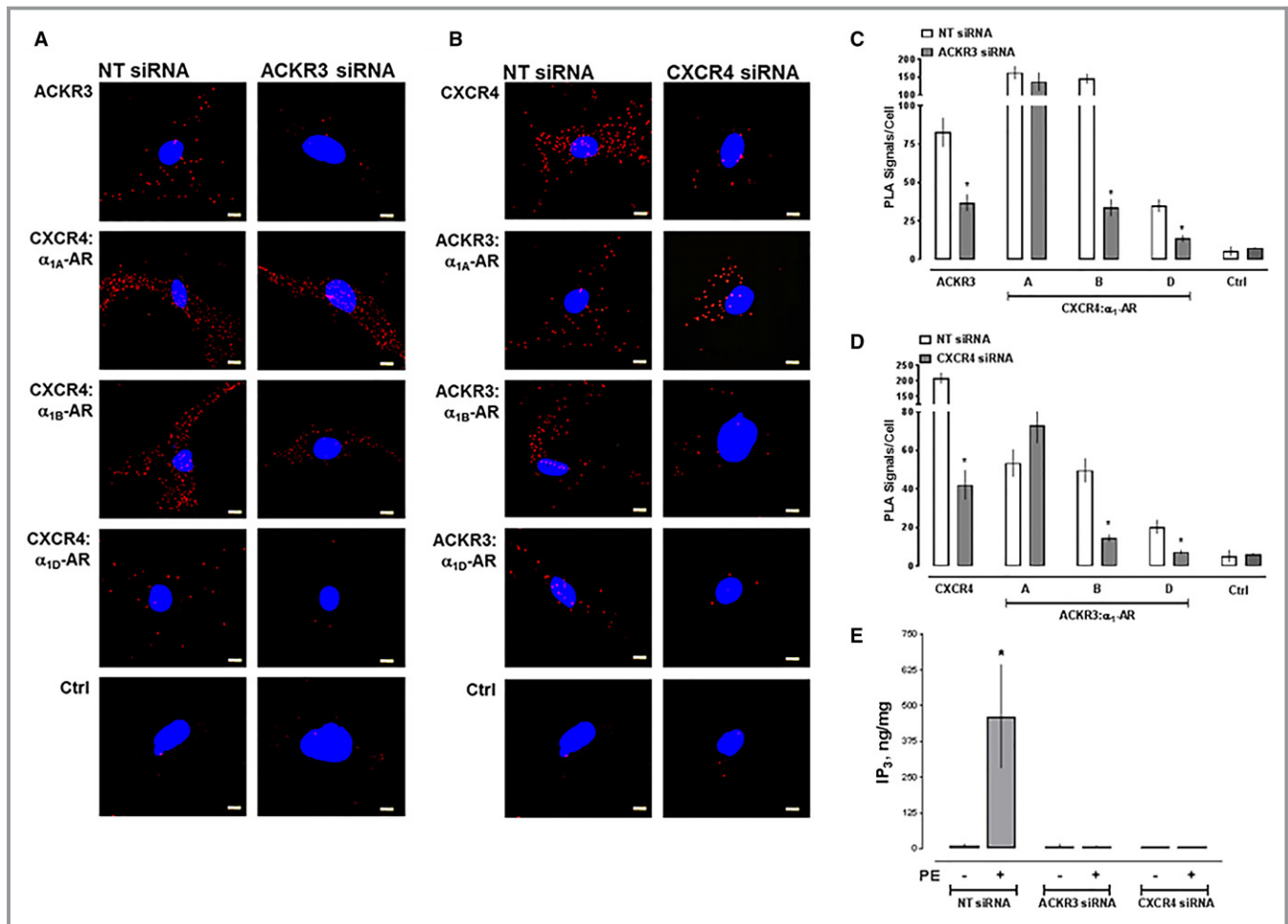


Figure 6. $\alpha_{1B/D}$ -Adrenergic receptors (ARs) form hetero-oligomeric complexes with the atypical chemokine receptor 3 (ACKR3): chemokine (C-X-C motif) receptor 4 (CXCR4) heteromer in human vascular smooth muscle cells (hVSMCs). A, Typical proximity ligation assay (PLA) images for the detection of ACKR3 and CXCR4: $\alpha_{1A/B/D}$ -AR heteromers in hVSMCs incubated 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 μ m. 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 μ m. 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 triphosphate (IP₃) production of hVSMCs incubated with NT, CXCR4, or ACKR3 siRNA upon stimulation with vehicle or 1 μ mol/L phenylephrine (PE). n=3 independent experiments. * P <0.05 vs vehicle.

complexes,^{4,8,17,18} 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 NT and ACKR3 siRNA are shown in Figure 6A and the quantification of the corresponding PLA signals from 3 independent experiments are shown in Figure 6C. Incubation of hVSMCs with ACKR3 siRNA reduced PLA signals for ACKR3 to $44 \pm 6\%$ of the signals for cells incubated with NT siRNA. As observed before (Figure 5C), ACKR3 knockdown did not significantly affect heteromerization between CXCR4 and α_{1A} -AR. ACKR3

knockdown, however, reduced signals corresponding to CXCR4: α_{1B} -AR and CXCR4: α_{1D} -AR to $23 \pm 4\%$ and $38 \pm 6\%$ of the signals of cells incubated with NT siRNA, respectively. Typical PLA images for the detection of CXCR4 and ACKR3: $\alpha_{1A/B/D}$ -AR heteromers after incubation of cells with NT and CXCR4 siRNA are shown in Figure 6B and the quantification of the corresponding PLA signals from 3 independent experiments are shown in Figure 6D. Incubation of hVSMCs with CXCR4 siRNA reduced PLA signals for CXCR4 to $20 \pm 3\%$ of the signals for cells incubated with NT siRNA. While signals for ACKR3: α_{1A} -AR were not affected after CXCR4 knockdown, signals corresponding to ACKR3: α_{1B} -AR and ACKR3: α_{1D} -AR

were reduced to $30\pm 3\%$ and $36\pm 5\%$ 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 β_2 -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, $\alpha_{1A/B/D}$ -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: $\alpha_{1B/D}$ -AR complexes but did not affect signals for ACKR3: α_{1A} -AR complexes. The TM4 peptide analog reduced PLA signals for ACKR3: α_{1B} -AR complexes without affecting ACKR3: $\alpha_{1A/D}$ -AR interactions. In contrast, the TM7 peptide analog significantly increased the number of PLA signals for ACKR3: α_{1A} -AR complexes but did not alter signals corresponding to ACKR3: $\alpha_{1B/D}$ -AR heteromers. While PLA

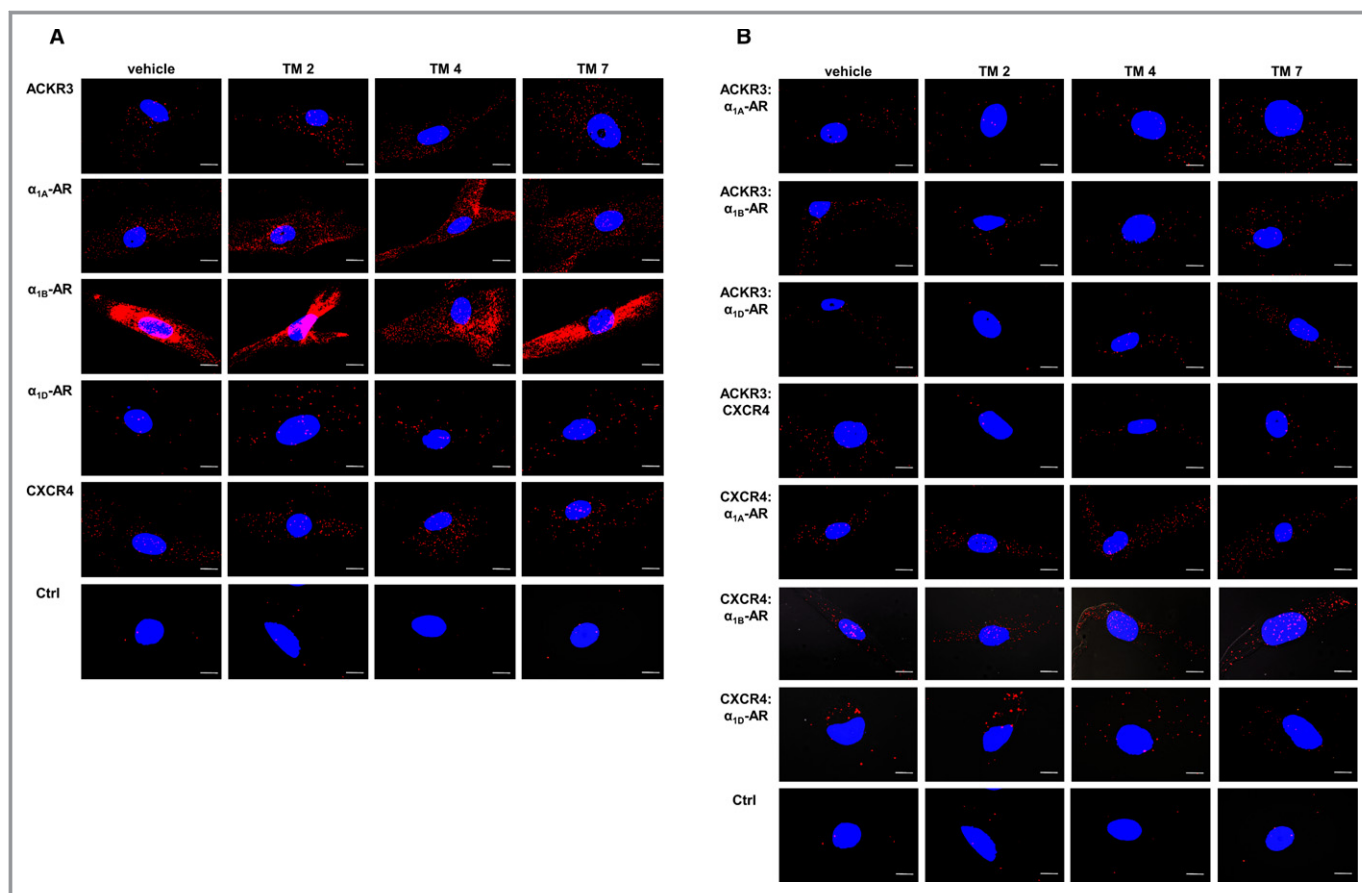


Figure 7. Peptides derived from transmembrane domains of atypical chemokine receptor 3 (ACKR3) alter receptor heteromerization in human vascular smooth muscle cells (hVSMCs). hVSMCs were treated with vehicle or transmembrane (TM) 2/4/7 peptide analogs ($10\ \mu\text{mol/L}$, 30 minutes at 37°C), washed 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= $10\ \mu\text{m}$. 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= $10\ \mu\text{m}$. AR indicates adrenergic receptor; CXCR4, chemokine (C-X-C motif) receptor 4.

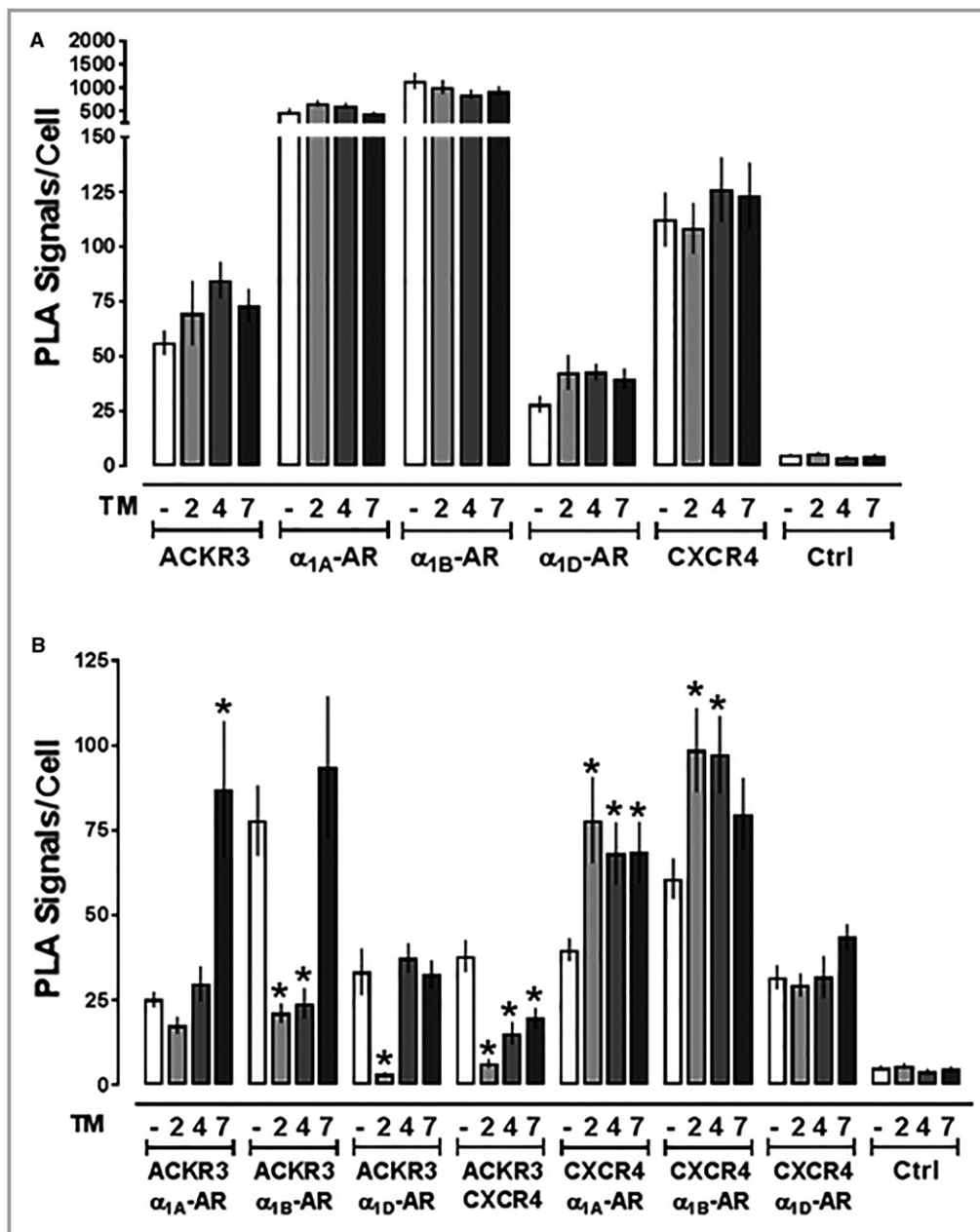


Figure 8. Peptides derived from transmembrane domains of atypical chemokine receptor 3 (ACKR3) alter receptor heteromerization in human vascular smooth muscle cell (hVSMC)—proximity ligation assay (PLA) signal quantification. A, Quantification of PLA signals per cell for the detection of individual receptors, 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.

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: α_{1A} -AR. Furthermore, the TM2 and TM4 peptides also increased signals for CXCR4: α_{1B} -AR complexes but did not affect signals for CXCR4: α_{1D} -AR complexes. The TM7 peptide did not significantly affect signals for CXCR4: α_{1B} -AR or CXCR4: α_{1D} -AR complexes.

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 α_{1B} -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

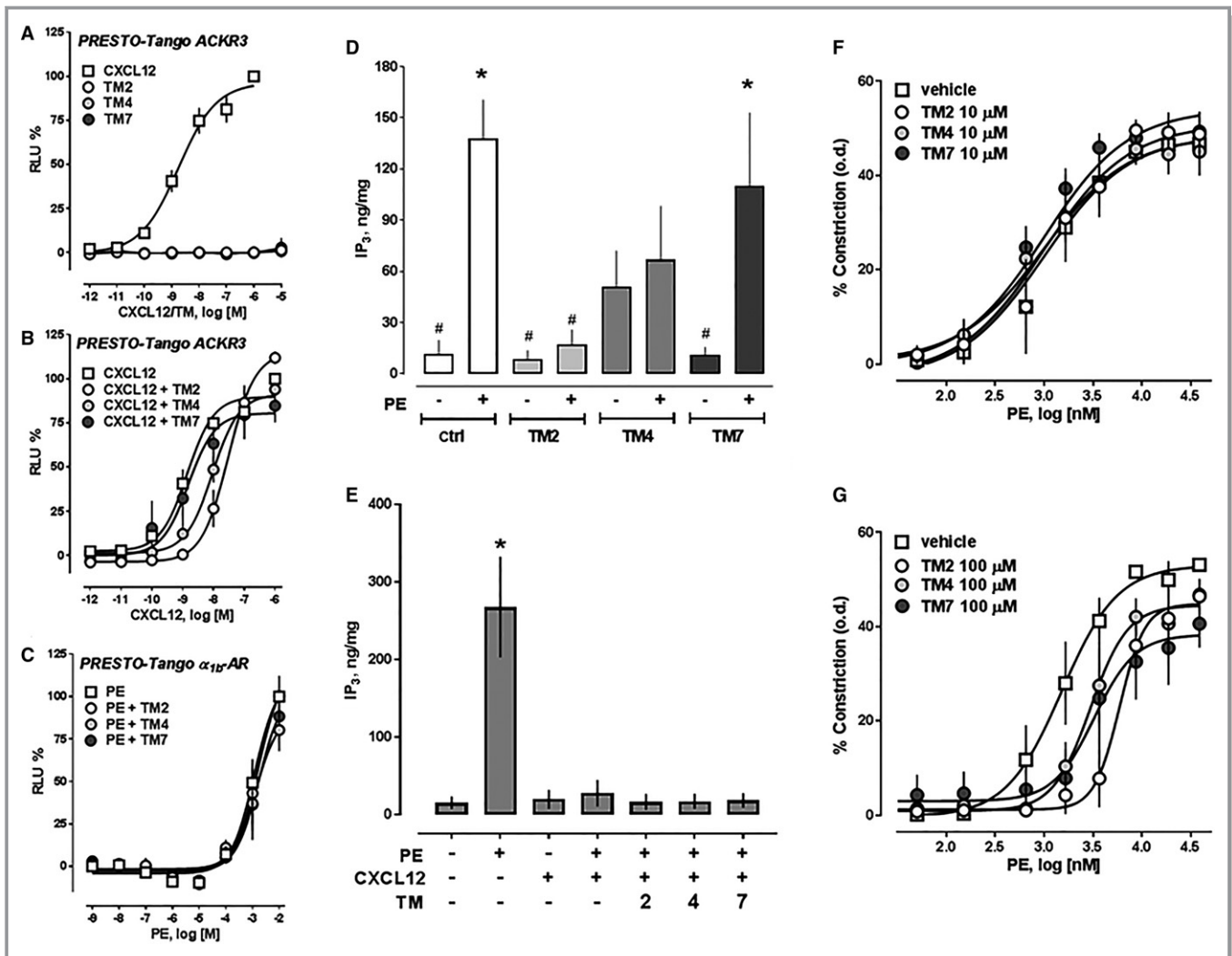


Figure 9. Peptides derived from transmembrane domains of atypical chemokine receptor 3 (ACKR3) modulate receptor function. A, β -arrestin 2 recruitment assay (PRESTO-Tango) for ACKR3. Cells were treated with chemokine (C-X-C motif) ligand 12 (CXCL12) or transmembrane (TM) 2/4/7 peptide analogs. RLU (%): relative luminescence units in percentage of the RLU after treatment with 1 μ mol/L CXCL12 (=100%). n=3 independent experiments. B, β -arrestin 2 recruitment assay (PRESTO-Tango) for ACKR3. Cells were treated with CXCL12 or CXCL12 plus 10 μ mol/L of the TM2/4/7 peptide analogs. RLU (%): relative luminescence units in % of the RLU after treatment with 1 μ mol/L CXCL12 (=100%). n=4 independent experiments. C, β -arrestin 2 recruitment assay (PRESTO-Tango) for α_{1b} -adrenergic receptor (AR). Cells were treated with phenylephrine (PE) or PE plus 10 μ mol/L of the TM2/4/7 peptide analogs. RLU (%): relative luminescence units in percentage of the RLU after treatment with 10 μ mol/L PE (=100%). n=3 independent experiments. D, Inositol trisphosphate (IP₃) production was measured in human vascular smooth muscle cells (hVSMCs) pretreated with vehicle or the TM2/4/7 peptide analogs (10 μ mol/L, 30 minutes at 37°C) and then stimulated with vehicle or 1 μ mol/L PE for 5 minutes. * P <0.05 vs vehicle/no PE; # P <0.05 vs vehicle plus PE, n=4 independent experiments. E, IP₃ production was measured in hVSMCs pretreated with vehicle or the TM2/4/7 peptide analogs (10 μ mol/L, 30 minutes at 37°C) plus vehicle or CXCL12 (1 μ mol/L, 15 minutes at 37°C) and then stimulated with vehicle or 1 μ mol/L PE for 5 minutes. * P <0.05 vs vehicle only, n=3 independent experiments. F and G, Pressure myography with rat mesenteric arteries. Arteries were pressurized to 80 mm Hg and pretreated with vehicle, 10 μ mol/L (F) or 100 μ mol/L (G) of the TM2/4/7 peptide analogs. Increasing concentrations of PE were then added to the vessel bath and dose-response curves generated. % o.d. indicates outer artery diameter in percentage of the outer diameter in the absence of PE. n=3 independent experiments.

to ACKR3 (EC₅₀ 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 α_{1b} -AR (Figure 9C).

Next, we tested whether the TM peptide analogs interfere with signaling of endogenous α_1 -AR in hVSMCs. While the TM7 peptide analog did not influence PE-induced IP₃ production in hVSMCs, the TM2 and TM4 peptide analogs inhibited this response (Figure 9D). Next, we reevaluated the effects of CXCL12 on PE-induced IP₃

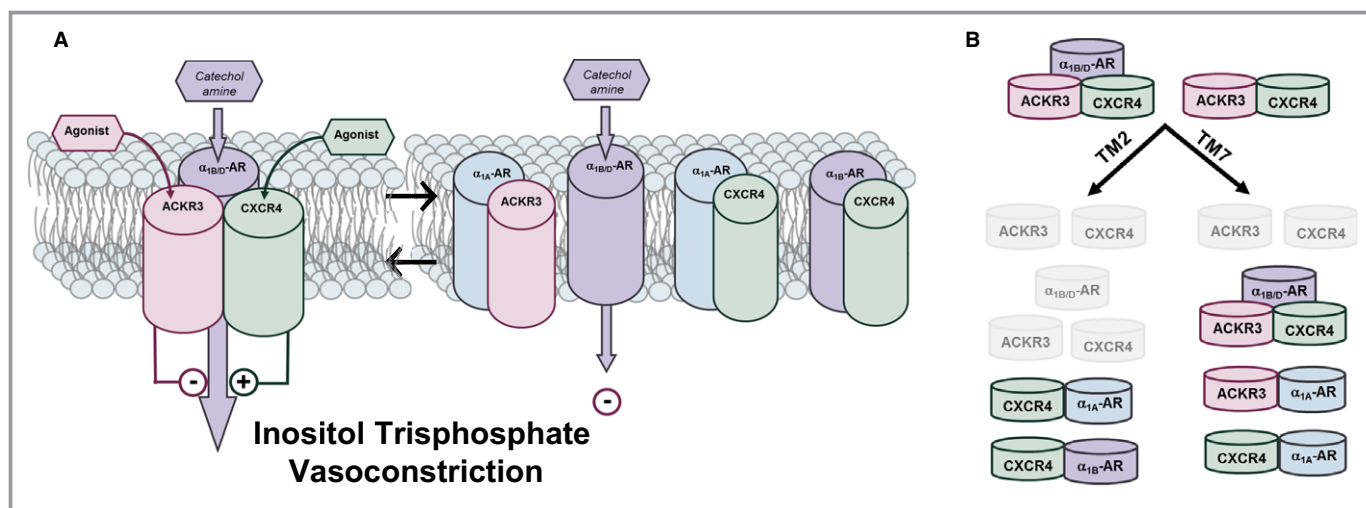


Figure 10. Hetero-oligomerization of α_1 -adrenergic receptor (AR) with atypical chemokine receptor 3 (ACKR3) and chemokine (C-X-C motif) receptor 4 (CXCR4)—working model. A, Hetero-oligomeric complexes between $\alpha_{1B/D}$ -AR and the ACKR3:CXCR4 heteromer are responsible for $\alpha_{1B/D}$ -AR signaling on catecholamine stimulation in human vascular smooth muscle cells (hVSMCs). Activation of ACKR3 inhibits, whereas activation of CXCR4 sensitizes this response via allosteric modulation of $\alpha_{1B/D}$ -AR within the hetero-oligomeric receptor complex. Disruption of the $\alpha_{1B/D}$ -AR:ACKR3:CXCR4 hetero-oligomeric 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 α_{1A} -AR:ACKR3 and α_{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.

production after incubation of hVSMCs with the TM peptides (Figure 9E). As observed in Figure 2B, PE-induced IP₃ production was abolished after pretreatment with CXCL12 alone and after pretreatment with a combination of CXCL12 and the individual TM peptides.

To assess whether the effects of the TM peptide analogs on PE-mediated signaling events in cells correspond to their effects on vascular smooth muscle function, we then performed pressure myography experiments with isolated rat mesenteric arteries (Figure 9F and 9G). The TM peptide analogs did not affect PE-induced vasoconstriction when tested at a concentration of 10 $\mu\text{mol/L}$ (Figure 9F). When tested at a 10-fold higher concentration (Figure 9G), the TM2 peptide analog significantly reduced the potency of PE to induce vasoconstriction (EC_{50} for PE: vehicle, $1.8 \pm 0.5 \mu\text{mol/L}$; TM2, $6.3 \pm 0.3 \mu\text{mol/L}$ [$P < 0.05$ versus vehicle]; TM4, $3.0 \pm 0.7 \mu\text{mol/L}$ [$P > 0.05$ versus vehicle]; and TM7, $4.2 \pm 1.7 \mu\text{mol/L}$ [$P > 0.05$ versus vehicle]).

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 α_1 -AR-mediated IP₃ production is consistent with the effects of the ACKR3 agonists on α_1 -AR-mediated constriction of isolated resistance arteries that we previously described and confirms functional cross-talk between ACKR3 and α_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 $\alpha_{1/2}$ -AR that we previously observed, our current findings indicate that ACKR3 also heteromerizes with α_{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 heteromeric CXCR4:ACKR3 and CXCR4: $\alpha_{1A/B}$ -AR complexes and inhibits α_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: $\alpha_{1B/D}$ -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 IP₃ 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 β -arrestin 2 recruitment to recombinant α_{1b} -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 α_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: α_{1A} -AR heteromerization and that CXCR4 knockdown did not affect ACKR3: α_{1A} -AR heteromerization. Furthermore, all TM peptide analogs interfered with ACKR3:CXCR4 heteromerization but increased CXCR4: α_{1A} -AR heteromers. As we have previously shown that CXCR4 knockdown in hVSMCs reduces CXCR4: α_{1A} -AR heteromers,⁸ these observations suggest that α_{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: α_{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: α_{1A} -AR heteromerization may be favored over the formation of other ACKR3 heteromers and ACKR3 protomers or homodimers. Reduction of ACKR3: α_{1A} -AR heteromerization would then only occur when the relative abundance of ACKR3 falls below the number of α_{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

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, eg 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: α_{1A} -AR complexes, α_1 -AR-mediated signaling on PE stimulation was abolished under both conditions. Furthermore, the TM7 peptide increased the number of ACKR3: α_{1A} -AR heteromers on hVSMCs but did not affect PE-induced α_1 -AR function. PE-induced IP₃ production, however, was not sensitive to the selective α_{1A} -AR antagonist 5'-methylurapidil but could be inhibited with a pan α_1 -AR antagonist and with selective $\alpha_{1B/D}$ -AR antagonists. This suggests that the relative contribution of α_{1A} -AR to the combined α_1 -AR response is limited under our experimental conditions, which is consistent with the relative functional relevance of α_1 -AR subtypes previously reported.^{32–34} Thus, we cannot currently comment on the functional role of α_{1A} -AR:ACKR3 heteromers. Similarly, possible functions of ACKR3: α_{2B} -AR heteromeric complexes remain to be determined in future studies.

Conclusions

We show that recombinant and endogenous ACKR3 form heteromeric complexes with α_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 α_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 α_1 -AR, CXCR4, and ACKR3 could provide new pharmacological approaches to regulate vascular function in disease conditions.

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Disclosures

None.

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