Heteromerization of chemokine (C-X-C motif) receptor 4 with \(\alpha_{1A/B}\)-adrenergic receptors controls \(\alpha_{1}\)-adrenergic receptor function

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Recent evidence suggests that chemokine (C-X-C motif) receptor 4 (CXCR4) contributes to the regulation of blood pressure through interactions with \(\alpha_{1}\)-adrenergic receptors (ARs) in vascular smooth muscle. The underlying molecular mechanisms, however, are unknown. Using proximity ligation assays to visualize single-molecule interactions, we detected that \(\alpha_{1A/B}\)-ARs associate with CXCR4 on the cell surface of rat and human vascular smooth muscle cells (VSMC). Furthermore, \(\alpha_{1A}\)-AR could be coimmunoprecipitated with CXCR4 in a HeLa expression system and in human VSMC. A peptide derived from the second transmembrane helix of CXCR4 induced chemical shift changes in the NMR spectrum of CXCR4 in membranes, disturbed the association between \(\alpha_{1A/B}\)-AR and CXCR4, and inhibited Ca\(^{2+}\) mobilization, myosin light chain (MLC) 2 phosphorylation, and contraction of VSMC upon \(\alpha_{1}\)-AR activation. CXCR4 silencing reduced \(\alpha_{1A/B}\)-AR:CXCR4 heteromeric complexes in VSMC and abolished phenylephrine-induced Ca\(^{2+}\) fluxes and MLC2 phosphorylation. Treatment of rats with CXCR4 antagonists (CXCL12, ubiquitin) reduced the EC\(_{50}\) of the phenylephrine-induced blood pressure response three- to fourfold. These observations suggest that disruption of the quaternary structure of CXCR4 and depletion of the heteromeric receptor complexes by CXCR4 knockdown inhibit \(\alpha_{1}\)-AR-mediated function in VSMC and that activation of CXCR4 enhances the potency of \(\alpha_{1}\)-AR agonists. Our findings extend the current understanding of the molecular mechanisms regulating \(\alpha_{1}\)-AR and provide an example of the importance of G protein-coupled receptor (GPCR) heteromerization for GPCR function. Compounds targeting the \(\alpha_{1A/B}\)-AR:CXCR4 interaction could provide an alternative pharmacological approach to modulate blood pressure.

CXCL12 | ubiquitin | AMD3100 | phenylephrine | blood pressure

Chemokine (C-X-C motif) receptor 4 (CXCR4) is a G protein-coupled receptor (GPCR) that is essential during development. Animals lacking CXCR4 are not viable and demonstrate defects of the hematopoietic and cardiovascular system (1). After birth, CXCR4 is expressed in many tissues, including the heart and vasculature, and fulfills multiple functions in the immune system, such as regulation of leukocyte trafficking, stem cell mobilization, and homing (2, 3). Moreover, CXCR4 is involved in various disease processes, such as HIV infection, cancer metastasis, and tissue repair (3–5).

In addition to these established functions, recent observations suggest that CXCR4 also contributes to the regulation of hemodynamics and blood pressure. Treatment with the CXCR4 antagonists AMD3100 and AMD3465 reduced blood pressure in experimental models of pulmonary arterial and systemic hypertension (6, 7). We have shown previously that AMD3100 reduces hemodynamic stability and blood pressure during the cardiovascular stress response to traumatic and hemorrhagic shock, whereas selective activation of CXCR4 with the noncongestive agonist ubiquitin improves hemodynamic stability and increases systemic blood pressure after traumatic, hemorrhagic, and endotoxic shock (8–13). Because in vivo pharmacological targeting of CXCR4 did not affect myocardial function, these findings suggested that effects of CXCR4 on hemodynamics and blood pressure are mediated via modulation of vascular function (9).

Accordingly, we observed that CXCR4 activation enhances and sensitizes vasoconstriction of isolated mesenteric arteries and veins in response to \(\alpha_{1}\)-adrenergic receptor (AR) activation with phenylephrine (PE) (9). As these effects were independent of the vascular endothelium, interactions between CXCR4 and \(\alpha_{1}\)-AR in vascular smooth muscle likely constitute the physiological basis for these observations (9). The molecular mechanisms underlying interactions between CXCR4 and \(\alpha_{1}\)-AR in vascular smooth muscle, however, remain unknown.

Crosstalk between GPCRs is a widely recognized principle that expands the physiological repertoire of GPCR-mediated signaling.

\(\alpha_{1}\)-Adrenergic receptors are important for the regulation of vascular function and are targeted clinically for blood pressure control. Here, we provide evidence that \(\alpha_{1A/B}\)-adrenergic receptors (AR) form heteromeric complexes with chemokine (C-X-C motif) receptor 4 (CXCR4) on the cell surface of vascular smooth muscle cells. We show that disruption of \(\alpha_{1A/B}\)-AR:CXCR4 heteromeric complexes inhibits \(\alpha_{1}\)-AR-mediated functions in vascular smooth muscle cells and that treatment with CXCR4 agonists enhances the potency of the \(\alpha_{1}\)-AR agonist phenylephrine to increase blood pressure. These findings extend the current understanding of the molecular mechanisms regulating \(\alpha_{1}\)-AR and provide an example of G protein-coupled receptor heteromerization with important functional implications. Compounds targeting the \(\alpha_{1A/B}\)-AR:CXCR4 interaction could provide an alternative pharmacological approach to modulating blood pressure.

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signaling events and functions (14–19). Receptor crosstalk can be attributed to a variety of molecular mechanisms, including receptor hetero-oligomerization (14–23). The formation of homo- and/or hetero-oligomeric complexes among GPCRs is thought to be important for many aspects of GPCR function (22–24).

CXCR4 has been shown to associate with multiple chemokine receptors in various expression systems (3, 25–28). ARs are also known to be able to form heteromeric receptor complexes (29–35), and recent evidence suggests that AR may also be able to form heteromeric complexes with chemokine receptors (36–38). Thus, we studied whether α1-AR and CXCR4 may interact on the cell surface of vascular smooth muscle cells through the formation of heteromeric receptor complexes.

Here, we provide evidence that heteromeric receptor complexes between α1A-AR and CXCR4 and between α1B-AR and CXCR4 are constitutively expressed in rat and human vascular smooth muscle cells (VSMC). We show that disruption of the quaternary structure of the heteromeric receptor complex by targeting transmembrane helix (TM) 2 of CXCR4 and depletion of heteromeric receptor complexes by CXCR4 knockdown inhibit α1-AR agonist-induced key signaling events and contraction of VSMC. Furthermore, we show that treatment with CXCR4 agonists increases the potency of the α1-AR agonist PE to increase blood pressure in vivo. Our observations suggest that α1-AR function in VSMC is controlled through the formation of heteromeric α1A/B-AR-CXCR4 complexes.

Results and Discussion

α1A/B-AR Associates with CXCR4 on the Cell Surface of Vascular Smooth Muscle Cells. We sought to evaluate whether heteromeric complexes between α1-AR and CXCR4 are expressed on the cell surface of VSMC. Thus, we used proximity ligation assays (PLA) to visualize individual receptors and receptor–receptor interactions (39–41). PLA have been previously used to observe individual proteins and interactions between individual endogenous proteins at a single-molecule resolution (39). We first used PLA to detect CXCR4 and α1A-AR in a format suited for high sensitivity detection of a single protein. As a positive control, we also tested for atypical chemokine receptor (ACKR) 3 (formerly known as RDC1 and CXCR7), which is known to be able to form heteromeric complexes with CXCR4 (3, 28). Toll-like receptor (TLR) 9 was used as a negative control receptor that is unlikely to be associated with α1A-AR. All individual receptors could be visualized by PLA in the rat vascular smooth muscle cell line A7r5, on freshly isolated aortic rat VSMC, and on primary human aortic VSMC (Fig. 1A). When PLA was used

![Fig. 1](https://www.pnas.org/cgi/doi/10.1073/pnas.1417564112)

**Fig. 1.** α1A-AR and CXCR4 are in close proximity on the cell surface of rat and human vascular smooth muscle cells. (Scale bars: 10 μm.) phase: phase contrast. PLA: PLA fluorescence signal. PLA/DAPI: merged PLA/DAPI signals. Merge: merged phase-contrast/PLA/DAPI signals. (A) Detection of α1A-AR, CXCR4, ACKR3, and TLR-9 on A7r5 cells and on rat and human VSMC by PLA. Images are representative of three independent experiments. (B) Detection of receptor–receptor associations on A7r5 cells and on rat and human VSMC by PLA. Ab ctrl.: omission of the primary antibody. Images are representative of three independent experiments.
to visualize protein–protein interactions at a single-molecule level, we validated the proximity of CXCR4 and ACKR3 in native cells (28) and also detected signals suggesting close proximity of α1A-AR–CXCR4 in A7r5 cells and in rat and human VSMC (Fig. 1B). In contrast, PLA signals for α1A-AR–TLR9 interactions were not detectable (Fig. 1B).

To define whether all α-ARs associate with CXCR4, we then evaluated human VSMC for interactions between α1-AR and α2-AR subtypes with CXCR4. All α1-AR and α2-AR subtypes were detectable on human VSMC in PLA when used to detect individual receptors (Fig. 2A). Quantification of PLA signals per cell suggested that α1B-ARs were more frequently expressed on human VSMC than all other α-AR subtypes and CXCR4 (Fig. 2C). We then screened human VSMC for possible interactions between CXCR4 and α-AR subtypes and detected PLA signals corresponding to interactions between CXCR4 and α1A-AR and between CXCR4 and α1B-AR (Fig. 2 B and D). Images from PLA signals corresponding to interactions between the receptors at a lower magnification (40×) are shown in Fig. S1. The number of PLA signals per cell corresponding to interactions between all other α-AR subtypes and CXCR4 were not distinguishable from the number of signals obtained in negative control experiments (Fig. 2D). Moreover, 3D reconstruction of the PLA signals from z-stack images confirmed that PLA signals corresponding to α1A-AR:CXCR4 and α1B-AR:CXCR4 are localized on the cell surface of VSMC (Fig. 2E).

To confirm direct physical interactions between α1A/B-AR and CXCR4, we then used a HeLa expression system to perform coimmunoprecipitation analyses of receptor interactions. HeLa cells were cotransfected with FLAG-CXCR4, hemagglutinin (HA)-α1A-AR, or HA-ACKR3 (= positive control), followed by immunoprecipitation of cell homogenates with anti-FLAG. HA-α1B-AR and HA-ACKR3 were detected in FLAG-CXCR4 immunoprecipitates, but not in control samples that expressed either receptor alone (Fig. S2). To further consolidate these observations, we next performed coimmunoprecipitation analyses of endogenous receptor interactions in human VSMC. As shown in Fig. 2F, α1A-AR and α1B-AR were detectable in CXCR4 immunoprecipitates, whereas α2C-AR was not detectable. These findings suggest that α1A/B-AR and CXCR4 physically interact in human VSMC and that the observed proximity between the receptors corresponds to direct receptor–receptor interactions.

**A Peptide Derived from Transmembrane Domain 2 of CXCR4 Disrupts α1A/B-AR:CXCR4 Complexes and Inhibits α1-AR Function in Vascular Smooth Muscle Cells.** Disruption of transmembrane domains of GPCRs with transmembrane domain-derived peptides can inhibit receptor function and affect receptor dimerization through

![Fig. 2. Screening for proximity of α-AR subtypes with CXCR4 and coimmunoprecipitation analyses of endogenous receptor interactions in human vascular smooth muscle cells. (A) Detection of α-AR subtypes in human VSMC using PLA. Ab ctrl.: omission of the primary antibodies. Phase: phase contrast. PLA: PLA fluorescence signal. PLA/DAPI: merged PLA/DAPI signals. Images are representative of three independent experiments. (Scale bar: 10 μm.) (B) Screening for associations between α-AR subtypes and CXCR4 on human VSMC by PLA. Ab ctrl.: omission of one primary antibody. Phase: phase contrast. PLA: PLA fluorescence signal. PLA/DAPI: merged PLA/DAPI signals. Images are representative of three independent experiments. (Scale bar: 10 μm.) (C) and (D) Quantification of the number of PLA signals per cell for individual (C) and receptor associations (D), as in A and B, respectively. Eleven randomly selected nonoverlapping vision fields were analyzed for each condition. n = 3. *P < 0.05 vs. ctrl. (E) Three-dimensional representations of α1A/AR:CXCR4 (Top) and α1B/AR:CXCR4 (Bottom) interactions in human VSMC. Deconvolved images were generated from z-stack images (n = 20; thickness: 0.5 μm, bottom to top). Images show merged PLA/DAPI signals. (F) Human VSMC were lysed and CXCR4 was immunoprecipitated (IP) followed by Western blotting (WB) to detect CXCR4 (Top Left), α1A-AR (Bottom Left), α1B-AR (Bottom Right), and α2C-AR (Top Right) in the IP samples. IP control: precipitate after incubation of cell lysates with nonreactive resin. PS: protein standards. The white light images are overlaid at the corresponding position of the standard proteins. Images are representative of n = 3.
interference with the correct assembly of the target membrane protein (42, 43). X4-2-6, a peptide derived from TM2 of CXCR4, has previously been shown to inhibit CXCR4 function (43). Therefore, we then evaluated whether X4-2-6 affects the association of CXCR4 with α1A/B-AR and ACKR3 on VSMC by PLA. X4-2-6 reduced the association between α1A/B-AR and CXCR4 and between α1B-AR and CXCR4 on the cell surface of human VSMC, compared with R3-2-1, a peptide derived from TM2 of chemokine (C-C motif) receptor 3 (Fig. 3 A and B). PLA signals corresponding to CXCR4:ACKR3, however, were not affected by X4-2-6 (Fig. 3 A and B). We observed the same effects of X4-2-6 in PLA experiments with A7r5 cells (Fig. S3 A and B). As assessed by fluorescence-activated cell sorting (FACS) analyses, X4-2-6 did not influence cell-surface expression of the individual receptors, suggesting that X4-2-6 disrupts physical interactions between CXCR4 and α1A/B-AR in the cell membrane without affecting receptor expression levels (Fig. 3C).

To assess whether disruption of the α1A/B-AR:CXCR4 association influences α1-AR-mediated signaling, we tested the effects of X4-2-6 on PE-induced intracellular Ca\(^{2+}\) mobilization and myosin light chain (MLC) 2 phosphorylation (Ser19) in VSMC. As shown in Fig. 3D, X4-2-6 inhibited PE-induced intracellular Ca\(^{2+}\) mobilization in A7r5 cells. X4-2-6, however, did not affect Ca\(^{2+}\) mobilization in response to arginine vasopressin (Fig. 3E), suggesting specificity of the observed effects for α1-AR.

Because pretreatment of A7r5 cells with the selective CXCR4 inhibitor AMD3100 did not affect PE-induced Ca\(^{2+}\) mobilization (Fig. 3F), effects of X4-2-6 cannot be attributed to the inhibition of CXCR4-mediated signaling. As observed in A7r5 cells, X4-2-6 also attenuated Ca\(^{2+}\) mobilization in response to

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Fig. 3. X4-2-6 disrupts α1A/B-AR:CXCR4 complexes and inhibits α1-AR function in vascular smooth muscle cells. (A) Human VSMC were incubated with 100 nM of X4-2-6 or R3-2-1 (= control) for 15 min at room temperature. Interactions between α1A/B-AR and CXCR4 and between CXCR4 and ACKR3 were visualized by PLA. Images show merged PLADAPI signals and are representative of three independent experiments. (Scale bar: 50 μm.) (B) Quantification of the number of PLA signals per cell for receptor interactions after X4-2-6 or R3-2-1 treatment, as in A. Twenty randomly selected nonoverlapping vision fields were analyzed per experiment; n = 3. *P < 0.05 vs. cells incubated with R3-2-1. (C) FACS analysis of the cell-surface expression of CXCR4 (Left), α1A/B-AR (Center), and α1B-AR (Right) after vehicle (thick red line) or X4-2-6 (100 μM, thick blue line) treatment. Gray area: unstained cells. Thin blue line: IgG control. (D) Ca\(^{2+}\) fluxes in A7r5 cells upon addition of PE (10 μM). Arrows: Time points when drugs were added. X4-2-6: 100 μM. AB: assay buffer. n = 3–8. (E) Ca\(^{2+}\) fluxes in A7r5 cells upon addition of arginine vasopressin (aVP: 100 μM). Arrows: Time points when drugs were added. X4-2-6: 100 μM. AB: assay buffer. n = 3. (F) Ca\(^{2+}\) fluxes in A7r5 cells upon addition of PE (10 μM). Arrow: Time point when drugs were added. AMD3100: 10 μM. AB: assay buffer. n = 3. (G) Ca\(^{2+}\) fluxes in human VSMC upon addition of PE (1 mM, arrow). Human VSMC were preincubated with or without 100 μM X4-2-6 for 15 min. n = 3. (H, Top) Western blot (WB) analyses of MLC2 phosphorylation (pMLC) after stimulation (45 min at 37 °C) of human VSMC with PE (1 mM). Before PE stimulation, cells were preincubated with (+) or without (−) 100 μM X4-2-6 (15 min at 37 °C). Western blotting with anti-GAPDH was used as a protein loading control. (Bottom) Quantification of the chemiluminescence signals after human VSMC stimulation. Data are expressed as percentage of untreated cells [PE (−), X4-2-6 (−)]. n = 4. *P < 0.05 vs. untreated cells. (I, Top) PLA analyses of pMLC2 phosphorylation after PE stimulation and X4-2-6 treatment, as in E. No PLA signal could be detected in nonpermeabilized cells (np). (Bottom) Quantification of the number of PLA signals per cell after treatment of human VSMC. For each condition and experiment, 11 randomly selected nonoverlapping vision fields were analyzed; n = 3. *P < 0.05 vs. untreated cells. (J) X4-2-6 attenuates rat mesenteric artery smooth muscle cell contraction in response to PE. Freshly isolated cells were treated with or without 100 μM X4-2-6 for 15 min at room temperature and then stimulated with 10 μM PE. (Top) Representative images of cells before (PE−) and after (PE+) exposure to PE. (Scale bar: 10 μm.) (Bottom) Quantification of the number of cells contracting in response to PE. *P < 0.05 X4-2-6 treated (+) vs. untreated (−) cells. n = 3.
PE in human VSMC (Fig. 3G). Furthermore, X4-2-6 inhibited Ca\(^{2+}\)/calmodulin-dependent MLC2 phosphorylation upon PE stimulation of human VSMC, as assessed by Western blotting (Fig. 3H). To confirm results from Western blotting experiments, we then used PLA to visualize and quantify intracellular phosphorylated MLC2 (Fig. 3I). Consistent with the intracellular localization of phospho-MLC2, we detected PLA signals only when VSMC were permeabilized before incubation with anti-phospho MLC2. Quantification of the number of PLA signals per cell showed the same magnitude of PE-induced MLC2 phosphorylation as determined by Western blot analyses and confirmed the inhibition of PE-induced MLC2 phosphorylation by X4-2-6 (Fig. 3H). As these data indicated that X4-2-6 inhibits key signaling events in the pathway mediating \(\alpha_1\)-AR–induced VSMC contraction, we then tested whether X4-2-6 also influences contraction of freshly isolated rat mesenteric artery smooth muscle cells upon exposure to PE. As shown in Fig. 3I, pretreatment of cells with X4-2-6 reduced the number of cells contracting upon PE stimulation from 71 ± 6% to 46 ± 9%. The observation that the inhibitory effects of X4-2-6 on PE-induced signaling events in human aortic VSMC were more pronounced than effects of X4-2-6 on PE-induced contraction of freshly isolated VSMC from mesenteric arteries could be explained by distinct functional roles of the \(\alpha_1\)-AR subtypes that have been observed among various vascular beds (44).

Because our observations suggested that X4-2-6 functions as an \(\alpha_1\)-AR antagonist, we then evaluated whether X4-2-6 can also interact with \(\alpha_1\)-AR using NMR spectroscopy. We used reductively methylated membranes prepared from cells overexpressing either \(\alpha_1\)-AR or CXCR4 to closely mimic native conditions for receptor folding and interactions with the plasma membrane. A similar approach has recently been used to provide structural insight into ligand regulation of the extracellular surface of the \(\beta_2\)-AR (45). The overlaid \(^{13}\)C–\(^{1}\)H heteronuclear single quantum coherence (HSQC) spectra of \(\alpha_1\)-AR with and without PE are shown in Fig. 4, Upper Left. Addition of 10 μM PE induced chemical shift changes that are indicative of a global structural rearrangement of the receptor induced by ligand binding. No chemical shift changes in the spectrum of \(\alpha_1\)-AR were induced by addition of 10 μM X4-2-6 (Fig. 4, Upper Right). To address the possibility that \(^{13}\)CH\(_3\) probes on \(\alpha_1\)-AR do not report on X4-2-6 binding, we added 10 μM PE to \(\alpha_1\)-AR in the presence of 10 μM X4-2-6 (Fig. 4, Lower Left). PE-induced chemical shift changes in \(\alpha_1\)-AR were similar to those in the absence of X4-2-6. When X4-2-6 was added to CXCR4, significant chemical shift changes in the spectrum of CXCR4 were detected, indicating peptide-receptor interactions (Fig. 4, Lower Right). Because X4-2-6 also caused loss of proximity between \(\alpha_1\)-AR and CXCR4 in VSMC, these data suggest that X4-2-6 binding to CXCR4 induces structural rearrangements of the receptor that disrupt the quaternary heteromer interface. Provided that PLA signals for native CXCR4:ACKR3 represent direct receptor interactions, the observation that the proximity between CXCR4 and ACKR3 was not affected by X4-2-6 points toward TM2 of CXCR4 as part of a specific \(\alpha_1\)-AR:CXCR4 heteromer interface, which does not participate in the formation of the CXCR4:ACKR3 interface.

Although crystal structures of GPCR heteromers are currently not available, crystallographic structures of GPCR homodimers revealed several different interfaces. The main interface of the CXCR4 homodimer is localized at TM5 and TM6, whereas several other GPCR homodimers form interfaces that also include TM2 (46–50). Thus, a TM2 contact site in CXCR4 could permit receptor heteromerization without interfering with the constitutive CXCR4 homodimerization (46).

Furthermore, as X4-2-6 did not interfere with \(\alpha_1\)-AR and did not affect ligand-induced conformational changes of \(\alpha_1\)-AR in membranes, off-target effects of X4-2-6 on \(\alpha_1\)-AR or PE appear unlikely to account for the inhibitory effects of X4-2-6 on signaling events upon \(\alpha_1\)-AR activation. Thus, these data led to the hypothesis that \(\alpha_1\)-AR:CXCR4 heteromers are a prerequisite for \(\alpha_1\)-AR function in VSMC.

**CXCR4 Silencing Inhibits \(\alpha_1\)-AR Function in Vascular Smooth Muscle Cells.** To test the hypothesis that CXCR4:\(\alpha_1\)-AR heteromeric complexes are required for \(\alpha_1\)-AR function, we aimed to reduce CXCR4:\(\alpha_1\)-AR heteromerization by reducing CXCR4 expression in human VSMC with RNA interference. Fig. 5A shows a typical Western blot with anti-CXCR4 with cell homogenates from human VSMC transfected with nontargeting (NT) or CXCR4-targeted siRNA. As expected, anti-CXCR4 recognized multiple bands in human VSMC transfected with NT siRNA, which likely correspond to proteolytically processed, ubiquitylated, or glycosylated forms of CXCR4 (51–55). The most abundant receptor species after transfection of human VSMC with NT siRNA were detectable at migration positions corresponding to 48–60 kDa. The intensities of these bands were reduced after transfection of the cells with CXCR4 siRNA (Fig. 5A). As estimated by FACS analyses, CXCR4 cell-surface expression was reduced by 69 ± 7% (\(n = 4\)) after transfection of human VSMC with CXCR4 siRNA, compared with cells transfected with NT siRNA (Fig. 5B). Cell-surface expressions of \(\alpha_1\)-AR were not affected by CXCR4 siRNA (Fig. 5B).

When CXCR4 expression was quantified by PLA (Fig. 5C), transfection of human VSMC with CXCR4 siRNA resulted in 62 ± 8% reduction of CXCR4 cell-surface expression, compared with cells transfected with NT siRNA.

Next, we analyzed the expression of \(\alpha_1\)-AR:CXCR4 and ACKR3:CXCR4 heteromers by PLA. Compared with human VSMC after transfection with NT siRNA, CXCR4 siRNA silencing reduced the PLA signals for \(\alpha_1\)-AR:CXCR4 (Fig. 5D), \(\alpha_1\)-AR:CXCR4 (Fig. 5E), and ACKR3:CXCR4 (Fig. 5F) heteromers by 90%, 60%, and 59%, respectively. The finding that the degree of reduction of CXCR4 and of the \(\alpha_1\)-AR:CXCR4 and ACKR3:CXCR4 heteromers on the cell surface after siRNA silencing was comparable argues for a 1:1 receptor:receptor stoichiometry. Notably, PLA signals for \(\alpha_1\)-AR:CXCR4 heteromers were almost completely depleted after CXCR4 silencing. Several explanations may account for this observation, such as alteration of the receptor heteromerization equilibrium in the plasma membrane or PLA signals resulting from the association of CXCR4 with other receptors that form heteromeric complexes with \(\alpha_1\)-AR, i.e., during receptor clustering (56).

**Fig. 4.** X4-2-6 induces chemical shift changes in the NMR spectrum of CXCR4 in membranes. Superimposition of \(^{1}\)H–\(^{13}\)C HSQC NMR spectra of reductively methylated membrane preparations of (Upper Left) \(\alpha_1\)-AR (blue) and \(\alpha_1\)-AR treated with 10 μM PE (red); (Upper Right) \(\alpha_1\)-AR (blue) and \(\alpha_1\)-AR treated with 10 μM X4-2-6 (red); (Lower Left) \(\alpha_1\)-AR (blue) and \(\alpha_1\)-AR treated with 10 μM X4-2-6 and 10 μM PE (red); and (Lower Right) CXCR4 (blue) and CXCR4 treated with 10 μM X4-2-6 (red).

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To assess the functional consequences of CXCR4 silencing on α1-AR function in human vascular smooth muscle cells, we then measured PE-induced Ca
2+
 fluxes and MLC2 phosphorylation. We detected that CXCR4 silencing inhibits α1-AR function in human VSMC, as shown in Fig. 5. (A) CXCR4 was silenced with siRNA in human VSMC followed by Western blotting of whole-cell lysates with anti-CXCR4 and anti-GAPDH. NT: nontargeting. PS: white light image of prestained protein standards. (B) FACS analysis of CXCR4 (Left), α1A-AR (Center), and α1B-AR (Right) expression on the cell surface of human VSMC after transfection with NT (red line) and CXCR4 (blue line) siRNA. Gray line: IgG control. Gray area: unstained cells. (C–F) Representative PLA images (merged PLA/DAPI) and quantification of PLA signals for CXCR4 (C), α1A-AR:CXCR4 (D), α1B-AR:CXCR4 (E), and ACKR3:CXCR4 (F) after CXCR4 silencing, as in A and B. For each condition and experiment, 11 randomly selected nonoverlapping vision fields were analyzed; n = 3. *P < 0.05 vs. cells transfected with NT siRNA. (Scale bars: 10 μm.) (G) PE (1 mM; arrow)-induced Ca
2+
 fluxes in human VSMC after CXCR4 silencing, as in A and B. Open circles: cells transfected with NT siRNA. Gray squares: cells transfected with CXCR4 siRNA. For each experiment (n = 3), the fluorescence ratio was calculated from 50 individual cells in each vision field. (H) Western blot (WB) analyses of MLC2 phosphorylation (pMLC) after PE stimulation, as in Fig. 3E, in human VSMC after CXCR4 silencing, as in A and B. Western blotting with anti-GAPDH was used as a protein loading control. (I) Quantification of the chemiluminescence signals after VSMC stimulation as in H. Data are expressed as a percentage of unstained cells transfected with NT siRNA. n = 4. *P < 0.05 vs. unstained cells transfected with NT siRNA.

CXCR4 Agonists Increase the Potency of PE to Increase Blood Pressure. To evaluate whether CXCR4 influences α1-AR function in vivo, we then tested the pharmacological modulation of CXCR4 on α1-AR function in vivo in rats. Under normal hemodynamic conditions, animals received a single injection of the cognate CXCR4 agonist CXCL12, the noncognate CXCR4 agonist ubiquitin, or the CXCR4 antagonist AMD3100, followed by increasing doses of PE. As a quantifiable marker of the integrated blood pressure response to each dose of PE, we then determined the area under the mean arterial blood pressure (MAP) curve for each dose of PE and generated dose–response curves. As shown in Fig. 6, the EC
50
 of PE after vehicle treatment was 664 ng/kg (95% confidence interval: 346–1273 ng/kg), and the maximal area under the MAP curve was 396 mmHg × s (95% confidence interval: 335–458 mmHg × s). CXCL12 and ubiquitin pretreatment reduced the EC
50
 of PE 3.9- and 3.5-fold, respectively. Whereas AMD3100 pretreatment did not affect the EC
50
 of PE, AMD3100 antagonized the effects of CXCL12 and ubiquitin. None of the CXCR4 ligands influenced the potency of PE. These findings suggest that CXCR4 activation enhances the potency of PE in vivo.

The observation that AMD3100 did not affect PE-induced effects in normal animals is consistent with the effects of AMD3100 on PE-induced Ca
2+
 mobilization in VSMC in the present study and with the previous observation that AMD3100 did not influence PE-induced vasoconstriction in pressure myography experiments (9). As depletion of α1A/AR:CXCR4 heteromers by X4-26 and CXCR4 knockdown inhibited PE-induced responses in VSMC, our observations of AMD3100 suggest that heteromeric complex formation per se, independent of ligand occupation or the activation status of CXCR4, controls α1-AR function. Similarly, ligand unoccupied ghrelin receptor has been reported to modulate dopamine receptor subtype-2 function via formation of heteromeric ghrelin receptor and dopamine receptor subtype-2 complexes (58). In addition, the finding that CXCR4 agonists enhanced the potency of PE in vivo is in agreement with previous observations from pressure myography experiments with isolated arteries (9). This indicates that ligand activation of CXCR4 further sensitizes α1-AR responses and could be explained through allosteric effects of CXCR4 on α1A/AR within the α1A/AR;CXCR4 complex when CXCR4 transitions into an activated configuration upon agonist binding.
CXCR4 agonist treatment enhances the potency of PE to increase blood pressure. Rats were treated with vehicle, CXCL12, ubiquitin, or AMD3100 alone or with AMD3100 plus CXCL12 or ubiquitin (750 nmol/kg each), followed by increasing doses of PE at 5-min intervals. For each dose of PE, the area under the MAP curve was calculated, and dose–response curves were generated. LogEC50 vehicle vs. CXCL12: P = 0.0007; vehicle vs. ubiquitin: \( P = 0.0003 \). CI. confidence interval. There were no differences in MAP at baseline among the individual groups (MAP (mmHg, mean ± SEM); vehicle (n = 5): 89 ± 3; CXCL12 (n = 3): 87 ± 6; ubiquitin (n = 4): 89 ± 3; AMD3100 (n = 4): 97 ± 2; AMD3100/CXCL12 (n = 3): 87 ± 3; AMD3100/ubiquitin (n = 3): 85 ± 2; \( P > 0.05 \) among groups).

α₁AR-AR: CXCR4 and CXCR4:ACKR3 heteromers appear to be constitutively expressed in VSMC, and pharmacological activation of CXCR4 and ACKR3 has been reported to result in opposite effects on PE-induced vasoconstriction in isolated arteries (9). This implies that CXCR4 and also ACKR3 function as modulators of α₁-AR. Whereas the molecular mechanisms through which ACKR3 influences α₁-AR function remain to be determined, the observed effects of ubiquitin in the present study are in agreement with the findings that ubiquitin functions as a noncognate CXCR4 agonist that does not bind to ACKR3 (51, 52, 59–63). The observation that CXCL12, which has a much higher affinity for ACKR3 than for CXCR4 (64), also enhanced the potency of PE in normal animals in vivo in the present study, whereas CXCL12 previously desensitized PE-mediated vasoconstriction of isolated arteries and reduced blood pressure during hemorrhagic shock (9), suggests that effects of CXCL12 depend on the relative functional contribution of CXCR4 and ACKR3 within the specific experimental or (patho)physiological environment (65).

Because CXCR4 antagonists have been reported to reduce blood pressure in experimental models of pulmonary arterial and systemic hypertension and during hemorrhagic shock (6, 7, 9), these findings point toward a pathophysiologic role of CXCR4 agonists during blood pressure regulation under disease conditions that are associated with increased catecholamine release, such as shock or hypertension. Interestingly, ubiquitin has been described to be stored along with catecholamines in secretory chromaffin granules in the adrenal gland and to be released into the circulation upon stimulation of chromaffin cells (66), which may reflect a physiological linkage between CXCR4 and adrenergic receptor function.

Conclusively, our data suggest that endogenous α₁B-AR: CXCR4 heteromers are constitutively expressed on VSMC, that the heteromeric receptor complex is important for α₁-AR function, and that ligand activation of CXCR4 further sensitizes α₁-AR. Such a regulation of α₁-AR function could be explained through allosteric interactions between α₁B-AR and CXCR4 within the heteromeric receptor complex, which could provide the physiologic advantage that α₁-AR, and subsequently vascular function, can be selectively regulated to allow fine-tuning of blood pressure in the systemic circulation or in different vascular beds (67, 68). Furthermore, our observations provide an example that signaling events, which have been considered as characteristic intracellular consequences following activation of a homomeric GPCR, reflect a biochemical fingerprint of a GPCR heteromer (69). We believe that our observations extend the current understanding of the molecular mechanisms regulating α₁-AR function in VSMC and that compounds targeting the α₁B-AR:CXCR4 interaction could provide an alternative pharmacological approach to modulate vascular function and blood pressure.

Materials and Methods

Cells and Cell Lines. A7r5 cells (rat aortic vascular smooth muscle cell line) and human aortic VSMC were obtained from the American Type Culture Collection. HeLa cells were as described (70). Rat aortic and mesenteric artery VSMC were isolated from male Lewis and Sprague–Dawley rats as described elsewhere (71–73). All procedures involving rats were in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of Loyola University Chicago.

A7r5, rat, and human VSMC were cultured in high-glucose Dulbecco’s Modified Eagle Medium, 10 mg/mL sodium pyruvate, 2 mM L-glutamine, 10% (vol/vol) FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin.

Proteins, Peptides, and Reagents. PE, arginine vasopressin, and AMD3100 were from Sigma-Aldrich. CXCL12 was as described (9, 74) and also obtained from Protein Foundry. Ubiquitin was purchased from R&D Systems. X4-2, a peptide derived from the second transmembrane helix of CXCR4, was prepared as described (43); R3-2 (LILFTLTPFWHYVRGHNNWFGHDD-DDPEG2-NH2), a peptide derived from the second transmembrane helix of chemokine receptor CCR3, was designed and produced similarly to the X4-2 peptide and used as a control. Solid-phase synthesis on a 433A Applied Biosystems Peptide Synthesizer using Fmoc amino acid derivatives was used for the production of the peptides. After cleavage with 87.5% (vol/vol) trifluoroacetic acid containing 5% (vol/vol) water, 5% (vol/vol) thioanisol, and 2.5% (vol/vol) triisopropylsilane, the peptides were purified by reverse phase HPLC using an Atlantis C3 column (Agilent Technologies). The peptide structure and purity was confirmed by ion-spray mass-spectrometry combined with HPLC.

Reductive Methylation of Membrane Preparations. ChemiSCREEN membrane preparations of α₁AR and CXCR4 were purchased from EMD Millipore. Reductive methylation of the membrane preparations was performed as described previously (75). In brief, 20 μl of 1 M borane–ammonia complex and 4 μL of 13C formaldehyde were added to 1 mL of membrane preparation. This mixture was incubated with stirring for 2 h at 4 °C. The addition of borane–ammonia and formaldehyde was repeated, and the mixture was incubated with stirring for an additional 2 h. Ten microliters of 1 M borane–ammonia complex were then added and the mixture was incubated at 4 °C overnight with stirring. The reaction was then stopped by adding 110 μL of 2 M Tris HCl (pH 7.6). Thereafter, the membrane preparations were dialyzed in PBS and used for NMR experiments.

Heteronuclear Single Quantum Coherence NMR. H-1/C-13 HSQC NMR experiments were carried out on a 900-MHz Bruker Avance Spectrometer equipped with a cryogenic probe. Data were processed and analyzed using the NMRPipe software.

Communoprecipitation Analyses of Receptor Interactions. HeLa cells were transiently transfected with DNA encoding FLAG-CXCR4 or empty expression vector pcDNA and pcDNA, HA-ACKR3, or HA-AR1b, using Transit-LTI transfection reagent, similar to previously published protocols (70). Twenty-four hours later, cells were collected in 1.0 mL immunoprecipitation buffer (20 mM NaPO₄, pH 6.5, 150 mM NaCl, 1% (vol/vol) Triton-X 100, 10 μg/mL leupeptin, 10 μg/mL aprotinin, and 10 μg/mL pepstatin A) and incubated at 4 °C for 30 min. Cells were sonicated and centrifuged, and 500 μg of the clarified lysates were incubated with an anti-FLAG polyclonal antibody (Sigma) to immunoprecipitate FLAG-CXCR4 followed by immunoblotting to detect bound HA-ACKR3 or HA-α₁β-AR, similar to previously published protocols (76).
Comunoprecipitation experiments with human VSMC were performed using the Thermo Scientific Pierce communoprecipitation kit according to the manufacturer's protocol. Anti-α1A-AR and anti-α2A-AR antibodies were purchased from Abcam. Forty micrograms of anti-CXCR4 was incubated with 50 μL AminoLink Plus coupling resin for 2 h. Three hundred micrograms of cell lysates was precleared with 25 μL of the control agarose resin slurry (30 min at 4 °C). Immobilized anti-CXCR4 resin and nonreactive resin (= control) were incubated with precleared lysate overnight at 4 °C. After incubation, the resins were washed three times with 200 μL IP Iysis/wash buffer, and protein was eluted using 50 μL of elution buffer. Samples were analyzed by Western blotting.

CXCR4 Gene Silencing by RNA Interference. CXCR4 siRNA gene silencing was performed as described previously (51). In brief, VSMC were grown in 1 mL Acelli siRNA delivery media per well (Thermo Scientific Dharmacon in 12-well plates (Nunc). Commercially available Acelli CXCR4 siRNA was reconstituted with 1× siRNA buffer to a stock concentration of 100 μM. Cells were then transfected with 10 nmol CXCR4 siRNA and incubated for 72 h at 37 °C, 5% (v/v) CO2. Acelli NT siRNA pool was used as negative control. After 72 h, cells were assayed for receptor cell-surface expression and used for signaling experiments.

Proximity Ligation Assays. Duolink proximity ligation assays were performed as described previously (40, 41). In brief, VSMC and A7r5 cells were grown and fixed on eight-well tissue culture slides. For the visualization of individual receptors, slides were blocked with 3% (v/v) BSA in PBS and incubated with rabbit anti-CXCR4 (ab2074, Abcam) (1:400), rabbit anti-ACKR3 (LS-B1815, LSBio) (1:400), rabbit anti-α1A-AR (ab137123, Abcam) (1:400), rabbit anti-α2A-AR (ab169523, Abcam) (1:400), rabbit anti-α1B-AR (ab84402, Abcam) (1:400), rabbit anti-α3A-AR (SAB4500548, Sigma) (1:400), mouse anti-α2A-AR (ab21768, Abcam) (1:400), mouse anti-α3A-AR (ab167433, Abcam) (1:400), or rabbit anti-TRL9 (ab25577, Abcam) (1:400) at 37 °C for 2 h in a humidifying chamber. Slides were then washed and incubated (1 h at 37 °C) with mouse secondary-antibody antibodies conjugated with plus and minus Duolink II PLA probes (1:5). Slides were washed again and then incubated with ligation-ligase solution (30 min at 37 °C) followed by incubation with amplification-polymerase solution (2 h at 37 °C). Slides were then mounted with minimal volume of Duolink II mounting medium or 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, 20–24 randomly selected nonoverlapping vision fields were analyzed, and the mean from all experiments was calculated.

Deconvolution 3D Imaging. Z-stack images were collected (from bottom to top) using identical acquisition parameters with a DeltaVision wide-field fluorescent microscope (Applied Precision, GE) equipped with a digital camera (CoolSNAP HQ; Photometrics), using a 1.4-numerical aperture 100× objective lens. Excitation light was generated using the Insight SSL solid-state illumination module (Applied Precision, GE), and images were deconvolved with the SoftWoRx deconvolution software (Applied Precision, GE). Following deconvolution, images were quantified by Imairis (Bitplane software) using the Surfaces feature function, generating surfaces around red puncta, as described (77). Three-dimensional views of images were generated using Surpass mode of Imairis software (78).

Calcium Assays. Intracellular Ca2+ in A7r5 cells was measured using the Fluo-4 NW Calcium Assay Kit (Molecular Probes), as described (51, 52, 79). Intracellular Ca2+ in human VSMC was measured using the ratiometric Ca2+ indicator dye Fura-2. VSMC were grown on 15-mm round coverslips for 3 d until 80–100% confluence. Coverslips were washed twice with modified Krebs solution (135 mM NaCl, 5.9 mM KCl, 1.5 mM CaCl2, 1.2 mM MgCl2, 11.5 mM glucose, 11.6 mM Heps, pH 7.3) and then incubated in the same solution with 5 mM Fura-2-AM, 0.1% BSA, and 0.02% Pluronic F127 detergent for 60 min at room temperature in the dark. Cells were then washed twice and incubated in the dark in control medium for 0.5–2 h. All experiments were performed at room temperature in the presence of continuous perfusion of bath solution (1× Hanks’ balanced salt solution, 20 mM Heps) at a rate of 2 mL/min. Cell images were acquired using C Imaging System (Compix Inc.) with an Olympus 1× 71 inverted epifluorescence microscope (10× fluorescence objective) and Simple PCI software (Vers.5.3.1.). Fura-2 fluorescence (340 and 380 nm excitation, 510 nm emission) was measured every 2 s for 1–2 min before application of PE, followed by 5 min after PE application. Background fluorescence was determined at the end of each experiment by quenching Fura-2 fluorescence with 2 μM ionomycin and 6 mM MnCl2 for 2 min and subtracted from individual wavelength measurements before calculating 340.380 nm fluorescence ratios. For Ca2+ flux analyses, 50 individual cells in each field were continuously monitored, and the corrected 340/380 fluorescence ratio was calculated for each cell and averaged among all cells in the vision field.

FACS Analyses. FACS analyses of receptor cell-surface expression were performed as described previously (51, 79). Cells were labeled with polyclonal rabbit anti-CXCR4 (ab2074, Abcam), polyclonal rabbit anti-α1B-AR (ab137123, Abcam), and polyclonal rabbit anti-α2A-AR (ab169523, Abcam) in combination with anti-rabbit FITC-conjugated goat IgG (ab6717, Abcam). Rabbit IgG (GWB-3274CD, R&D Systems) in combination with FITC-conjugated anti-rabbit goat IgG (ab6717, Abcam) was used as a negative control. The geometric fluorescence intensities of at least 3 × 104 cells were recorded and analyzed using the FlowJo software (Tree Star).

Western Blots. Western blotting with mouse monoclonal anti-phospho-MLC2 (S19) (Cell Signaling), rabbit polyclonal anti-CXCR4 (ab2074, Abcam), rabbit polyclonal anti-α1A-AR (ab137123, Abcam), rabbit polyclonal anti-α2A-AR (ab169523, Abcam), and mouse anti-α2A-AR (ab176433, Abcam) in combination with anti-rabbit HRP-conjugated whole antibody (GE Healthcare) was performed as described (52, 79). Rabbit anti-GAPDH (Cell Signaling) in combination with anti-rabbit IgG horseradish peroxidase-linked whole antibody (GE Healthcare) was used as a protein loading control.

Vascular Smooth Muscle Cell Contraction Assay. Freshly isolated VSMC from mesenteric arteries were dispensed onto a glass coverslip base of the recording chamber and allowed to adhere for at least 15 min at room temperature. All experiments were performed with continuous perfusion of the bath solution containing 140 mM NaCl, 5.36 mM KCl, 1.2 mM MgCl2, 2 mM CaCl2, 10 mM Heps, and 10 mM d-glucose (pH 7.3), 298 mosm/L. Images were acquired using a C Imaging System (Compix Inc.) with an Olympus 1× 71 inverted epifluorescence microscope (10× objective, phase contrast) and Simple PCI software (Vers.5.3.1.) every 2 s for 1 min before the application of 10 μM PE and for an additional 3 min after PE application. The total number of the cells and the number of the cells that contracted in response to PE were counted in each field. For each experiment, cells from a single animal were tested in triplicate or quadruplicate.
In Vivo Testing of PE Responsiveness. Male Sprague–Dawley rats (300–325 g body weight, Harlan) were anesthetized with 2.5% (vol/vol) isoflurane, and a central venous catheter and an arterial line were placed in the femoral vessels. After stable baseline conditions were achieved, animals received an i.v. bolus injection of the CXCR4 ligands (AMD3100, CXC12, or ubiquitin; 750 nmol/kg in 0.5 mL of normal saline). Five minutes later, i.v. bolus injections of increasing doses of PE (5 ng/kg–40 μg/kg) in 0.5 mL of normal saline were administered at 5-min intervals. In blocking experiments, AMD3100 was administered 5 min before CXC12 or ubiquitin treatment. MAP was recorded at 10-s intervals for the duration of the experimental period. At the end of the experimental period, animals were euthanized (isoflurane inhalation, bilateral pithotomy). For each dose of PE, data under the MAP curve were calculated using the GraphPad-Prism 6 software, and dose–response curves were generated.

Data Analyses. Data are expressed as mean ± SEM from n independent experiments that were performed on different days. Data were analyzed using the GraphPad-Prism 6 software. Unpaired Student’s t test or one-way analyses of variance with Bonferroni’s multiple comparison post hoc test for multiple comparisons. Dose–response curves were generated using nonlinear regression analyses. Best-fit values were compared with the extra sum-of-squares F test. A two-tailed P < 0.05 was considered significant.

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