Published in final edited form as:
J Cardiovasc Pharmacol. 2010 November ; 56(5): 548–559. doi:10.1097/FJC.0b013e3181f713fe.

β_2-Adrenergic Receptor Signaling in the Cardiac Myocyte is Modulated by Interactions with CXCR4

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Abstract

Chemokines are small secreted proteins with chemoattractant properties that play a key role in inflammation, metastasis, and embryonic development. We previously demonstrated a non-chemotactic role for one such chemokine pair, stromal cell-derived factor-1α (SDF-1α) and its G-protein coupled receptor (GPCR), CXCR4. SDF-1/CXCR4 are expressed on cardiac myocytes and have direct consequences on cardiac myocyte physiology by inhibiting contractility in response to the non-selective β-adrenergic receptor (βAR) agonist, isoproterenol. Due to the importance of β-adrenergic signaling in heart failure pathophysiology, we investigated the underlying mechanism involved in CXCR4 modulation of βAR signaling. Our studies demonstrate activation of CXCR4 by SDF-1 leads to a decrease in βAR-induced PKA activity as assessed by cAMP accumulation and PKA-dependent phosphorylation of phospholamban (PLB), an inhibitor of SERCA2a. We determined CXCR4 regulation of βAR downstream targets is β_2AR dependent. We demonstrated a physical interaction between CXCR4 and β_2AR as determined by co-immunoprecipitation, confocal microscopy and BRET techniques. The CXCR4-β_2AR interaction leads to G-protein signal modulation and suggests the interaction is a novel mechanism for regulating cardiac myocyte contractility. Chemokines are physiologically and developmentally relevant to myocardial biology and represent a novel receptor class of cardiac modulators. The CXCR4-β_2AR complex could represent a hitherto unknown target for therapeutic intervention.

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Conflict of Interest Disclosure: None of the authors have any conflict of interest to disclose.

Keywords

Chemokines; Beta adrenergic receptors; G Protein Coupled Receptors; Dimerization; Heart failure

Introduction

SDF-1α (also known as CXCL12) is an inflammatory chemotactic cytokine with CXCR4 being its predominant receptor. In various hematopoietic and metastatic cells, this leads to modulation of intracellular calcium stores and alteration of the cytoskeletal architecture inducing chemotaxis. Importantly, the SDF-1/CXCR4 signaling axis is increasingly being investigated for non-chemotactic functions including regulation of neurotransmitter release, hepatic fibrosis, and thrombosis [1–3]. Our laboratory has previously shown activation of CXCR4 on the cardiac myocyte negatively modulates contractile function in response to isoproterenol (ISO), a non-selective βAR agonist. Activation of βAR is one of the major regulators of cardiac contractile function [4]. βAR signaling is integral in regulating myocardial function in both healthy and pathological states and any modulator of βAR signaling, such as CXCR4 activation, may have profound implications both for understanding cardiac pathophysiology and for clinical interventions in heart disease.

Controversies exist over the protective versus apoptotic effects of SDF-1 and CXCR4 [5–10] in cardiac injury models. Several studies have reported SDF-1 exerts cardioprotective effects following myocardial infarction [5–7]. Saxena et al. demonstrated the protective benefits of SDF-1 in a murine model of myocardial infarction (MI) using direct myocardial injections of SDF-1 post-MI [5]. This approach led to increased progenitor cell recruitment, cardiac regeneration and maintenance of cardiac function. Dai et al recently reported chronic AMD3100 administration leads to increased scar size and adverse ventricular remodeling in murine models of chronic MI further suggesting the beneficial effect of the CXCR4 axis post-myocardial infarction [10]. However, in contrast, Jujo et al. described CXCR4 blockade augmented bone marrow progenitor cell recruitment, enhanced the neovasculature and reduced mortality post-MI in the mouse [9] In addition, Agarwal et al recently described a cardiac specific deletion of CXCR4 in the mouse model of MI. The lack of CXCR4 in the myocardium had no effect on infarct size or remodeling mechanism [11]. Finally, our laboratory has shown adenoviral over-expression of CXCR4 worsens cardiac function in a rat model of ischemia/reperfusion (I/R) injury [8] via augmentation of TNFα expression and inflammation. There are fundamental differences between experimental approaches and the cardiac injury models from these studies, all potentially contributing to the contrasting results. Therefore, it is essential to further address the possibility of an autocrine/paracrine mechanism wherein CXCR4, present on the cardiac myocyte surface, modulates functional responses to myocardial stresses.

Recently, several published reports have indicated SDF-1 and CXCR4 have neuromodulatory functions [12,13]. The possibility of SDF-1 having non-inflammatory roles in the CNS is supported by observations that SDF-1α can modulate calcium and potassium currents in CXCR4+ neurons [1,12,14]. Furthermore, SDF-1α was shown to negatively modulate the firing pattern of vasopressin neurons and counteract vasopressin release through CXCR4 activation [1]. Neurons, like cardiac myocytes, are terminally differentiated, excitable cells which make translating these observations to the heart intriguing. CXCR4 activation is reported to decrease ISO-induced voltage gated L-type Ca^{2+} channel activity and negatively modulated myocyte contractile function [15]. Consistent with the SDF-1/CXCR4 modulatory role in the CNS, we report that SDF-1/CXCR4 also has a prominent modulatory role in cardiac myocyte function.

In heart failure, βARs undergo profound remodeling in the myocardium, including an increase in β_2AR expression with increased G-alpha i ($G\alpha_i$) activity [16]. Interestingly, in the setting of heart failure both CXCR4 and SDF-1 are increased in the myocardium, as well as increased plasma concentration of SDF-1 [17,18].Therefore, with our previous knowledge of CXCR4-βAR regulation, we explored a possible signal interaction between CXCR4 and β_2AR, the main βAR isoform expressed in the failing myocardium [16]. The mechanism by which SDF-1/CXCR4 can negatively modulate β_2AR signaling pathways is described.

Materials and Methods

Isolation of Adult Rat Ventricular Myocytes (ARVM)

ARVM were isolated from adult rat hearts as described previously [15]. Briefly, rat hearts were excised and the aorta quickly cannulated. The heart was perfused with a low calcium Tyrode's buffer followed by an enzyme solution containing collagenase and protease. The heart was minced, filtered, and resuspended in Tyrode's solution, and cultured in medium M199 containing proper supplements.

Western blot analysis

Membrane and tissue homogenates were prepared as described [19]. Proteins were resolved on 7.5% or 10% SDS-PAGE gels (Prosieve 50 solution, FMC) followed by transfer to ECL Hybond nitrocellulose membranes (Amersham Pharmacia Biotech, Piscataway, NJ). Membranes were probed with designated antibodies.

Immunoprecipitation and immunoblotting

Cardiac myocytes were cultured for 24 h in serum-free medium before treatments. For immunoprecipitation, cardiac myocytes were pretreated for 5 min with SDF-1 (100 ng/ml) and followed by ISO (10 μM) for designated times prior to lysis with RIPA buffer (50 mM Tris, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1mM sodium orthovanadate, 1mM sodium fluoride, protease inhibitors). 1 mg of total protein lysate was incubated overnight (4° C) with specific antibodies followed by incubation with protein A/G-plus agarose beads (3 h, 4°C). Beads were then collected by centrifugation, washed, boiled in Laemmli's buffer, subjected to SDS-polyacrylamide gel electrophoresis and immunoblotted with antibodies to the specific protein of interest. Immunoreactive bands were visualized by the ECL detection kit on Kodak ML light films.

Antibodies and Reagents

Antibodies against the following proteins were utilized: CXCR4 (1:500, Abcam, Cambridge, MA), β_2AR (1:1000, Santa Cruz, Santa Cruz, CA), Active-conformational β_2AR antibody (provided by Dr. Lakshmi Devi, Mount Sinai School of Medicine), β_1AR (1:1000, Santa Cruz), phospholamban (1:500, Abcam), phospho-phospholamban (1:500, Upstate, Billerica, MA), ERK42/44 (1:1000, Cell Signaling), phospho-ERK 42/44 (1:1000, Cell Signaling), p38 MAPK (1:1000, Cell Signaling), phospho-p38 MAPK(1:1000, Cell Signaling), Akt (1:1000, Cell Signaling), phospho-Akt^{ser473} (1:1000, Cell Signaling), CGP 20712A (1 μM, Sigma Aldrich), ICI 118551 (1 μM, Sigma Aldrich, St. Louis, MO), H89 (10 μM, Calbiochem Gibbstown, NJ), DT2 (1 μM, provided by Dr. Wolfgang Dostmann, University of Vermont), or ODQ (3 μM, Sigma Aldrich).

cAMP and PKG Activity Assay

Cardiac myocytes were isolated as described above and were plated at 1×10^4 cells/cm^2. When utilizing the CXCR4 inhibitor AMD3100 (10 μM), the cells were pretreated for 45 minutes. Cardiac myocytes were exposed to the β_2AR inhibitor, ICI 118551 (1 μM) for 30 min

pretreatment. Additionally, cardiac myocytes were exposed to the PKA inhibitor, H89 (10 μM), PKG inhibitor DT2 (1 μM), or the guanylate cyclase inhibitor ODQ (3 μM) was used for 5 min pretreatment. Cardiac myocytes were then treated with SDF-1 (100 ng/ml) for 5 min which was followed by ISO (10uM) for 10 min at 37°C. We utilized SDF-1 at 100ng/mL due to our previously published data which indicate this concentration negatively regulates L type calcium channel activity in response to ISO. SDF-1 treatment at 100ng/mL also approximates previously published literature on the activation of CXCR4-dependent signaling pathways in a number of other cell-types [20,21]. Cells were assessed for cAMP accumulation using a colorimetric immunoassay (R&D system, Minneapolis, MN) as described by the manufacturer. PKG activity was determined by colorimetric immunoassay (CycLex, Nagano, Japan) following manufacturer instructions [22]. All the inhibitors were dissolved in water or DMSO (H89= soluble in H_2O (25 mg/ml) stock, DT2= soluble in H_2O (10mM), ODQ= soluble in DMSO (5mg/ml) and AMD3100= soluble in H_2O). The diluents used were also tested in control groups. There were no significant effects observed as compared to control (untreated cells) (data not shown).

Diaminofluoresceins (Nitric Oxide Synthase) assay

Cardiac myocytes were seeded onto 96-well flat-bottomed culture plate and loaded with DAF2 prior to treatment. Treatment with H89 (10 μM), DT2 (1 μM), ODQ (3 μM) for 5 min was followed by exposure to SDF-1 (100ng/ml) for 5 min followed by either SDF-1/ISO/H89, SDF-1/ISO/DT2, or SDF-1/ISO/ODQ, respectively for 10 min at 37°C. Fluorescence was measured using a Spectramax M5 (Molecular Devices) plate reader that is calibrated for excitation at 485 nm and emission at 538 nm [22].

ELISA for Activated β_2AR

ARVMs were plated on laminin-coated 24-well plates at 1×10^4 cells/cm^2. After 24 h, cells were washed with phosphate-buffered saline (PBS) and incubated with or without SDF-1 followed by ISO 5 min later in 50 mM Tris-Cl, pH 7.5, or in isotonic HEPES buffer (10 mM HEPES containing 300 mM sucrose and 0.2 mM EDTA, pH 7.4) for 5 min at 37°C. Cells were quickly rinsed and fixed with ice-cold methanol for 10 min at −20°C. This treatment was included to help reduce cell loss during multiple washings as determined by protein estimation or recognition by β_2AR active conformation antibody provided by Dr. Lakshmi Devi. ELISA was carried out as previously published [23].

Cell Based ELISA

HEK 293 cells were seeded in 96-well plates at 5×10^4 cells/well. 48 hrs post transfection, the cells were fixed with 4% formaldehyde and the endogenous peroxidase was quenched with 0.6% H2O2 in PBS-Triton for 20 min, washed three times in PBS- Triton, blocked with 10% fetal calf serum in PBS-Triton for 1 h and incubated overnight with various primary antibody in PBS containing 5% BSA at 4°C. Next day, cells were washed and incubated with secondary antibody (peroxidase-conjugated goat anti-rabbit antibody, dilution 1:100) in PBS-Triton with 5% BSA for 1 h at room temperature and washed three times with PBS-Triton for 5 min and twice with PBS. Subsequently the cells were incubated with a solution containing 0.4 mg/ml OPD, 11.8 mg/ml Na2HPO4, 7.3 mg/ml citric acid and 0.015% H2O2 for 15 min at room temperature in the dark. The reaction was stopped with 25μl of 1M H2SO4, and absorbance at 405nm was determined [24].

Bioluminescence Resonance Energy Transfer (BRET) Assay

BRET experiments were performed as previously described [25]. Briefly, the β_2AR and CXCR4 fusion proteins used for BRET studies were β_2AR-*R*Luc, β_2AR-YFP, CXCR4-YFP, and CXCR4-*R*Luc (courtesy of Dr. Michel Bouvier, University of Montreal) [26,27]. Briefly,

48 h after plasmid transfection, HEK 293 cells were detached and washed 3 times with PBS. Cells dispensed onto a 96-well optiplate (Packard, Meriden, CT) as described previously [25]. The cells were incubated with ISO (10μM), SDF-1 (100ng/mL) or pretreated with SDF-1 for 5 min followed by ISO and SDF-1 (ISO+SDF-1) for 5 min at 25°C. Coelenterazine H luciferase substrate (5μM final, Invitrogen, Eugene, OR) and readings were taken with a lumino/fluorometer Fusion™ (Packard, Meriden, CT) using two filter settings (*R*Luc filter, 485 ± 10 nm; YFP filter, 530 ± 12.5 nm). The BRET ratio was determined as the difference between the emission ratio of Rluc only transfected cells (530nm/480nm) and Rluc+YFP transfected cells (530nm/480nm) under the various treatment conditions.

[^{35}S]GTPγS binding assay

Membranes from isolated ARVMs were prepared and used in a [^{35}S]GTPγS binding assay as described previously [28]. Cells were permeabilized and the membrane fraction was isolated by centrifugation. Next, the membrane fraction (~5 μg/μl protein) was washed with the GTPγS assay buffer and incubated in the same buffer containing 100 μM GDP, 0.1 nM [^{35}S] GTPγS and then treated with 100ng/ml SDF-1 in the absence or presence of 10 μM ISO in a final volume of 1 ml. After incubation for 1 h at 30°C, the membranes were collected on Whatman GF/B filters (Schleicher and Schuell, Keene, NH, U.S.A.) with a Brandel cell harvester (Brandel, Gaithersburg, MD, U.S.A.). The membranes were washed three times with ice-cold 50 mM Tris-Cl pH 7.5, and radioactivity detected using a scintillation counter. Data were analyzed using Prism 2.0 (Graph Pad, San Diego, CA, U.S.A.).

Assessment of SDF-1 on β_2AR-mediated Intracellular Ca^{2+} Levels

ARVMs were isolated and loaded with the Ca^{2+} indicator, Fura-2AM (3μM, 15min). The cells were washed and allowed to equilibrate for 15 min in a light-sealed, temperature-controlled chamber (37°C) mounted on an inverted microscope (Zeiss). Cells were then pretreated with or without SDF-1 (100ng/mL) for 5 min followed by treatment with either diluent (control), ISO (10 μM), or SDF-1 (100ng/mL), in the absence or presence of the β_1 or β_2 blocker, CGP 20712A (1 μM) and ICI 118551 (1 μM) respectively. Intracellular calcium was continuously recorded over 20 minutes using a dual excitation spectrofluorometer (Ultraview Imaging System, Perkin Elmer). Intracellular calcium was determined using the ratio of fluorescence emissions of 505 nm excited by wavelengths 340 nm and 380 nm [29].

Confocal Microscopy

ARVMs were pretreated with SDF-1 (100ng/mL) for 5 min followed by treatment with either diluent (control), ISO (10 μM), or SDF-1 (100ng/mL) for an additional 5 minutes. Cells were fixed and immunostained using anti-CXCR4 and anti-β_2AR antibodies. Primary Abs were substituted with murine IgG followed by FITC conjugate as a negative control. Primary antibodies were visualized with appropriate secondary conjugated to FITC (CXCR4) or texas red (β_2AR). A merged signal (yellow) is indicative of colocalization of both receptors.

Animal use

All experimental animal studies have been conducted under protocols reviewed and approved by Mount Sinai School of Medicine Animal Care and Use Committee (in accordance with protocols approved by local IACUC06-1440) and to adhere to accepted NIH guidelines for animal experimentation.

Statistical Analysis

Data represent mean ± SEM from at least 3 independent experiments and were analyzed using student *t* tests unless otherwise specified. A *P* value of less than 0.05 was used as the criterion for statistical significance.

Results

CXCR4 Negatively Regulates β-adrenergic Induced cAMP-PKA Pathway

In order to assess the modulation of βAR signaling by CXCR4, we examined the effects of SDF-1 on βAR-mediated cAMP accumulation in cultured adult rat ventricular myocytes. Activation of βAR by ISO increased cAMP production at 10 minutes and 90 minutes. In comparison, activation of CXCR4 by SDF-1 prior to ISO treatment substantially diminished cAMP accumulation (Fig. 1A–B). Additionally, the protein kinase A (PKA) inhibitor, H89, but not the PKG inhibitor DT2, abolished the decrease in cAMP induced by activation of CXCR4 prior to ISO treatment suggesting involvement of a PKA dependent pathway (Fig. 1A). The inhibitors (H89, DT2) alone or in combination with ISO had no effects on cAMP accumulation as compared to control (untreated cells) or ISO only treated cells (data not shown). Pretreatment with AMD3100 (10μM), a bicyclam antagonist of CXCR4 blocked the decrease in cAMP induced by activation of CXCR4 prior to ISO treatment (Fig. 1B) thus supporting the involvement of CXCR4 in modulation of βAR mediated G-protein activity. In contrast, activation of CXCR4 had no effect on ISO-mediated increases in protein kinase (PKG) and nitric oxide synthase (NOS) activity (Fig. 1C–D). Nitric oxide (NO) can modulate cardiac contractility by accelerating relaxation mechanisms, PKG and NOS were assessed as potential intracellular mechanisms underlying SDF-1 negative modulatory effects on contractile function [30]. These results indicate that activation of CXCR4 negatively modulates the cAMP-PKA pathway following βAR activation.

CXCR4 Inhibition of Isoproterenol Induced Phospholamban Phosphorylation is β_2AR-dependent

Isoproterenol agonism of either β_1 and β_2-adrenergic receptors on the cardiac myocyte induces cAMP production and enhances contractility. We explored if CXCR4 uses an inhibitory mechanism specific to β_1 or β_2AR signaling. Phosphorylation of PLB^{ser16} has been shown to affect contractility in adult cardiac myocytes by preventing the ability of phospholamban to inhibit SERCA2a activity [31]. The activation of CXCR4 by SDF-1 prior to ISO treatment reduced the levels of phosphorylated PLB^{ser16} compared to myocytes treated with ISO only. The CXCR4 antagonist, AMD3100, blocked the CXCR4-mediated decrease in ISO-induced PLB phosphorylation further validating the role of a CXCR4-βAR regulatory mechanism (Fig. 2A). Importantly, the β_2AR-specific antagonist, ICI 118551, reversed CXCR4-mediated negative modulation of PLB^{ser16} phosphorylation (Fig. 2B). These results suggest a β_2AR selective inhibitory mechanism for CXCR4. The β_1AR-specific antagonist, CGP 20712A fully abrogated the PLB phosphorylation in response to ISO. Therefore any negative modulation by SDF-1 with CGP 20712A on PLB phosphorylation could not be determined (data not shown).

Next, we sought to identify if the negative modulatory effects of CXCR4 on βAR cAMP production is due to general adenylyl cyclase inhibition from increased CXCR4 Gαi activity or if it is specific to β_2AR. We performed a cAMP assay and assessed whether ICI 118551, a specific β_2AR antagonist, can negate the inhibitory effects of SDF-1 on ISO stimulation. Our results demonstrated SDF-1 fails to inhibit ISO induced cAMP accumulation when β_2AR is blocked suggesting the regulatory capacity of CXCR4 is dependent upon β_2AR activation (Fig. 2C). This is in agreement with our results demonstrating β_2AR inhibition abrogates the negative modulatory effects of SDF-1 on PKA-dependent phosphorylation of PLB^{ser16}. These results indicate CXCR4 modulation of βAR signaling is β_2AR dependent establishing the importance of how they may potentially regulate each other in the setting of heart failure.

CXCR4 Activation Limits β-adrenergic Diastolic [Ca^{2+}] Accumulation in the Cardiac Myocyte

We have reported previously CXCR4 activation decreased ISO-induced voltage gated L-type Ca^{2+} channel activity. Hence, we explored the possibility of CXCR4 regulating βAR induced

diastolic [Ca^{+2}] accumulation. In isolated adult rat ventricular myocytes, activation of CXCR4 reduces diastolic [Ca^{+2}] accumulation as a result of sustained ISO activation of βAR (Fig. 3A). Notably, CXCR4 activation alone does not affect diastolic calcium levels (Fig. 3A). The ability of CXCR4 activation to limit the acute ISO-induced stress response suggests it may do so by altering diastolic [Ca^{+2}]. In support of this, we show that 5 min after ISO treatment, diastolic [Ca^{+2}] levels are elevated, reaching a 6-fold increase over 15 min (Fig. 3). A significant reduction in the rate of rise of diastolic [Ca^{+2}] upon activation of both CXCR4 and βAR as compared to activation of βAR alone is observed (Fig. 3A). Additionally, the increase in diastolic [Ca^{+2}] in response to ISO was reduced by 63%+/−1% by the β_2AR antagonist, ICI 118551 (Fig. 3B). ICI 118551 had no affect on diastolic calcium accumulation without ISO stimulation (Fig.3B). Importantly, cardiac myocytes treated with ISO and the β_2AR antagonist, ICI 118551, with SDF-1 did not further attenuate diastolic [Ca^{+2}] supporting the potential of a β_2AR dependent mechanism (Fig. 3C). The effect of the β_1AR antagonist, CGP 20712A, on diastolic calcium accumulation was also assessed. CGP 20712A alone has no effect but in combination with ISO, it completely prevented diastolic calcium accumulation over 20 min (Fig. 3D). Consequently any negative modulation by SDF-1 with CGP 20712A on ISO-induced diastolic calcium accumulation can not be determined. This study focuses on a β_2AR dependent mechanism as ICI 118551 negates the inhibitory effects of SDF-1 on ISO mediated diastolic calcium responses.

CXCR4 Prevents the Active Conformation of β_2AR and Participates in a CXCR4-β_2AR Receptor Complex

GPCR heterodimerization has been shown to be a unique pharmacological mechanism regulating “outside-in” signaling [32]. We initially assessed a possible β_2AR interaction with CXCR4 utilizing conformational-sensitive antibodies to β_2AR which recognize the activated state of the receptor [23]. The N-terminal region of GPCRs undergoes conformational changes upon agonist binding and participates in receptor activation and transduction of signal. Antibodies directed against the N-terminus of activated β_2AR receptors by isoproterenol were used to indentify if CXCR4 activation can regulate receptor conformation. We have subsequently shown binding of ISO to β_2AR induces the active conformation of the β_2AR in adult rat ventricular myocytes using conformation specific antibodies [23]. The activation of CXCR4 by SDF-1 prior to ISO blocks β_2AR from entering the active, conformational state (Fig. 4A) indicating the ability of CXCR4 to affect the structural dynamics of the β_2-Adrenergic receptor.

Subsequently, we further explored a potential physical interaction between CXCR4 and β_2AR using co-immunoprecipitation (IP) in adult rat ventricular myocytes (Fig. 4B–C). CXCR4 was detected only in β_2AR immunoprecipitates and not in IgG immunoprecipitates (Fig. 4B). We observed a substantial increase in the levels of CXCR4 in β_2AR immunoprecipitates from cardiac myocyte lysates treated with both ISO and SDF-1 as compared to myocytes treated with either ligand alone (Fig. 4C). We also performed the reverse IP demonstrating a significant increase in β_2AR in CXCR4 immunoprecipate (Fig. 4D). Importantly, co-immunoprecipitation revealed a basal level of interaction in non-stimulated cells. These data suggest the existence of a CXCR4/β_2AR complex. To further support co-immunoprecipitation results, we used confocal microscopy to identify possible colocalization of CXCR4 and β_2AR (Fig. 4E). Cardiac myocytes were treated with SDF-1 and ISO as previously described and immunostained for CXCR4 and β_2AR. Upon treatment with both ligands we observe a clear increase in colocalizing signal (yellow) between CXCR4 and β_2AR on the cardiac myocyte surface.

In order to investigate if these two receptors can interact directly in an *in vitro* cellular assay, we used a BRET based approach. HEK 293 cells were transfected with one or a combination

of CXCR4-*R*luc and β_2AR-YFP plasmids. The BRET ratio indicated there is a significant basal level of interaction between CXCR4-β_2AR (0.23+/−0.04) and this interaction tends to increase upon the presence of both ligands (0.42+/−0.09, $p<0.075$) (Fig. 5A). The specificity of the BRET assay in assessing whether the two receptors are in close enough proximity to interact was compared to our positive control of β_2AR-YFP and β_2AR-*R*luc, known to homodimerize, and with receptor pairs that have been shown not to dimerize [23], as observed with cells expressing CCR5-*YFP* and mu-opioid-*R*luc (Fig. 5A). The levels of expression of each receptor, CXCR4-*R*luc and β_2AR-YFP were determined using two independent assays, western blot and a cell based ELISA (Fig. 5B–C). The BRET results indicate CXCR4 and β_2AR are within 100Å of each other; however, we cannot exclude the possibility of other membrane associated proteins facilitating these receptor interactions. Therefore, we are continuing to investigate the potential of a direct CXCR4-β_2AR heterodimer. In summary, these results indicate CXCR4 and β_2AR participate in a macromolecular complex which may be the basis for CXCR4 modulation of ISO mediated responses in the cardiac myocyte.

CXCR4 regulation of downstream targets of βAR signaling

We examined the impact of Gαi-coupled CXCR4 activation on ISO induced G-protein-mediated signaling. First, we measured Gαs activity utilizing the [^{35}S]GTPγS binding assay. We observed a decreased ISO-mediated [^{35}S]GTPγS binding (Fig. 6A) with CXCR4 activation suggesting Gαs suppression in concordance with our cAMP studies. The addition of pertussis toxin (PTX) to cardiac myocyte treatment allowed us to determine the contribution of Gαi from CXCR4-βAR co-activation. We assessed how CXCR4 affects βAR mediated the phosphorylation of PLB^{ser16} and kinase signaling including extracellular signal-regulated kinase (ERK), stress activated kinase p38, and the serine-threonine kinase Akt^{ser473}. We show SDF-1 enhances ISO-mediated Erk1/2 and Akt^{ser473} phosphorylation, yet decreases p38 activation at 5 min. PTX blocked the negative effects of SDF-1 pretreatment on ISO-mediated phosphoprylation at PLB^{ser16} (Fig. 6B) and also abrogated the increase in phospho-ERK and phospho-Akt^{ser473} (Fig. 6C). Interestingly, PTX treatment in cardiac myocytes stimulated by SDF-1 and ISO had no effect on phospho-p38 MAPK levels (Fig. 6C). Based on our hypothesis of SDF-1 modulation of β_2AR signaling, we assessed whether ICI 118551 can prevent the actions of SDF-1 on ISO-mediated phosphorylation of ERK, Akt^{ser473} and p38. ICI 118551 was shown to reverse the SDF-1 negative effect on p38 activation. However, ICI 118551 could not negate the SDF-1 effect on phospho-ERK or phospho-Akt^{ser473} (Fig. 6C) suggesting CXCR4 regulation of βAR downstream signals is not entirely dependent on β_2AR.

Discussion

The β-adrenergic signaling cascade is central in the pathogenesis of heart failure. As the myocardium transitions to symptomatic failure, βARs undergo profound remodeling with an increase in the β_2: β_1 AR ratio and increased Gαi activity [4]. This leads to a decrease in the inotropic reserve of the myocardium and hastens the development systolic dysfunction and heart failure onset. β-adrenergic receptor blockers are the most widely used therapy to maintain systolic function in patients with CHF. Therefore, we are interested in understanding additional mechanisms which regulate βAR function. In this study, we show CXCR4 activation modulates β_2AR signaling in adult rat ventricular cardiac myocytes. CXCR4 is rapidly expanding beyond its role as a chemotactic factor and has been shown to be a key mediator of calcium handling in non-migratory neurons and cardiac myocytes [1,12,14,15]. We demonstrated CXCR4 and β_2AR physically interact which leads to a decrease in PKA-dependent phosphorylation of PLB^{ser16} in a β_2AR dependent manner. Moreover, data obtained with active conformation-specific β_2AR antibodies and the use of the β_2AR specific antagonist, ICI 118551, indicate SDF-1/CXCR4 activation affects β_2AR structure and downstream signaling. These results

suggest an intriguing possibility of targeting CXCR4-β_2AR in order to develop a novel class of therapeutics to treat heart failure.

A growing body of evidence currently supports GPCR super family members can form hetero- or homodimers [33]. CXCR4 is able to not only homodimerize, but to heterodimerize with chemokine receptors CCR2 and CXCR7 [34]. However, chemokine receptor heterodimerization is not restricted to other isoforms of chemokine receptors. Recent studies by Pello et al. show the δ-opioid receptor (DOR) can heterodimerize with CXCR4 in neurons [35] leading to unique signaling consequences in the cell. GPCR heterodimerization can lead to a change in the preference for G protein subunits and alter the efficiency of downstream signaling compared to the homodimer or the single receptor. This has been shown for μ and δ opioid receptors, where heterodimers signal through Gαz or β-arrestin whereas receptor homodimers signal via Gαi [36]. β_1 and β_2-adrenergic receptor heterodimerization provides synergy and sensitivity to βAR signaling [37] and, in essence, generates a new receptor subtype with specific signaling capabilities [38]. CXCR4 may potentially act similarly and the concept of heterodimerization makes CXCR4 modulation of β_2AR a potential mechanism for CXCR4 negative modulation on βAR pathways.

Cardiac myocyte β_2AR signaling induces the recruitment and activation of ERK, p38 and Akt pathways. These signaling mediators have the ability to influence cardiac myocyte contractility, growth, and survival [39–42]. It is well known the critical nature of CXCR4 in cardiac developmental processes [43], however, the exact role of CXCR4 on adult cardiac myocyte physiology is yet to be determined. We hypothesize CXCR4 is a part of a β_2AR regulatory complex influencing downstream βAR signaling and calcium homeostasis. We show PTX treatment blocks the increase in the phosphorylation of ERK1/2 and Akt^{ser473} following co-activation of CXCR4 and βAR. However, the β_2AR blocker, ICI 118551 did not inhibit the effect on phospho-ERK or phospho- Akt^{ser473}. Therefore, CXCR4 activates the pro-growth, pro-survival ERK1/2 and Akt via a Gαi dependent mechanism irrespective of β_2AR. However, ICI 118551 does reverse the SDF-1 negative effect on p38 activation by ISO. The deleterious effect of chronic βAR stimulation in the heart results in part from excessive Gαs-dependent adenylyl cyclase activation [44]. Importantly, β_2AR has a pro-survival function that can counteract ISO-induced βAR-Gαs dependent cell death [45]. βAR-Gαs signaling in the cardiac myocyte stimulates the p38 MAPK pathway defined as pro-apoptotic [46]. A recent study by Kovacs et al. suggests activation of phosphatidylinositol-3-kinase (PI-3K)-Akt and ERK2 pathways significantly contributes to the cardioprotective effects of the βAR blocker, Metoprolol [47]. This is significant because CXCR4 and βAR co-activation in the cardiac myocyte reduces p38 phosphorylation and enhances ERK and Akt pathways. Interestingly, Hu et al also reported the cardioprotective capacity for the SDF-1/CXCR4 axis against hypoxia/reoxygenation cell death. Pretreatment of cardiac myocytes with PD98059 (ERK inhibitor) and LY290042 (PI3K inhibitor) before SDF-1 treatment blocked the increase in myocyte viability demonstrating the importance of ERK and Akt pathways for SDF-1–mediated myocyte survival [6]. Furthermore, Saxena et al. also demonstrated the protective benefits of SDF-1 in a murine myocardial infarction model. Upon SDF-1 myocardial injection post-MI, they observed decreased scar size and enhanced Akt phosphorylation which coincided with decreased apoptosis in the myocardium [5]. This is further supported by the recent work by Dai et al reporting CXCR4 blockade diminished Akt activation, increased scar size and further worsened cardiac function post-MI [10]. Collectively, our data indicate that the CXCR4 and β_2AR interaction may have important implications on cardiac myocyte survival.

Conclusion

In summary, our data suggests there is a physical interaction between CXCR4 and β_2AR. The CXCR4-β_2AR molecular interaction may have physiological consequences resulting in the

activation of novel signaling pathways impacting cardiac myocyte calcium handling. The pathophysiology of heart failure is a multifactorial process, however, one important transition leading to symptomatic heart failure is βAR remodeling ultimately promoting β_2AR expression and signaling. Concomitantly, the SDF-1/CXCR4 chemokine axis is also upregulated in heart failure due to the hypoxic and inflammatory microenvironment [17]. Therefore, activation of CXCR4 may lead to modulating β_2AR signaling and play an important role in calcium homeostasis and heart failure disease progression. Thus, the CXCR4-β_2AR complex may represent a novel target for therapeutic intervention in heart disease.

Abbreviations

(βAR)	βadrenergic receptor
(PLB)	phospholamban
(GPCR)	G-protein-coupled receptor
($G\alpha_s$)	stimulatory G protein
($G\alpha_i$)	inhibitory G protein

Acknowledgments

We thank Drs. Monique Lagace and Michel Bouvier for providing us with the plasmids for the *in vitro* BRET studies. This work was supported, in whole or in part, by American Heart Association AHA0735576T (S. T. T.), NHLBI Ruth L. Kirschstein National Research Service Award 1F30HL096344-01 (T.J.L.), Mount Sinai School of Medicine Medical Scientist Training Program GM007280 (T.J.L.), National Institutes of Health Grants RO1HL7458 (A.D.S), and DA08863 and DA019521 (L.A.D).

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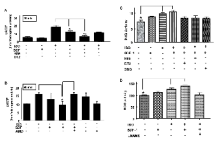

Fig. 1.

CXCR4 activation negatively modulates βAR signaling. (**A & B**) ARVMs were pretreated with or without SDF-1 (100 ng/mL) followed by treatment with diluent (control), ISO (10 μM), SDF-1 (100 ng/mL), in the presence or absence of a PKA inhibitor, H89 (10 μM), or a PKG inhibitor, DT2 (1 μM) and assessed for camp at 10 minutes. Additionally, a CXCR4 antagonist, AMD3100 was utilized and cAMP accumulation was assessed at 90 minutes *$p<0.01$, **$p<0.0001$. (**C**) PKG activity was measured by pretreating CM with or without SDF-1 followed by treatment with ISO (10 μM), SDF-1 (100ng/mL) in the absence or presence of either a PKA inhibitor, H89 (10 μM), a PKG inhibitor, DT2 (1 μM) or a soluble guanylate cyclase inhibitor, ODQ (3 μM). ISO alone significantly increased PKG activity compared to control and this is not significantly affected by the presence of SDF-1. All inhibitors reduced the effects of ISO with SDF-1 on PKG activity. Since there is no significant effect of SDF-1 alone on PKG activity this suggests that increases in PKG activity are due to βAR activation. (**D**) ISO treatment causes significant increases in NOS activity and this is not significantly affected by the presence of SDF-1. Cardiac myocytes were treated as described above in the absence or presence of L-NAME (0.1 μM) an inhibitor of NOS activity. Treatment with L-NAME reduced the ISO-mediated effects. Data are Mean ± SEM of 4 independent experiments.*$p<0.05$.

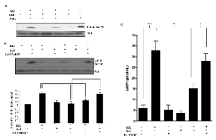

Fig. 2.

CXCR4-mediated negative modulation of PLB activation. (**A**) ARVMs were treated as in Figure 1B. Cells were harvested and protein lysates were immunoblotted to detect phospho-PLB^{ser16} and total PLB levels. (**B**) ARVMs were pretreated as in (**A**) in the absence or presence of the β_2AR antagonist, ICI 118551 (1 μM). Protein lysates were subjected to Western blot analysis to determine phospho-PLB^{ser16} and total PLB levels. *p< 0.05. (**C**) ARVMs were pretreated as in (**A**) in the absence or presence of the β_2AR antagonist, ICI118551 (1 μM) and cAMP accumulation was determined to assess whether blocking β_2AR negates the inhibitory effects of SDF-1 on ISO-induced cAMP accumulation. Data are Mean ± SEM of 3 experiments. *p<0.05, **p<0.01, ***p<0.001.

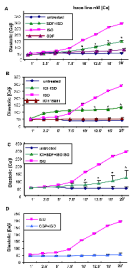

Fig. 3.

CXCR4 activation reduces the ISO-induced rise in diastolic calcium. **(A–D)** ARVMs were loaded with Fura-2AM and pretreated with or without SDF-1(100 ng/mL) followed by treatment with diluent (control), or ISO (100nM-1 μM), in the presence or absence of the β_2AR antagonist, ICI 118551 (1 μM), or the β_1AR antagonist, CGP 20712A (1 μM). Diastolic calcium concentrations were recorded over 20 minutes, *$p<0.05$.

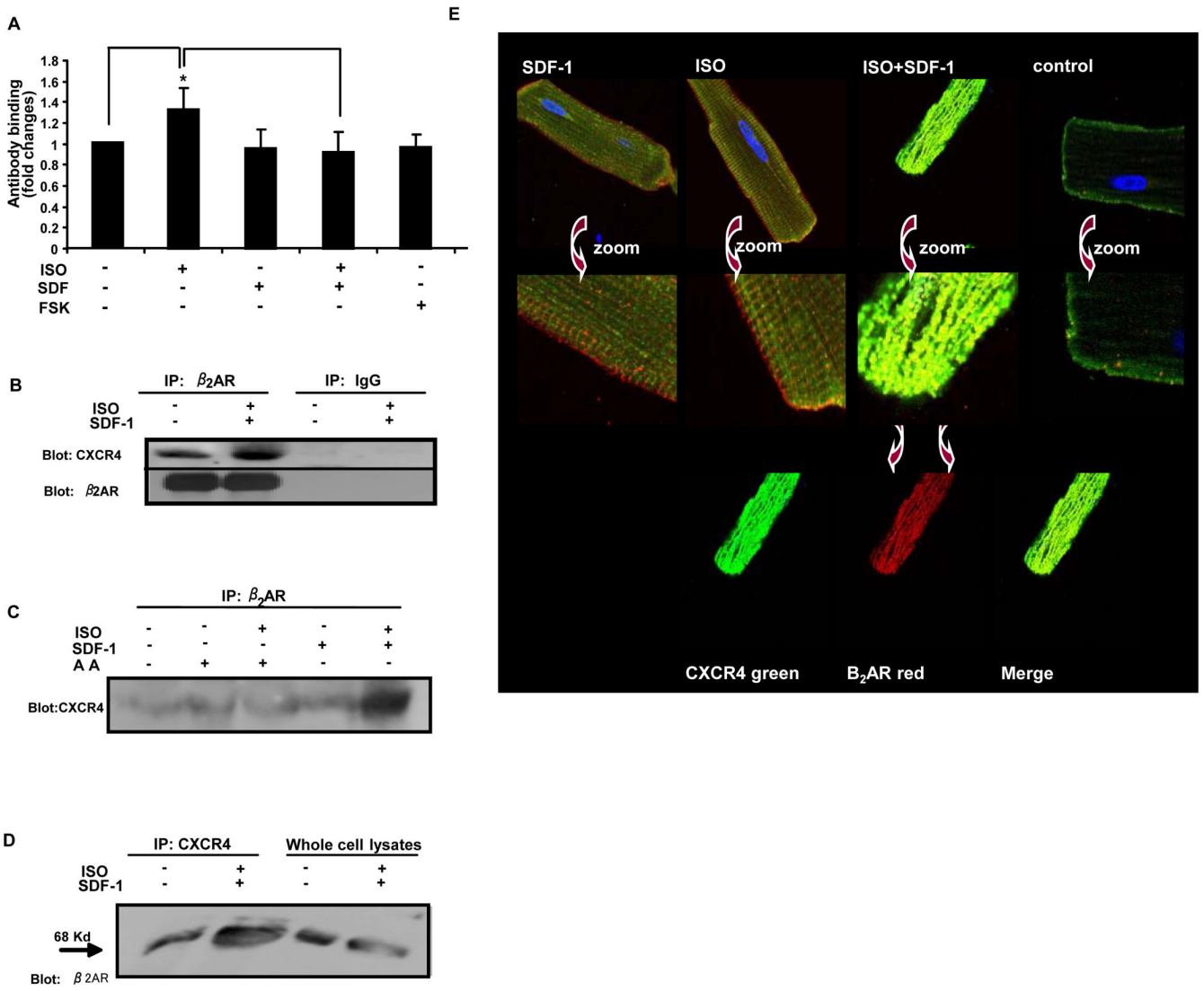

Fig. 4.

CXCR4 activation affects the conformational state of the β_2AR. (**A**) ARVMs were plated in 96 well plates and treated with either diluent, SDF-1 (100 ng/ml), ISO (10 μM) or ISO and SDF-1 for 5 min. The cells were fixed and analyzed by ELISA with conformationally specific antibody recognizing the activatedform of the β_2AR. The PKA-activating agent, forskolin (FSK) represents a receptor-independent activator of the β_2AR pathway and therefore used as a negative control. *$p<0.05$. CXCR4 and β_2AR form interacting complexes. (**B**) ARVMs were pretreated with or without SDF-1 (100 ng/mL) followed by treatment with diluent (control), ISO (10 μM), SDF-1 (100 ng/mL) and immunoprecipitated with either a β_2AR or IgG antibody followed by immunoblotting with anti-CXCR4 antibody. **(C)** A substantial increase in the basal levels of CXCR4 was observed in the β_2AR immunoprecipitates from cardiac myocyte lysates treated with both ISO and SDF-1. ARVMs were pretreated as in (**B**) and subjected to IP with a β_2AR antibody followed by immunoblotting with an anti-CXCR4 antibody. Ascorbic acid (AA, 300uM) was used as a vehicle for isoproterenol. **(D)** The reverse IP was performed with immunoprecipitated CXCR4 and immunoblotted with an anti-β_2AR Ab. Similar results were obtained in three independent experiments. In order to confirm the loading, 50 μg/mL of whole cell lysates with and without treatment were run on the gel and blotted for β_2AR antibody.

(**E**) CXCR4 and β_2AR are colocalized on the cardiac myocyte. ARVMs were pretreated as in (**B**) after 5 minutes treatment cells were fixed and immunostained for CXCR4 and β_2AR using FITC or texas red conjugate secondary antibodies, respectively. Confocal microscopic images were taken with CXCR4 antibody detected in green and β_2AR in red, merge images are shown yellow.

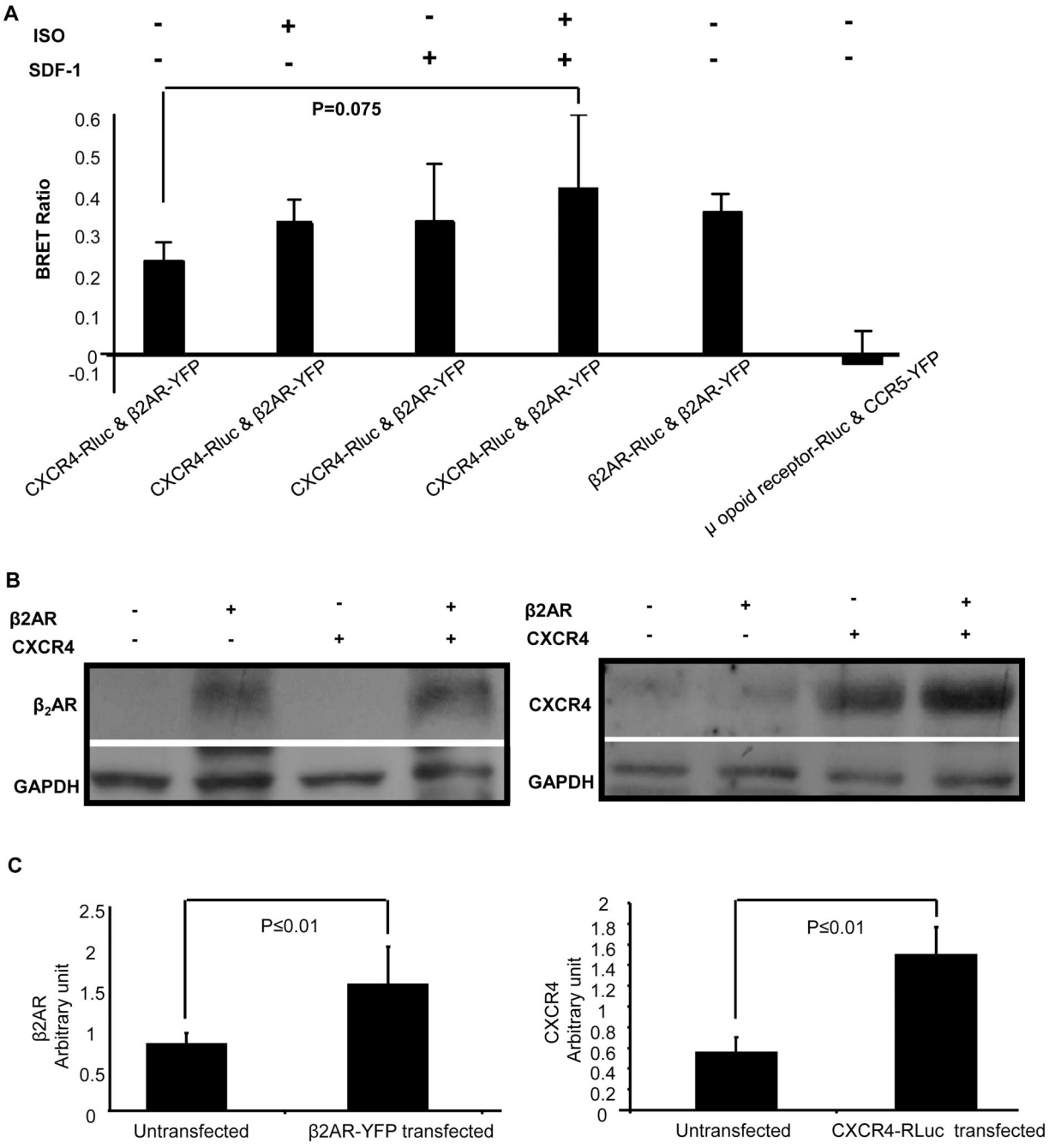

Fig. 5.

CXCR4 and β_2AR interact in HEK293 cells. **(A)** The BRET ratio from HEK-293 cells co-transfected with 2 μg of either a luciferase-tagged CXCR4 construct, a YFP and/or luciferase-tagged β_2AR construct, a YFP-tagged CCR5 or a luciferase-tagged μ-opioid receptor construct. Transfected cells were exposed to either diluent, SDF-1 (100 ng/ml), ISO (10 μM) or ISO and SDF-1. Homodimerization of cells expressing both the β_2AR-*R*luc and β_2AR-YFP represented a positive control whereas cells expressing CCR5-YFP and μ-opioid receptor-*R*luc, known not to dimerize act as a negative control. **(B & C)** The levels of expression of each receptor post-tranfection, CXCR4-*R*luc and β_2AR-YFP was determined using two independent assays: B)

western blot analysis and C) cell based ELISA (n=3). Representative blot of three independent experiments is shown. *$p<0.05$.

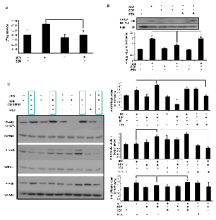

Fig. 6.

SDF modulates downstream targets of βAR signaling. (**A**) ARVM membranes were treated with ISO (10 μM) in the presence or absence of SDF-1 (100 ng/mL) and subjected to a [^{35}S] GTPγS binding assay. Data represents mean±SEM (n=3). *p<0.05. (**B**) CXCR4 effects on PLB is Gαi-mediated. ARVMs were treated as described in Fig. 1, in the presence or absence of PTX (200 ng/mL) and subjected to Western blot analysis to detect phospho-PLB^{ser16} and total PLB. Representative blot of four independent experiments is shown.*p<0.05. (**C**) CXCR4 effects on ISO-mediated ERK, Akt and P38 phosphorylation. ARVMs were pretreated with or without SDF-1 (100 ng/mL) followed by treatment with ISO (10 μM), in the presence or absence of PTX (200 ng/mL) the β_2AR antagonist, ICI118551 (1 μM) and immunoblotted to detect phospho ERK, phospho-Akt^{ser473} and phospho-P38. Representative blot and densitometric analysis of four independent experiments is shown. *p<0.05.