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CXCR4 modulates contractility in adult cardiac myocytes

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Abstract

The inflammatory response is critical to the development and progression of heart failure. Chemokines and their receptors are a distinct class of inflammatory modulators that may play a role in mediating myocardial dysfunction in heart failure. Levels of the chemokine CXCL12, also known as stromal cell-derived factor (SDF), and its receptor, CXCR4, are elevated in patients with heart failure, and we undertook this study to determine whether this chemokine system can directly affect cardiac function in the absence of leukocytes. Murine papillary muscles and adult rat cardiac myocytes treated with CXCL12, the only identified ligand of CXCR4, demonstrate blunted inotropic responses to physiologic concentrations of calcium. The negative inotropic effects on cardiac myocytes are accompanied by a proportional diminution of calcium transients. The effects are abrogated by AMD3100, a specific CXCR4 inhibitor. Overexpression of the receptor through adenoviral infection with a CXCR4 construct accentuates the negative inotropic effects of CXCL12 on cardiac myocytes during calcium stimulation. CXCR4 activation also attenuates beta-adrenergic-mediated increases in calcium mobilization and fractional shortening in cardiac myocytes. In electrophysiologic studies, CXCL12 decreases forskolin- and isoproterenol-induced voltage-gated L-type calcium channel activation. These studies demonstrate that activation of CXCR4 results in a direct negative inotropic modulation of cardiac myocyte function. The specific mechanism of action involves alterations of calcium channel activity on the membrane. The presence of functional CXCR4 on cardiac myocytes introduces a new target for treating cardiac dysfunction.

Keywords

Calcium; Chemokines; Chemokine receptors; Contractility; CXCL12; CXCR4; SDF

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1. Introduction

Chemokines and their receptors are a relatively new class of inflammatory mediators that regulate inflammation by directing leukocytes to sites of injury. Chemokines and their receptors are up-regulated in patients with heart failure and therefore have been implicated in the development of this disease (reviewed in [1]). Contractile dysfunction is a prominent feature in heart failure, and there is an inverse relationship between chemokine levels and cardiac performance [2]. However, it is unclear whether these findings are merely coincidental or whether chemokines actually play a mechanistic role in mediating myocardial dysfunction. Furthermore, *if* chemokines mediate cardiac dysfunction, it is not clear whether the mechanism is via inflammatory cells or whether chemokines, through their receptors on the cardiac myocyte (CM) surface, can directly affect myocardial function.

The function of chemokines initially was thought to be limited to their migratory effects on inflammatory cells. However, as knowledge of the physiologic roles for chemokines and their receptors has markedly expanded, it is now appreciated that chemokine receptors mediate a myriad of important biological effects by activating their receptors on organs in a manner *independent of inflammatory cells*. CXCR4 exemplifies this concept. It is present on the mammalian heart and appears to play a critical role in cardiovascular development, in the mobilization of hematopoietic precursors and in vasculogenesis [3,4]. CXCR4 is also a co-receptor for HIV infection and can modulate Ca^{2+} channel function in neuronal cell lines [5].

CXCR4 is one of several chemokine receptors that are up-regulated in patients with heart failure [2,6,7]. To our knowledge, it is the only chemokine receptor that has been shown to be up-regulated in the failing myocardium of patients by direct staining [6]. Other studies examining chemokine receptors in heart failure have analyzed whole heart specimens to assess mRNA levels and therefore cannot localize the cellular source of the receptor given that the heart is composed of heterogeneous cell types. In early cardiac allograft rejection, where myocardial dysfunction can be significant without an obviously discernible cause (such as significant myocyte necrosis), CXCR4 expression is also elevated [8–11].

Given the increased expression of CXCR4 on dysfunctional myocardium in a variety of diseases and the specificity of *CXCL12 (formerly known as stromal cell-derived factor (SDF-1))* for binding to CXCR4, this is an ideal model to demonstrate experimentally that a chemokine can directly affect myocardial function. The present study examines the effects of CXCR4 activation on myocardial contractility and explores the mechanisms at the cellular level. We demonstrate that CXCR4 negatively modulates myocardial contractility by binding its endogenous ligand, CXCL12. The mechanism of action involves an alteration of calcium (Ca^{2+}) metabolism in response to β-adrenergic and Ca^{2+} stimulation in CM. These studies identify a potentially new class of receptors to target in the treatment of cardiac dysfunction.

2. Materials and methods

2.1. Papillary muscle contractility measurements

Papillary muscles (PM) from 10-week-old male FVBN-1 mice (Jackson Laboratories) were excised, loaded onto a force-transducer (Grass Instruments) and immersed in a circulating tissue bath at 37 °C. PM were field stimulated with platinum electrodes at 2× pacing threshold amplitude with a pacing frequency of 0.3 Hz and a stimulus duration of 0.3 ms. PM were then equilibrated in a modified Tyrode solution (containing the following in mM: K^+, 5.9; Na^+, 135.0; Cl^-, 126.0; Ca^{2+}, 1.0; $HCO3^-$, 15.0; $PO4^-$, 1.2; $SO4^-$, 1.2; Mg^{2+}, 1.2) and stretched to the length of maximal tension development. Following a 1-h equilibration, PM were subjected to stepwise increasing concentrations of extracellular calcium $\left(Ca_e^{2+}\right)$ from a baseline of 1.0

mM to a maximal concentration of 5.0 mM. PM underwent this first Ca^{2+} challenge to establish baseline contractile function and confirm muscle viability. They were then re-equilibrated at baseline Ca_e^{2+}, treated for 5 min with either diluent (0.1% BSA) (control) or CXCL12 (125 ng/ml in 0.1% BSA) before undergoing a second Ca^{2+} challenge. Isometric tension data, measured by a force transducer, amplified and captured via Polyview data acquisition software (Grass Instruments), were expressed as % baseline tension at each concentration of Ca^{2+}. The normalized data were subjected to a non-linear regression analysis, fitted to a dose response curve and log ED_{50} was calculated. To limit inter-animal variability, each PM served as its own control.

2.2. Cardiac myocyte isolation

Ca^{2+}-tolerant rat ventricular CM were isolated via enzymatic dissociation, as described previously [12]. Briefly, rat hearts were perfused in a retrograde fashion for 5 min with oxygenated Krebs–Henseleit buffer (containing the following in mM: NaCl, 120.0; KCl, 4.7; KH_2PO_4, 1.2; $MgSO_4$, 1.2 and HEPES, 25.0; pH 7.40 at 37 °C). The perfusate was then switched to an enzyme solution containing collagenase 0.7 mg/ml (Worthington Type II, 258 u/mg) and hyaluronidase 0.5 mg/ml (Sigma Type II, 667 u/mg), and the heart was perfused for another 10–15 min. Ventricular tissue was then finely minced and shaken gently in enzyme solution for 20–30 min. CM were filtered through a nylon mesh, collected and made Ca^{2+}-tolerant over 15 min. CM were then re-suspended in ACCT medium consisting of DMEM (containing 2.0 mg/ml BSA, 50 IU/ml penicillin, 50 IU/ml streptomycin and the following in mM: L-carnitine, 0.4; creatine, 0.66; taurine, 0.62) and plated at a density of 2×10^4 cells/ml onto laminin-precoated coverslips (2 μg/cm^2, Invitrogen).

2.3. Intracellular calcium and contractility measurements

Freshly isolated rat CM plated onto laminin-coated coverslips as described above were loaded with fura 2-AM (Molecular Probes), superfused with 1.2 mM Ca^{2+} Tyrode's solution, pH 7.40 at 37 °C, and electrically stimulated with a biphasic pulse (0.5 Hz, 50% above threshold). Cells included in the study were rod-shaped with a clear striation pattern and were quiescent in the absence of electrical stimulation. Contraction amplitude and intracellular Ca^{2+} transients were recorded online using a dual excitation spectrofluorometer and video-edge detection system (Ionoptix), as described previously [13].

During the Ca^{2+} stimulation protocol, CM were subjected to stepwise increases in Ca_e^{2+} from 1 mM to 8 mM. CM were treated with either diluent (0.1% BSA) (control) or CXCL12 (125 ng/ml) for 5, 8 and 60 min and assessed for fractional shortening and Ca^{2+} transients. Response to isoproterenol (ISO) was measured by increasing concentrations of ISO in a stepwise fashion from 0.001 μM to 10 μM. The concentration of Ca_e^{2+} was kept constant at 2.0 mM during the ISO stimulation.

The bicyclam AMD3100 was obtained from The National Institute of Health AIDS Research and Reference Reagent Program. During the blocking experiments, this compound was added to the chamber bathing the isolated myocytes to achieve a final concentration of 10 mM, prior to adding CXCL12.

2.4. Construction of recombinant adenoviruses and overexpression of CXCR4

The methods used to construct the recombinant adenovirus have been described previously by our group [14]. Briefly, the backbone vector, which contains most of the adenoviral genome (pAd.EASY1), was used and the recombination performed in *Escherichia coli.* CXCR4 cDNA was subcloned into the adenoviral shuttle vector (pAd.TRACK), which uses the

cytomegalovirus long-terminal repeat as a promoter. pAd.TRACK also has a concomitantly expressed green fluorescent protein (GFP) under the control of a separate cytomegalovirus promoter. The adenoviruses were propagated in 293 cells. The titer of stocks used for these studies measured by plaque assays was 4×10^{10} pfu/ml for CXCR4-expressing adenovirus (Ad.CXCR4) with particle/pfu ratios of 4:1. These recombinant adenoviruses were tested for the absence of wild-type virus by polymerase chain reaction of the early transcriptional unit E1. Subsequently, CM were incubated with Ad.CXCR4 at a multiplicity of infection (MOI) of 100. Fractional shortening and Ca^{2+} transients then were measured as previously described for the uninfected CM.

2.5. Fluorescent immunostaining

Adult rat CM, isolated as described above, were fixed with 4% formaldehyde and double stained with an anti-sarcomeric α-actinin monoclonal antibody (Ab) (Sigma, 1:2000) and an anti-CXCR4 polyclonal Ab (eBioscience, 1:200). Primary antibodies were detected with either an anti-mouse-Cy3 secondary Ab for α-actinin or an anti-rabbit-Cy2 secondary Ab (Rockland, Gilbertsville, PA) for CXCR4. Human CM were isolated as previously described [15] and stained as described for the rat CM. Images were collected using a Leica DMI 6000 (Leica Microsystems) fluorescence microscope. Non-specific staining for CXCR4 was assessed by omission of the primary Ab and examination of the sections in the presence of the secondary Ab alone.

2.6. Whole-cell patch clamp measurements

Adult rat ventricular CM, isolated as described above, were suspended in a high potassium medium (PSS solution containing the following in mM: KCl, 50.0; glucose, 10.0; HEPES, 10.0; taurine, 20.0; $MgCl_2$, 3.0; L-glutamic acid, 50.0; EGTA, 0.5; KH_2PO4, 20.0) and stored at 4 °C. Isolated CM suspension was inoculated onto an acid-treated 12-mm round glass cover-slip placed at the bottom of the recording chamber. After the cells settled on the coverslip, viable CM were identified morphologically. To study inward Ca^{2+} currents using Ca^{2+} as the charge carrier, extracellular buffer (containing the following in mM: NaCl, 140.0; $MgCl_2$, 0.5; $CaCl_2$, 1.0; HEPES, 5.5; CsCl, 5.4; pH 7.4) was used. Whole-cell patch electrodes were pulled from Kimax glass pipette (Fisher Scientific Inc), with a series resistance of 1.0–1.5 MΩ when filled with the intracellular solution (containing the following in mM: TEA–Cl, 20.0; HEPES 5.0; EGTA, 5.0; CsCl, 130.0; ATP 5.0; GTP 0.01; pH 7.4). Whole-cell patch-clamp recordings were carried out with an EPC-9 amplifier and Pulse/PulseFit software package (HEKA Electronic). Data analyses were carried out with Pulsefit software.

2.7. Statistical Analysis

Experimental data were presented as the mean±SE. Student's *t* test was used in the analysis of paired and unpaired means. Unless indicated, all values were significant at $p<0.05$. Blocking experiments was performed on three consecutive CM and representative data are presented.

3. Results

3.1. CXCR4 activation decreases PM contractility

Control and CXCL-12-treated papillary muscles were subjected to a first Ca^{2+} challenge to establish baseline function. They were then equilibrated at baseline Ca_e^{2+} prior to a second Ca^{2+} challenge. Prior to the second Ca^{2+} challenge, control PM were treated for 5 min with either diluent or CXCL12. Control PM demonstrated similar Log ED_{50} during the second (1.5 ±0.13) and first (0.90±0.23) Ca^{2+} challenges ($p>0.05$) (Fig. 1A). In contrast, exposure to CXCL12 significantly blunted the PM response to Ca^{2+}. After exposure to CXCL12, PM demonstrated a significantly higher Log ED_{50} during the second calcium challenge (2.4±0.14)

when compared to the first calcium challenge (1.4±0.15) ($p<0.05$). There was no significant difference between the average cross-sectional areas of Control PM (0.08±0.04 mm^2) and PM treated with CXCL12 (0.11±0.06 mm^2)($p=0.20$). Histologic examination demonstrated no significant inflammatory infiltrate (data not shown).

3.2. CXCR4 decreases CM contractility in response to Ca^{2+} and isoproterenol (ISO)

To determine whether the blunted Ca^{2+} response of PM can be explained by an alteration of CM function, we examined the effect of CXCL12 on isolated rat CM. The average percent increase in shortening of CM treated for 5 min with CXCL12 was significantly decreased at 4 mM (4.5±0.4%) and at 8 mM (5.4±1.0%) Ca_e^{2+} when compared to Control CM at 4 mM (5.8 ±0.6%) and at 8 mM (6.7±0.9%) Ca_e^{2+} ($p<0.05$) (Fig. 2A). Similarly, the average peak Ca^{2+} transient of CM treated for 5 min with CXCL12 was significantly decreased at 4 mM Ca_e^{2+} (427±40 nM) and at 8 mM (583±97 nM) Ca_e^{2+} when compared to Control CM at 4 mM (601 ±94 nM) and at 8 mM (699±110 nM) Ca_e^{2+} ($p<0.05$) (Fig. 2B). Similar results were obtained after 10 min exposure to CXCL12 (Figs. 2A and B). At 1 mM and 2 mM Ca_e^{2+}, there was no difference in fractional shortening or Ca^{2+} transient between Control and CM exposed for either 5 or 10 min to CXCL12. Interestingly, CM exposed for 60 min to CXCL12 demonstrated no diminution in response to 4 mM and 8 mM Ca_e^{2+} compared to control. This suggested that CXCR4 may be down-regulated, or desensitized in response to prolonged agonist exposure, a common mechanism of chemokine receptor regulation [16] (Figs. 2A and B). To verify that CXCL12 also decreased contractility in response to a physiologically relevant positive inotrope, isoproterenol (ISO), CM were exposed to increasing ISO concentrations. Pretreatment with CXCL12 significantly decreased CM shortening in response to β-receptor stimulation with ISO (Fig. 2C). There was no difference at the lowest dose (0.001 μM) of ISO. These experiments demonstrate that the CXCR4-mediated decrement in contractility is not limited to Ca^{2+} stimulation.

3.3. CXCR4 is present on the surface of CM

To confirm the expression of CXCR4 in adult CM, isolated rat and human CM were plated on slides and then stained with an anti-CXCR4 Ab and analyzed by fluorescent microscopy. As shown in Fig. 3 (top panel), CXCR4 was present on or near the surface of adult rat ventricular CM. The images were captured at various planes (data not shown) and the pattern of expression on the surface appeared linear (arrowheads, inset A) with some clustering of receptors. There was no detectable staining with omission of the CXCR4 Ab (inset B). Significant CXCR4 is detectable on human CM (bottom panel). Human CM appeared to display more localization of CXCR4 antigen at the Z-disks as distinguished by α-actinin staining.

3.4. CXCL12 effects on CM contractility are mediated through CXCR4

The effects of CXCL12 on CM function are mediated by CXCR4. Pre-incubation with the bicyclam AMD3100, a highly selective CXCR4 antagonist [17,18] completely abolished the negative effect of CXCL12 on CM shortening and Ca^{2+} transients (Figs. 4A and B). This suggests that the observed effects on CM are CXCR4 receptor mediated.

3.5. Infection of CM with an adenoviral CXCR4 vector increases its expression

To study the efficacy of adenoviral infection of CXCR4 on CM CXCR4 expression, CM were assessed by fluorescent microscopy at 24 (Fig. 5A) and 48 h (Fig. 5B) after infection with increasing concentrations (MOI) of Ad.CXCR4. CXCR4 was expressed in a concentration-dependent fashion at both 24 and 48 h post-infection. No significant toxicity was noted with

Ad.CXCR4 infection up to 20 MOI. At this dose, there was no difference in the number of rod-shaped CM compared to a sample without infection.

3.6. Overexpression of CXCR4 increases the negative modulation of CM contractility

To further establish that the CXCL12-induced changes in myocyte function are CXCR4 mediated, CM expressing Ad.CXCR4 were studied for their response to CXCL12 exposure. CM expressing Ad.CXCR4 demonstrated accentuated decreases in fractional shortening and Ca^{2+} transients (Figs. 6A and B) in response to CXCL12 when compared to uninfected CM treated with CXCL12 ($p<0.05$). The effects of CXCR4 overexpression on fractional shortening and Ca^{2+} transients were statistically significant at all Ca_e^{2+} concentrations except 4 mM and 8 mM, where it reached near significance ($p=0.06$). The vector itself has been previously demonstrated to have no intrinsic effect on CM contractility [13].

3.7. CXCL12 attenuates cAMP-induced Ca^{2+} channel activation in CM

To study the possible mechanisms by which CXCR4 modulates CM function, we employed whole-cell patch clamp techniques to measure L-type Ca^{2+} channel activity of CM freshly isolated from rat left ventricles. With 1.0 mM $CaCl_2$ in the bath, and a step voltage protocol from a holding potential of −50 mV, the inward current (Fig. 7A) displayed characteristic slow inactivating kinetics, sensitivity to cadmium (Cd) and an I-V plot with a peak inward current near 0 mV (Fig. 7B). These data fit the profile of voltage-gated L-type Ca^{2+} channels.

To determine whether CXCL12 modulates the L-type Ca^{2+} channels, forskolin (FSK), a direct adenylyl cyclase activator, was used to stimulate Ca^{2+} currents. As expected, FSK alone increased Ca^{2+} currents in CM (n=5) by 45.6±4.5% (Fig. 7C, upper panel). To test the effects of CXCL12, in modulating the FSK-induced Ca^{2+} currents, initial FSK exposure was followed by either CXCL12 or diluent (control) prior to a second FSK challenge. Data from a typical experiment where CXCL12 was applied prior to the second FSK challenge are shown in Fig. 7C, lower panel. Treatment with CXCL12 markedly attenuated the Ca^{2+} current increase by FSK (FSK-CXCL12-FSK, 12.8±6.4%, $n=12$, $p<0.01$) compared to control (FSK-CON-FSK, 44.4±7.8%, $n=5$, $p>0.05$) (Fig. 7D).

To determine whether CXCL12 also causes a marked decrement in Ca^{2+} current increase in response to ISO, similar experiments were carried out as described for FSK. ISO, a β-adrenergic receptor (βAR) agonist, signals through the G_s protein pathway resulting in an increase intracellular cAMP through adenylate cyclase activation. Ca^{2+} channel activity in CM was significantly increased by ISO and was fully blocked by Cd (Fig. 8A). ISO alone increased Ca^{2+} currents by 223±42% ($n=7$) whereas pretreatment with CXCL12 (Fig. 8B) markedly attenuated the ISO-induced Ca^{2+} channel activity (68±14% (CXCL12+ISO, $n=11$, $p<0.001$)), as summarized in Fig. 8C.

Of note, CXCL12 (100 ng/ml, 5–6 min) alone did not produce significant changes on the basal inward Ca^{2+} current before and after stimulation (−6.8±5.3%, $n=11$) (data not shown).

4. Discussion

Heart failure is a clinical syndrome characterized by the inability of the heart to produce an adequate cardiac output [19]. A key contributor to the dysfunction may be depressed myocardial contractility [20,21]. Studies that have examined the function of cardiac myocytes (CM) isolated from failing hearts suggest that a defect in CM contractility may contribute to the overall cardiac dysfunction [22]. The responsible agents or mechanisms, however, remain incompletely characterized.

Inflammatory molecules may be agents of myocardial depression in heart failure. The hypothesis that inflammation plays a role in cardiac dysfunction was first suggested from animal [23] and human studies of sepsis (reviewed by van der Poll *et al*. [24]). Sepsis-associated cardiac dysfunction was thought to be mediated, in part, by circulating or "humoral" myocardial depressants that were subsequently identified as predominantly inflammatory cytokines (including tumor necrosis factor-1alpha (TNF-1α), interleukin-1beta, (IL-1β) [25], IL-6 [26], IL-2 [27] and interferon-gamma (IFN-γ)). These ideas were supported by numerous animal [28–30] and by in vitro studies [25,31,32]. However, clinical trials (reviewed by Anker *et al*. [33]), that used "targeted" approaches to neutralize cytokines, such as TNF, were unsuccessful in treating patients with moderate to advanced heart failure.

Chemokines are a distinct class of inflammatory molecules that are also elevated in heart failure. This class of receptors is present on the myocardium and vascular smooth muscle cell types and is capable of mediating important biological processes in a manner that is independent of inflammatory cells [34]. Recently, several studies have demonstrated that circulating chemokine levels, including that of CXCL12, are elevated in animals and humans with cardiac dysfunction [35–37]. Therefore, chemokines have been proposed as mediators of cardiac dysfunction. However, the mechanism of chemokine-mediated cardiac dysfunction is unknown.

We hypothesized that the role of chemokines and their receptors in the cardiac response to inflammation may be quite distinct from that of cytokines' given the wide degree of structural, functional and regulatory differences between the two classes of molecules. Therefore, there is a rationale for assuming that treatment outcomes for cytokine-directed therapies of heart failure may not be predictive of chemokine receptor-directed modalities. Damas *et al.* [6] demonstrated that a variety of chemokine receptors was increased in the failing human myocardium compared to non-failing specimens. Although the CM is the predominant cell type in cardiac tissue, a potential drawback of this study is that the entire ventricle or atrium was processed and used for RNA protection assay analysis so that the relative contribution of each resident cell type to the elevated chemokine receptor levels could not be determined. Seino *et al*. [35] demonstrated that CCL2 (monocyte chemoattractant protein (MCP-1)) RNA levels are detectable in human myocardial biopsies and in TNF-α1-treated neonatal rat CM. These studies postulated that the mechanisms for chemokine effects on myocardial function were predominantly paracrine responses to circulating leukocytes [7,38]. Our study demonstrates that the chemokine CXCL12 can directly depress contractility of the myocardium.

In the present study, normal PM exposed to CXCL12 demonstrated a blunted response to Ca^{2+} stimulation. The concentrations of CXCL12 used in the experiments are similar to those used in measuring the physiologic effects of CXCL12 in a variety of cell types such as epithelial cells [39,40], lymphocytes [41] and neurons [42]. Histological examination of the PM demonstrated a paucity of inflammatory cells. Therefore, it was unlikely that inflammatory cells mediated the effect of CXCL12 on PM contractility. However, because the papillary muscle is composed of heterogeneous cell types, the effects of CXCL12 could not be ascribed to one particular cell type based on studies employing multi-cellular preparations.

The blunted Ca^{2+} response of papillary muscles is explained in part by a direct effect of CXCL12 on CM. CM exposed to CXCL12 demonstrated a significantly decreased shortening in response to both Ca^{2+} and ISO stimulation at higher concentrations. At lower concentrations of Ca^{2+} (1 mM and 2 mM) and ISO (0.001 μM) CXCL12 exposure did not cause decreased CM shortening. This was consistent with the observation that whereas the failing myocardium shows normal baseline function, the contractile reserve is diminished [43]. The lack of CXCL12 effect on CM shortening at normal Ca_e^{2+} and low ISO concentration imply that in patients with normal left ventricular function, CXL12 may not have a direct negative effect on

contractility. However, in conditions such as heart failure where there is sympathetic activation, it is possible that the presence of CXCL12 induces a negative inotropic effect.

CXCL12 imparts its effects on CM through interaction with its exclusive receptor CXCR4. A highly specific CXCR4 antagonist, AMD3100, abrogated the depressed contractile response to Ca^{2+}, and overexpression of CXCR4 by adenoviral infection of CM depressed the contractile response to Ca^{2+} treatment even further in the presence of CXCL12. Ligand-induced receptor internalization is a general regulatory mechanism in chemokine signaling. During Ca^{2+} stimulation, we observed that CM returned to normal contractile response after prolonged CXCL12 exposure, which is consistent with receptor desensitization. These observations demonstrate that the effect of CXCL12 is mediated through its interaction with CXCR4.

Immunofluorescent microscopy showed that there was a significant amount of CXCR4 on or near the surface of human and rat adult CM. The expression appeared to be linear and the receptors in certain sections appeared to be clustered, a finding consistent with reports of CXCR4 expression in other cell types [44]. Further studies are ongoing to determine the factors that regulate CXCR4 surface expression on CM. The immunohistochemical studies of CXCR4 on human CM demonstrated that CXCR4 expression on CM is not limited to murine species and therefore may be relevant to human diseases.

To determine the possible mechanisms by which CXCR4 activation decreases myocardial function, we examined Ca^{2+} mobilization within CM when exposed to CXCL12. During Ca^{2+} stimulation peak, Ca^{2+} transients were decreased in CM exposed to CXCL12 *at supraphysiologic concentrations of* Ca_e^{2+} *(4–8 mM), but not at basal* Ca_e^{2+} *levels (1–2 mM).* CXCL12 may modulate Ca^{2+} transients by altering L-type Ca^{2+} channel activity in response *to either calcium or an adrenergic challenge.* Patch clamp studies measuring L-type Ca^{2+} channel activity of individual CM demonstrated that CXCL12 decreased Ca^{2+} currents induced by either β-adrenergic with ISO or FSK stimulation *but CXCR4 activation did not have a significant effect on basal activity of the* Ca^{2+} *currents.* Together, these data suggested both an upstream effect of CXCR4 on L-type Ca^{2+} channels as well as a downstream effect that appears to be unrelated to β-adrenergic receptor stimulation. This is consistent with the findings of others wherein treatment of colonic epithelial cells with CXCL12 inhibited cAMP response only in the presence of FSK stimulation [45]. It appears that an important function of CXCR4 is to modulate cAMP-mediated events. In CM, this may have profound physiologic implications such as the modulation of contractility as demonstrated in the present studies.

A novel and exciting potential methodology to reverse myocardial remodeling associated with myocardial infarction [46] is regeneration of myocardium using bone marrow (BM)-derived mesenchymal cells, endothelial progenitor cells and endogenous cells [47–50]. Given its essential role in BM homing and recruitment [49,51], CXCL12 is a candidate to promote stem cells recruitment to the heart. Because CXCR4 may negatively impact myocardial function, strategies [52] that primarily employ up-regulation of CXCL12 may need to be examined more closely. Our results would caution that a therapeutic approach whereby myocardial CXCL12 is increased, in an effort to recruit circulating or endogenous stem cells in the setting of an ischemic injury, may result in a significant decrease in contractility.

In this study we have shown, for the first time to our knowledge, that CXCR4 is functional on CM. CXCR4 activation can directly inhibit myocardial contractile response to increases in Ca^{2+} and ISO concentrations through modulation of L-type Ca^{2+} channel activity. Manipulation of CXCR4 activity may serve as a new target in the treatment of cardiac dysfunction. Additionally, the discovery of functional chemokine receptors on CM may have broad implications on preexisting chemokine/chemokine receptor based stem cell therapies for cardiomyopathies.

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Fig 1.

Activation of CXCR4 blunts PM response to Ca^{2+}. Murine PM were subjected to two graded stepwise Ca^{2+} challenges separated by wash-out. PM exposed to diluent before the second Ca^{2+} challenge demonstrate similar responses when compared to the first challenge. (A) PM exposed to CXCL12 (125 ng/ml) demonstrated a blunted response during the second Ca^{2+} challenge (B).

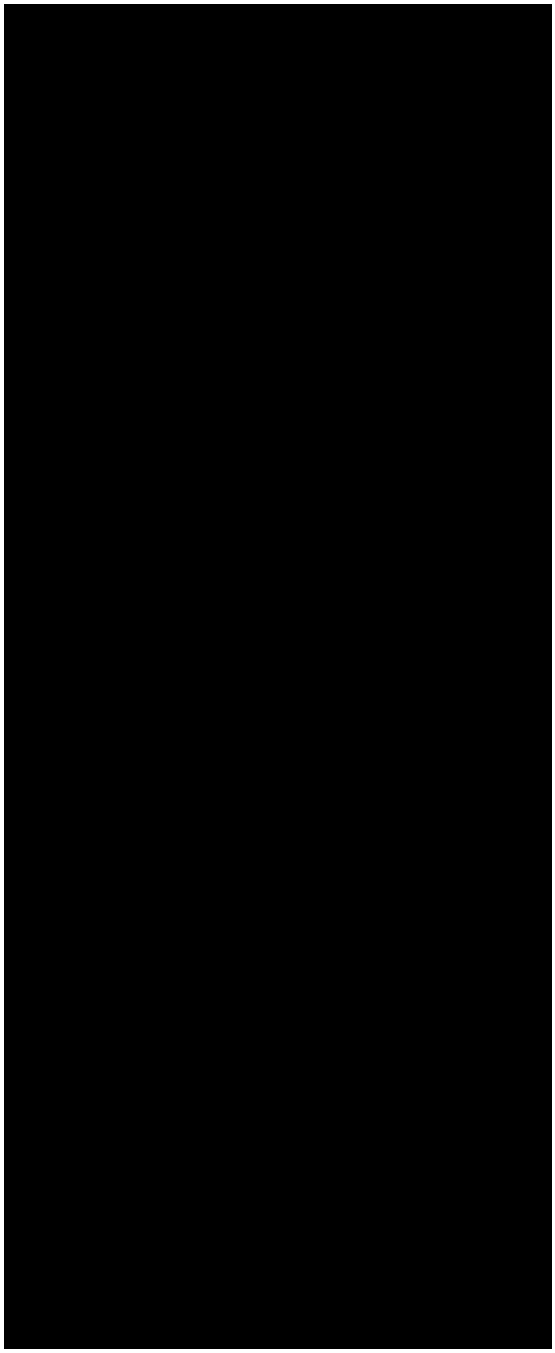

Fig 2.

Activation of CXCR4 decreases CM contractility. Adult rat CM were exposed to CXCL12 (125 ng/ml) for either 5, 10 or 60 min prior to a stepwise Ca^{2+} challenge (A, B) or to increasing ISO concentrations after a 5-min exposure to CXCL12 (C). In both experiments, control CM were exposed to diluent only. The average percent increase in shortening (A) and average peak Ca^{2+} transients (B) during the Ca^{2+} challenge were significantly lower in CM exposed for either 5 or 10 min to CXCL12. No difference in shortening or Ca^{2+} transient was detected in CM exposed for 60 min to CXCL12 (A and B). Percent increase in fractional shortening in the CM is shown as a function of increasing [ISO] (0.001–10 μM) (C). CXCL12 exposed CM

demonstrated a significant decrease in shortening response. ISO experiments were performed in triplicate.

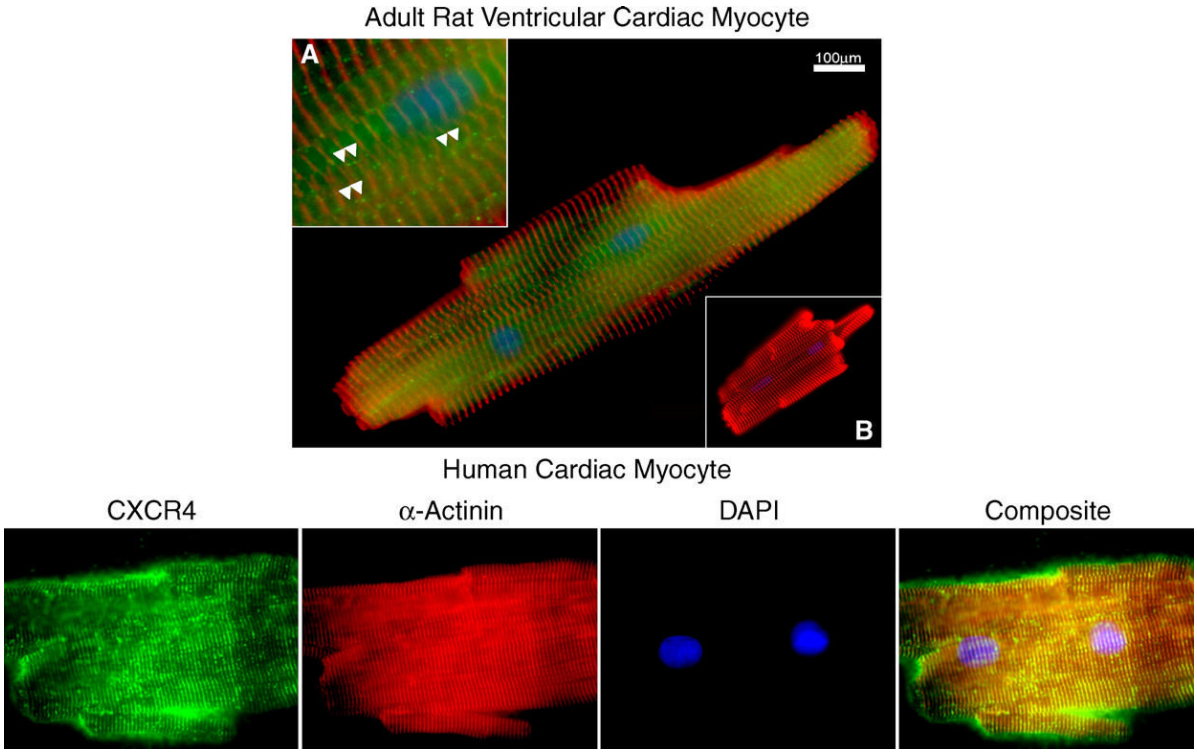

Fig 3.

CXCR4 is present on adult rat CM. Alpha-actinin (red), CXCR4 chemokine receptor (green) and DAPI-stained nuclei (blue) were visualized in rodent and human myocytes. A single rat CM was stained one h after isolation with a polyclonal anti-rat CXCR4 Ab (green) and an anti-α-actinin Ab (red) and examined by fluorescent microscopy (top panel). Higher magnification (inset A) reveals a punctate distribution of CXCR4 on the surface (arrowheads). The CXCR4 Ab was omitted and only the fluoresceinated anti-rabbit-Cy2 Ab was applied (inset B). Normal human cardiac myocyte also demonstrate a similar pattern of CXCR4 chemokine receptor staining (bottom panel).

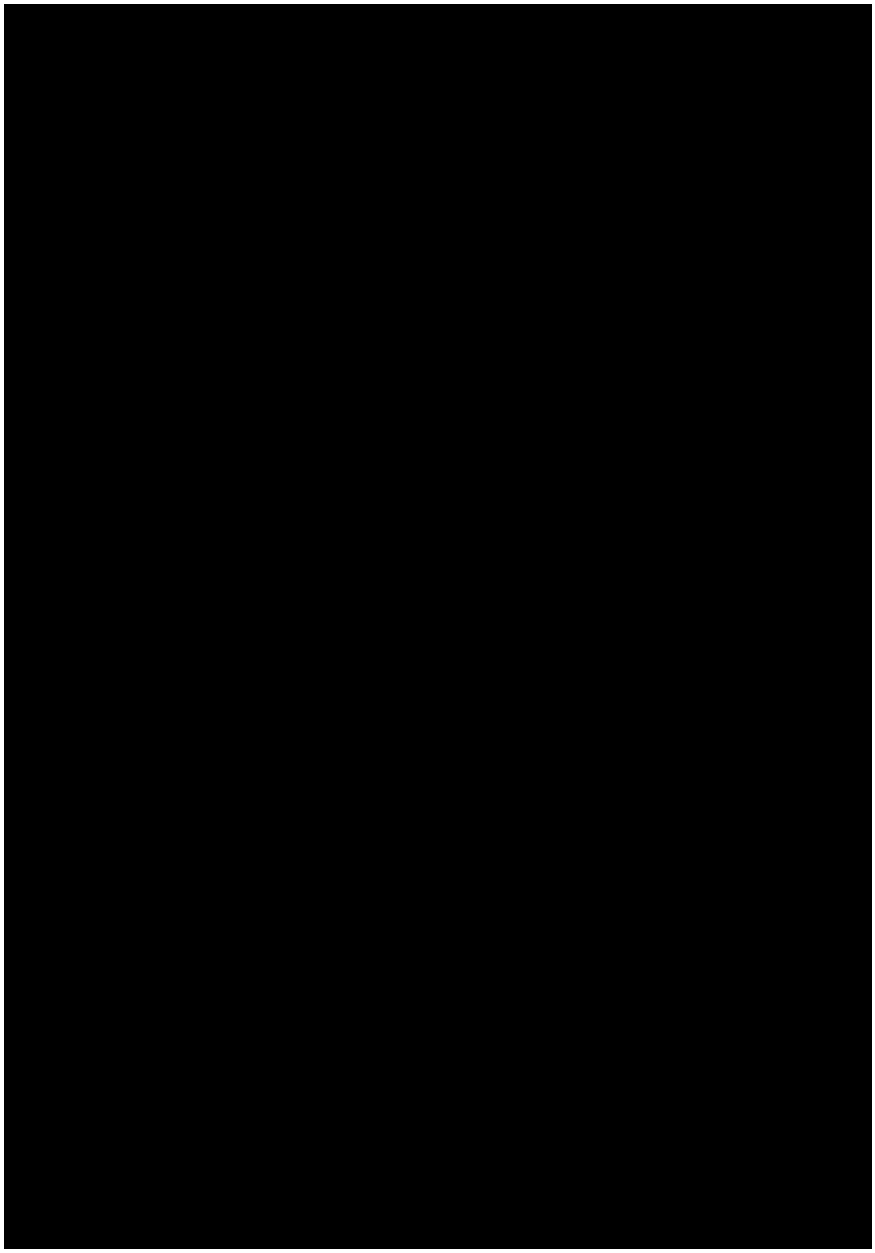

Fig 4.

Effects of CXCL12 on CM are mediated through CXCR4. Adult rat CM were pretreated with AMD3100 for 1 h and CXCL12 (125 ng/ml) for 10 min prior to a graded stepwise Ca^{2+} challenge. A representative tracing for fractional shortening (A) and peak Ca^{2+} transients during stimulation (B) are presented. Experiments were performed in triplicate.

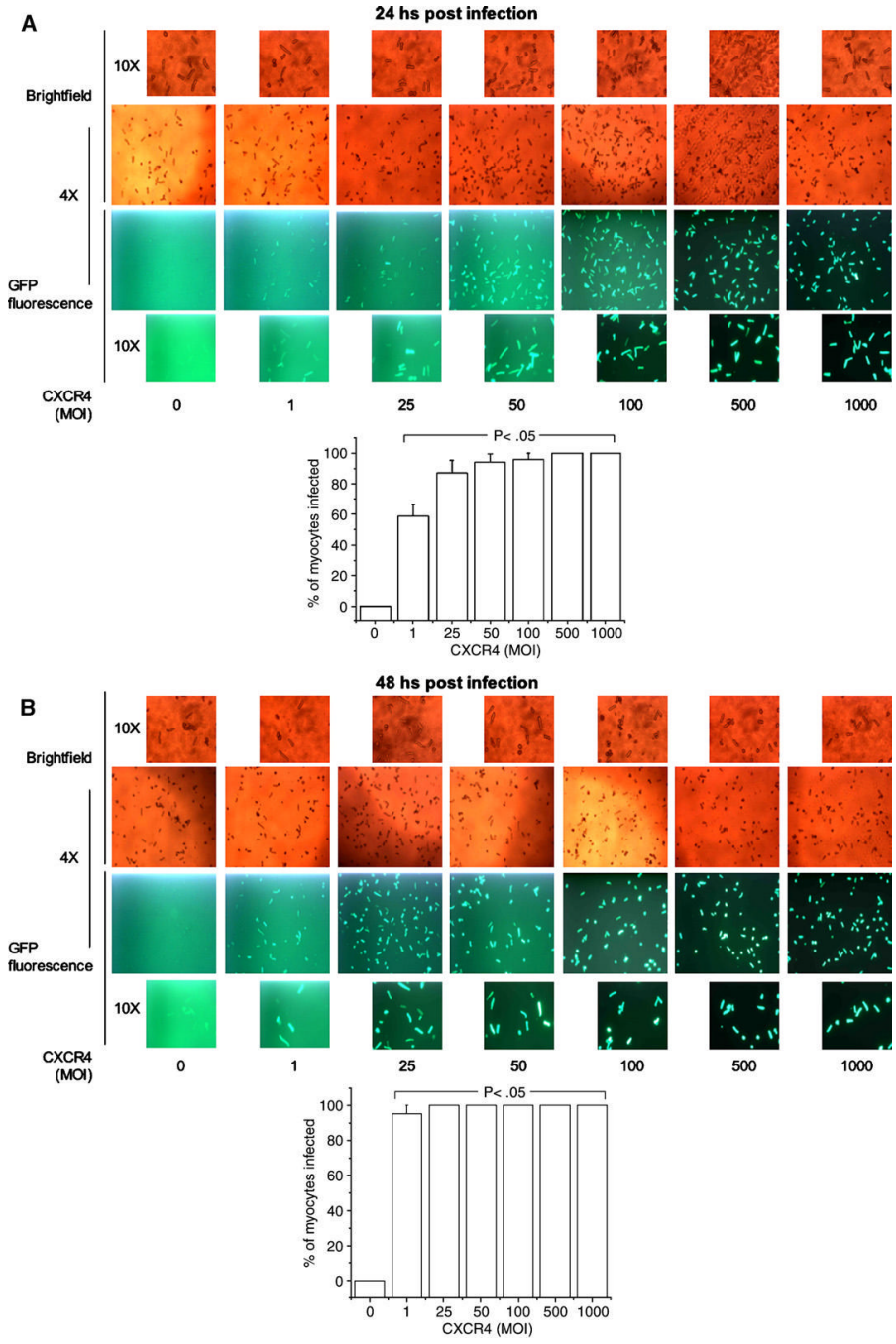

Fig 5.

Adenoviral infection significantly increases expression of CXCR4 on rat CM. Adult rat CM were visualized to detect GFP fluorescence 24 (A) or 48 h (B) after infection with Ad.CXCR4. Co-expression of GFP demonstrates visually that CXCR4 gene is being expressed in the cells. A dose–response infection was performed in order to show significant increase in CXCR4 expression with increased MOI. The percentage of infected CM was determined as the number of GFP positives cells against the total cells counted in the bright-field. Different magnifications were used for optimal visualization of the cells. The bar graphs show that there was a significant increase in CXCR4-infected CM even at the lowest MOI.

Fig 6.

Overexpression of CXCR4 decreases CM contractility. Both CM infected with Ad.CXCR4 and control uninfected CM were exposed to CXCL12 (125 ng/ml) and then subjected to a graded stepwise Ca^{2+} challenge. The average percent shortening (A) at 37 °C at 1 Hz and average peak Ca^{2+} transients (B) at increasing external Ca^{2+} are presented. *p<0.05 vs. uninfected control in the presence of CXCL12.

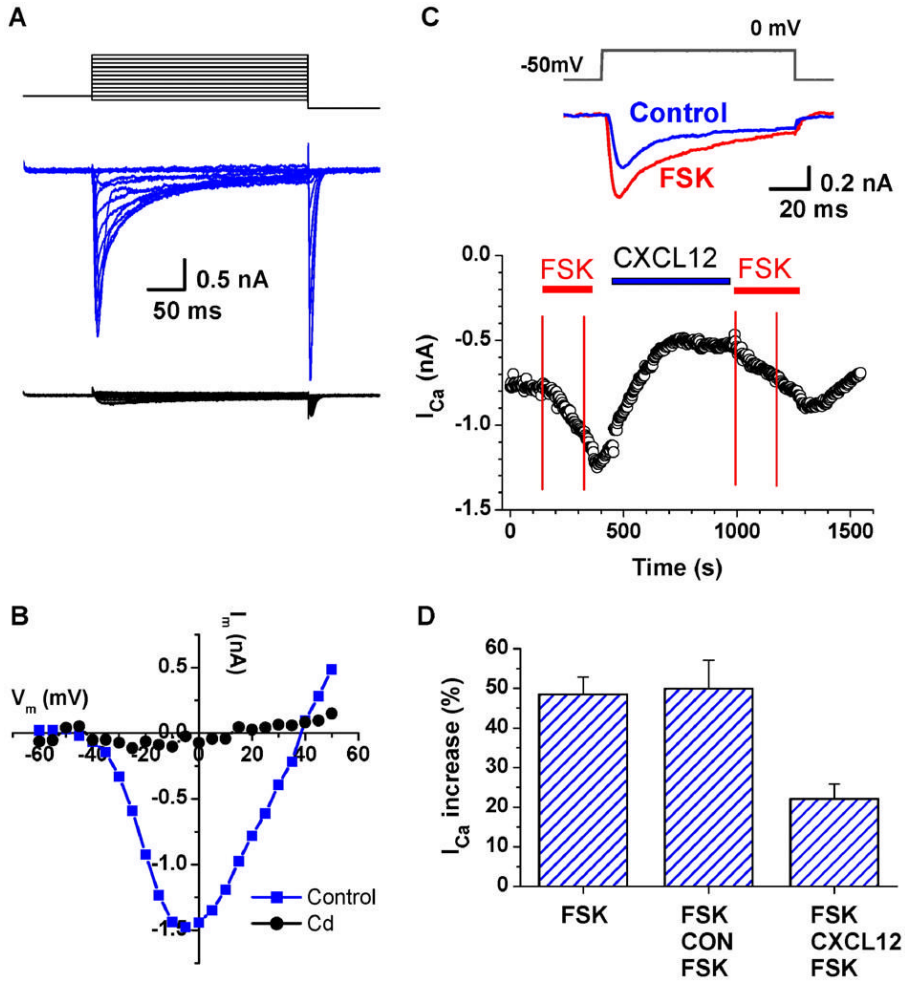

Fig 7.

CXCL12 modulates forskolin (FSK)-induced Ca^{2+} channel activation in CM. Whole-cell recording of inward Ca^{2+} currents from isolated rat ventricular CM with 1 mM $CaCl_2$ in the bath solution. L-type Ca^{2+} currents were activated with the step voltage clamp protocol shown in the top panel. Currents were mostly blocked by Cd (0.1 mM, lower panel in red) (A). I-V plot of the L-type Ca^{2+} current in control conditions (blue) and in 0.1 mM Cd (red) (B). Time course of Ca^{2+} current in response to the application of FSK (1 μm) and CXCL12 (100 ng/ml). The top panel illustrates the voltage protocol and typical current traces under each condition. The inward Ca^{2+} currents were activated by a repeating voltage step from −50 mV to 0 mV. Inward current values were measured at the inward peak. The bottom panel illustrates the relative changes of peak currents during 3-min applications of FSK (1 μM) indicated by the red horizontal lines. CM were exposed for approximately 5 min to either CXCL12 (FSK-CXCL12-FSK) or diluent (FSK-CON-FSK), as indicated by the blue horizontal line, before the second FSK application (C). Summary of the CXCL12 attenuation of the FSK-induced Ca^{2+} current stimulations is shown (*n*=5) (D).

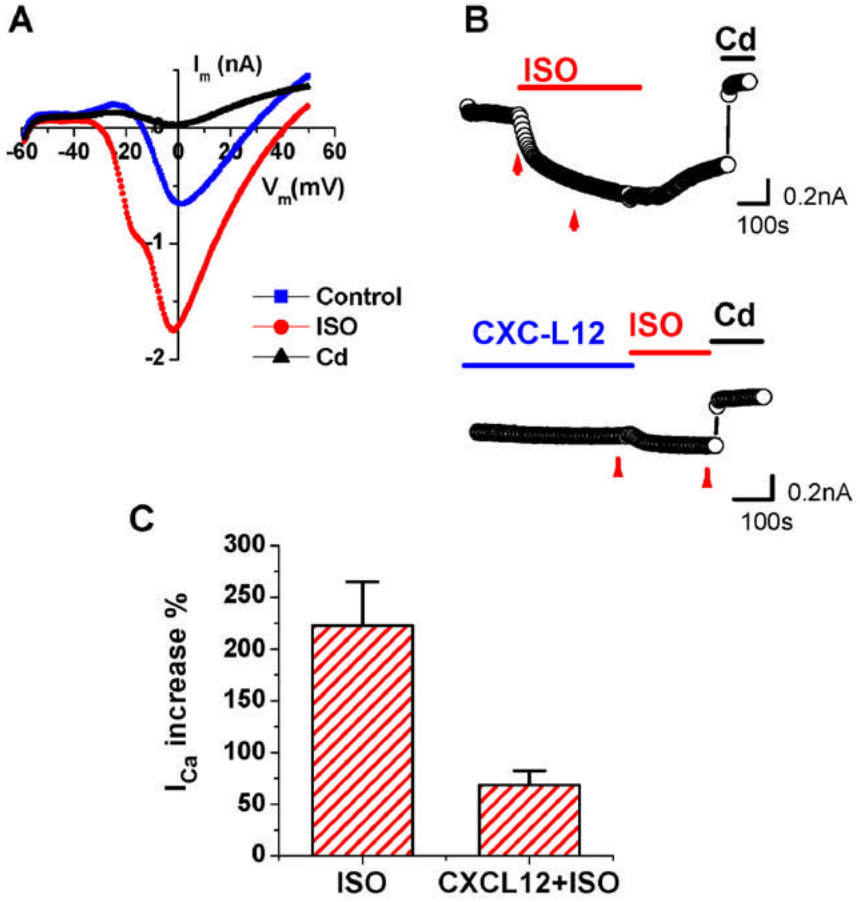

Fig 8.

CXCL12 modulates β-adrenergic Ca^{2+} channel activation in CM. Whole-cell inward Ca^{2+} currents were recorded from isolated adult rat ventricular CM with 1 mM Ca^{2+} in the bath solutions. Ca^{2+} currents were activated with a protocol ramping from −50 mV to +50 mV from a holding potential of −60 mV. I-V plots of Ca^{2+} currents against membrane potentials recorded in control condition (blue), ISO (1 μM, red) and in CdCl2 (0.1 mM, black) (A). Representative time course record of the Ca^{2+} current from CM treated with ISO alone (1 μM, top panel), or pre-incubated with CXCL12 (100 ng/ml) for 5 min prior to ISO treatment (lower panel). Applications of ISO, (1 μM), CXCL12 (100 ng/ml) and $CdCl_2$ (0.1 mM) are indicated by the bars. Data values for analysis were taken from the point before ISO and 200 s after ISO application, as indicated by the arrows in each panel B. Summary of the effects of CXCL12 on ISO-induced Ca^{2+} current increases (n=11) (C).