

G Protein-Coupled Receptor Kinase 3 and Protein Kinase C Phosphorylate the Distal C-Terminal Tail of the Chemokine Receptor CXCR4 and Mediate Recruitment of β -Arrestin*

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Abbreviations: AU, arbitrary units; β_2 AR, β_2 -adrenergic receptor; Bis I, Bisindolylmaleimide I; BRET, Bioluminescence Resonance Energy Transfer; BSA, bovine serum albumin; CK, casein kinase; CXCR, CXC chemokine receptor; DMEM, Delbecco's modified Eagle's medium; FBS, fetal bovine serum; G_i , $G\alpha_i\beta\gamma$ heterotrimer; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; GSK3, glycogen synthase kinase 3; ICL, intracellular loop; PBS, phosphate buffered saline; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; SDS, sodium dodecyl sulfate; TBS-T, Tris-buffered saline with 0.05% Tween-20

Abstract

Phosphorylation of G protein-coupled receptors (GPCRs) is a key event for cell signaling and regulation of receptor function. Previously, using tandem mass spectrometry, we identified two phosphorylation sites at the distal C-terminal tail of the chemokine receptor CXCR4, but were unable to determine which specific residues were phosphorylated. Here, we demonstrate that serines 346 and/or 347 (Ser-346/7) of CXCR4 are phosphorylated upon stimulation with the agonist CXCL12 as well as a CXCR4 pepducin, ATI-2341. ATI-2341, a Gi-biased CXCR4 agonist, induced more robust phosphorylation of Ser-346/7 compared to CXCL12. Knockdown of GRK2, GRK3 or GRK6 reduced CXCL12-induced phosphorylation of Ser-346/7 with GRK3 knockdown having the strongest effect, while inhibition of the conventional PKC isoforms, particularly PKC α , reduced phosphorylation of Ser-346/7 induced by either CXCL12 or ATI-2341. The loss of GRK3- or PKC-mediated phosphorylation of Ser-346/7 impaired the recruitment of β -arrestin to CXCR4. We also found that a pseudo-substrate peptide inhibitor for PKC ζ effectively inhibited CXCR4 phosphorylation and signaling, most likely by functioning as a non-specific CXCR4 antagonist. Together, these studies demonstrate the role Ser-346/7 plays in arrestin recruitment and initiation of receptor desensitization and provide insight into the dysregulation of CXCR4 observed in patients with various forms of WHIM syndrome.

Introduction

The chemokine receptor CXCR4 is critical for embryonic germ cell development, hematopoiesis, and directed chemotaxis [Ara et al., 2003; Doitsidou et al., 2002; Nagasawa et al., 1996] and is involved in the progression of a number of diseases. For example, CXCR4 acts as a co-receptor for T-cell tropic isolates of human immunodeficiency virus [Feng et al., 1996], mutations that either truncate or mutate the C-terminal tail of CXCR4 lead to a rare immunodeficiency characterized by *warts*, *hypogammaglobulinemia*, recurrent bacterial *infection*, and *myelokathexis* known as WHIM syndrome [Diaz & Gulino, 2005, Liu et al., 2012], and dysregulation of CXCR4 can lead to cancer progression and metastases [Muller et al., 2001, Busillo & Benovic, 2007].

Upon activation, G protein-coupled receptors (GPCRs) are rapidly phosphorylated, typically by members of the GPCR kinase (GRK) family [Krupnick & Benovic, 1998; Pitcher et al., 1998]. This triggers the recruitment of arrestins, which prevents further activation of their cognate G protein and leads to receptor desensitization, internalization and initiation of arrestin-dependent signaling [DeWire et al., 2007; Moore et al., 2007]. Recent studies have shown that GPCRs can be phosphorylated by multiple kinases and that individual kinases are often able to phosphorylate multiple sites [Tobin et al., 2008; Busillo et al., 2010; Doll et al., 2011; Nobles et al., 2011]. The C-terminal tail of CXCR4 contains 18 potential phosphorylation sites; 15 serines and 3 threonines. The agonist CXCL12 was previously shown to promote phosphorylation of Ser-339 in CXCR4 while phorbol esters and epidermal growth factor stimulation also led to phosphorylation of Ser-339, suggesting that it was a substrate for protein kinase C (PKC) [Woerner et al., 2005]. Using a combination of liquid chromatography tandem mass spectrometry (LC/MS/MS) and phospho-specific antibodies, we identified seven serine residues

that were phosphorylated following CXCL12 stimulation: Ser-321, Ser-324, Ser-325, Ser-330, Ser-339, a residue between Ser-346 and Ser-348, and either Ser-351 or Ser-352 [Busillo et al., 2010]. Moreover, we showed that Ser-330 and Ser-339 were phosphorylated by GRK6, while Ser-324/5 was primarily phosphorylated by PKC and GRK6 [Busillo et al., 2010]. Recently, Ser-346/7 was shown to be rapidly phosphorylated by GRK2 and GRK3 following CXCL12 stimulation [Mueller et al., 2013]. Moreover, phosphorylation of Ser-346/7 may be required for phosphorylation of Ser-324/5 and Ser-330 [Mueller et al., 2013]. In addition to the studies identifying specific phosphorylation sites, several studies have implicated GRK2 [Orsini et al., 1999; Jiminez-Sainz et al., 2006; Busillo et al., 2010], GRK3 [Balabanian et al., 2008; Tarrant et al., 2013; Diaz & Gulino, 2005; Busillo et al., 2010; Liu et al., 2012], GRK6 [Fong et al., 2002; McCormick et al., 2009], and PKC [Signoret et al., 1997; Orsini et al., 1999] as being involved in regulating CXCR4 activity and signaling.

Multiple kinases play a critical role in phosphorylating and/or regulating CXCR4 following activation. While there is some data to suggest specific roles for GRKs as mentioned above, the PKC isoforms that are involved in the phosphorylation and regulation of CXCR4 are still unclear. Furthermore, how site-specific phosphorylation is linked with the various downstream signaling pathways and cellular functions of CXCR4 still needs to be better defined. Agonist-promoted phosphorylation of GPCRs initiates the recruitment and high-affinity binding of arrestins [Shenoy & Lefkowitz, 2005]. In this regard, deletion of GRK6 and β -arrestin2 enhances CXCR4 function in mice [Fong et al., 2002] while knockdown of GRK6 and β -arrestin2 significantly enhances calcium mobilization following CXCR4 activation [Busillo et al., 2010]. It has also been shown that there is impaired recruitment of GRK6 and β -arrestin2 to a mutant CXCR4 that is associated with WHIM syndrome [McCormick et al., 2009] while overexpression

of β -arrestin2 was unable to rescue internalization of CXCR4 lacking the last 15 amino acids [Balabanian et al., 2008]. On the other hand, loss of GRK3, GRK6 or β -arrestin1 reduces ERK activation [Busillo et al., 2010], suggesting that receptor phosphorylation can also play a positive role in signal transduction following activation of CXCR4.

Together, these data suggest that GRK- and PKC-mediated phosphorylation of CXCR4 results in the differential recruitment of arrestins to promote desensitization and G protein-independent signaling [Fong et al., 2002; Busillo et al., 2010; Doll et al., 2011]. Previously, we were unable to determine the kinase(s) that mediated phosphorylation of the distal tail of CXCR4 [Busillo et al., 2010]. In this study, we sought to determine the kinase(s) responsible for phosphorylation of Ser-346 to Ser-348 and how site-specific phosphorylation of these residues affects the recruitment of β -arrestin. We show that GRK3- and PKC-mediated phosphorylation of Ser-346/7 contributes to the recruitment of β -arrestin2.

Materials and Methods

Materials. HEK293 cells were from Microbix Biosystems, Inc (Toronto, Canada) and Lipofectamine 2000 and Opti-MEM were from Invitrogen (Carlsbad, CA). Anti-GRK3 polyclonal rabbit antibodies and anti-PKC α and PKC β monoclonal mouse antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-CXCR4 monoclonal antibodies and anti- β -arrestin monoclonal antibodies were from BD Biosciences Pharmingen (San Diego, CA). Anti-GRK4-6 and anti-GRK2/3 monoclonal antibodies were from Millipore (Billerica, MA). GRK2 monoclonal and anti-CXCR4 phospho-serine 324/5 (pS324/5) antibodies were produced in our laboratory [Busillo et al., 2010], while anti-CXCR4 pS346/7 polyclonal antibody was previously reported and validated using a CXCR4-S346-348A mutant [Mueller et al., 2013]. Anti-flag-tagged monoclonal and polyclonal antibodies and anti- α -tubulin monoclonal antibodies were from Sigma. Anti-PKC ζ polyclonal rabbit antibodies were from Cell Signaling (Danvers, MA). The CXCR4 pepducin ATI-2341 was from Anchor Therapeutics (Boston, MA) or was synthesized by Peptide 2.0 (Chantilly, VA). On-TARGETplus siRNA reagents were from GE Dharmacon (Lafayette, CO).

Cell Culture and Transfection. HEK293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 25 mM HEPES, pH 7.2, and 0.1 mM nonessential amino acids in a 5% CO₂ incubator at 37°C. Cells stably expressing human CXCR4 were selected and maintained in complete DMEM supplemented with 0.8 mg/ml G418 and penicillin/streptomycin. For transfection of DNA or siRNAs, HEK293 cells were grown to ~70% confluency in 100-mm dishes and transfected with the indicated amount of DNA or 600 pmol of ON-TARGETplus siRNA using Lipofectamine 2000 in Opti-MEM. 24 hr later, cells were split into six-well plates for assay the next day.

Detection of CXCR4 Phosphorylation in HEK293 Cells Stably Expressing Flag-Tagged CXCR4 by Immunoblotting. Cells were plated into 12 well plates and cultured for 24 hr. Cells were then starved with serum-free DMEM for 4 hr prior to stimulation. Cells were then washed twice with ice-cold phosphate-buffered saline (PBS) on ice to stop the reaction, and lysed with buffer (20 mM HEPES, pH 7.2, 10 mM EDTA, 150 mM NaCl, 1% Triton X-100, one tablet each of mini-Complete protease inhibitor and PhosSTOP phosphatase inhibitor [Roche, Indianapolis, IN] per 10 ml) at 4°C on a rocker for 30 min. The lysates were cleared by centrifugation at 14,000 rpm in an Eppendorf centrifuge for 20 min at 4°C. For assays with kinase inhibitors, cells were pretreated with the indicated inhibitor or vehicle for 20 min prior to stimulation. Total protein in cell lysates was measured with Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). An equal amount of total protein was electrophoresed on a 10% SDS polyacrylamide gel, transferred to nitrocellulose, and immunoblotted with the following primary antibodies overnight at 4 °C: a monoclonal anti-CXCR4 (1:1000), a polyclonal anti-pS346/7-CXCR4 (1:500), a polyclonal anti-pS324/5-CXCR4 (1:1000), a polyclonal anti-GRK3 (1:500), a monoclonal anti-GRK4-6 (1:3000), a monoclonal anti-PKC α (1:1000), a monoclonal anti-PKC β (1:200), a polyclonal anti-PKC ζ (1:1000) or a monoclonal anti- β -arrestin1 (1:1000, detects both β -arrestin1 and 2). Blots were washed extensively with TBS-T (Tris-buffered saline plus 0.05% Tween 20), incubated with a horseradish peroxidase-labeled secondary antibody, washed with TBS-T, and proteins were detected using chemiluminescence. The blots were then stripped and reprobed using an anti-tubulin (1:7500) monoclonal antibody. Western-blot images were visualized and quantified using a LI-COR C-DiGit Blot Scanner using Image Studio Software (LI-COR, Lincoln, NE).

Bioluminescence Resonance Energy Transfer (BRET) Assay. For BRET studies, HEK293 cells were split into six-well plates 24 hr before transfection. Transient transfections were performed using Lipofectamine 2000. CXCR4-RLucII (10 ng) was co-transfected with GFP10- β -arrestin2 (100 ng). The total amount of DNA transfected in each well was adjusted to 2 μ g with pcDNA3. After overnight incubation, transfected cells were detached and seeded into a 96-well white plate (50,000 cells/well) that was pretreated with poly-L-ornithine hydrobromide (Sigma). The cells were then incubated with phenol red-free complete DMEM for an additional 24 hr before being tested. For BRET measurements, cells were washed once with 200 μ l of PBS plus glucose (Life Technologies, Grand Island, NY) and Deep Blue C (coelenterazine 400A) was added (final concentration of 2.5 μ M in PBS) 5 min before reading. Readings were collected using an Infinite F500 multidetector plate reader (Tecan, Mannedorf, Switzerland). The BRET signal was determined by calculating the ratio of the light intensity emitted by the GFP10- β -arrestin2 over the light intensity emitted by CXCR4-RLucII. The values were corrected by subtracting the background BRET signal detected when the CXCR4-RLucII construct was expressed alone. CXCL12 was added just before reading the BRET signal.

Measurement of Intracellular Calcium Mobilization. Calcium mobilization was performed as previously described [Iwata et al., 2005] with slight modification. In brief, HEK293 cells that stably express CXCR4 were harvested, washed twice with PBS, and resuspended at 5×10^6 cells/ml in Hanks' balanced salt solution (140 mM NaCl, 5 mM KCl, 10 mM HEPES, pH 7.4, 1 mM CaCl₂, 1 mM MgCl₂, and 1 mg/ml glucose) (Invitrogen) containing 0.025% bovine serum albumin (Hanks-BSA). The cells were then loaded with 2 μ M Fura-2 acetoxymethyl ester derivative (Fura-2/AM; Invitrogen, Carlsbad, CA) for 30 min at 37°C. The cells were washed once in Hanks' solution, resuspended in Hanks-BSA, incubated at room

temperature for 15 min, and washed twice in Hanks' solution. The cells were then resuspended in Hanks' at a concentration of 3×10^7 cells/ml. To measure calcium mobilization, 1.5×10^6 cells/1.6 ml in a quartz cuvette were pre-incubated with the designated concentrations of PKC ζ inhibitor for one min and then stimulated with the indicated agonists. Calcium mobilization was measured using excitation at 340 and 380 nm and emission at 510 nm in a fluorescence spectrometer (LS55; PerkinElmer Life and Analytical Sciences, Waltham, MA). Calibration was performed using 0.1% Triton X-100 for total fluorophore release and 15 mM EGTA to chelate free calcium. Intracellular calcium concentrations were calculated using a fluorescence spectrometer measurement program.

Statistics. Western-blot were quantified on a LI-COR C-DiGit scanner. Data are shown as the mean \pm SEM with p values determined by comparing the data from experimental versus control from at least three independent experiments using a two-tailed paired t test in Excel. Where noted, n equals the number of independent experiments that were performed.

Results

Phosphorylation of serine 346/7 following CXCL12 stimulation. Phosphorylation of GPCRs is a key regulatory event following agonist activation. The C-terminal tail of CXCR4 (Fig. 1A) contains 15 serine and 3 threonine residues, and at least 7 residues are phosphorylated following receptor activation [Busillo et al., 2010]. Truncation and alanine scanning mutagenesis indicated that a number of potential phosphorylation sites are important for receptor desensitization, internalization and degradation [Orsini et al., 1999; Marchese & Benovic, 2001]. Our previous mass spectrometry data suggested that one serine residue between Ser-346 and Ser-348 was phosphorylated following CXCL12 stimulation [Busillo et al., 2010], while recent data suggested that Ser-346/7 was rapidly phosphorylated following CXCL12 stimulation [Mueller et al., 2013]. Therefore, we sought to further characterize the CXCL12-promoted phosphorylation of CXCR4 in this cluster of serine residues (Ser-346 to Ser-348) in cells that stably express FLAG-tagged CXCR4. HEK293 cells stably expressing CXCR4 were stimulated with 50 nM CXCL12 for various times up to 60 min, and cell lysates were probed with pS346/7 antibodies to assess the kinetics of phosphorylation (Fig. 1B). Phosphorylation of Ser-346/7 was detected as early as 2 min, peaked by 5 min, and was returning to baseline levels by 60 min (Fig. 1B). These data demonstrate that Ser-346/7 is rapidly phosphorylated following receptor activation by CXCL12, and suggest that phosphorylation of these residues likely has an important function in receptor regulation.

Serine 346/7 is phosphorylated by GRKs and PKC. The distal tail of CXCR4 has been shown to be critical for arrestin recruitment following CXCR4 activation [Balabanian et al., 2008; McCormick et al., 2009; Busillo et al., 2010]. Our previous results suggested that either GRK2 or GRK3 could be responsible for phosphorylation of the distal tail of CXCR4 [Busillo et

al., 2010]. Indeed, a recent report suggested that Ser-346/7 is phosphorylated by GRK2/3, although siRNA-mediated knockdown of GRK2/3 did not completely eliminate the phosphorylation [Mueller et al., 2013]. Therefore, we wanted to determine the contribution of other kinases in Ser-346/7 phosphorylation following CXCL12 stimulation. Endogenous GRK2, GRK3 and GRK6 in HEK293 cells stably transfected with FLAG-CXCR4 were knocked down using GRK-selective siRNAs (Fig. 1C), and Ser-346/7 phosphorylation triggered by the agonist CXCL12 was detected using a site-specific phospho-antibody. To enhance our ability to inhibit agonist activated phosphorylation, cells were stimulated with a relatively low concentration of CXCL12 (5 nM). Phosphorylation of Ser-346/7 was reduced by ~50% at 5 min following knockdown of GRK3 as compared to control siRNA ($p < 0.01$), while knockdown of GRK2 or GRK6 had lesser effects (Fig. 1D). Knocking down GRK2 and GRK3 together did not further decrease phosphorylation of Ser-346/7 (Fig. 1D). In contrast, phosphorylation of Ser-346/7 following knockdown of GRK2, GRK3, or GRK6 was not significantly reduced at 15 min (Fig. 1D). These results suggest that while GRK2, GRK3 and GRK6 may be involved in the phosphorylation of Ser-346/7 following stimulation with CXCL12, GRK3 is the primary GRK involved in HEK293 cells.

The phosphorylation of GPCRs is often mediated by multiple kinases [Tobin et al., 2008]. Indeed, our data indicate that several GRKs are involved in the phosphorylation of Ser-346/7. However, agonist-promoted phosphorylation is still evident following GRK knockdown, suggesting that other kinases might also be involved (Fig. 1D). PKCs are ubiquitously expressed in mammalian cells and phosphorylate many proteins, including GPCRs [Newton, 1995; Orsini et al., 1999]. Using a broad spectrum PKC inhibitor (Bisindolylmaleimide I [Bis I]), we previously found that multiple PKCs contributed to phosphorylation of Ser-324/5 [Busillo et al.,

2010]. To determine if PKC isoforms play a role in the phosphorylation of Ser-346/7, HEK293 cells stably expressing FLAG-CXCR4 were pre-treated with Bis I (2.5 μ M) for 30 min prior to stimulation with CXCL12. Inhibition of PKC significantly reduced the basal phosphorylation of Ser-346/7 as well as CXCL12-promoted phosphorylation at 5 and 30 min (Fig. 1E). Thus, our results suggest that both GRK3 (Fig. 1D) and PKC (Fig. 1E) contribute to phosphorylation of Ser-346/7. We next evaluated the combined effect of GRK3 knockdown and PKC inhibition on Ser-346/7 phosphorylation. GRK3 knockdown or inhibition of PKC each reduced phosphorylation of Ser-346/7 by ~60% at 5 min while combined knockdown of GRK3 and PKC inhibition reduced CXCL12-promoted phosphorylation of Ser-346/7 by >80% at 5 min (Fig. 1F). These data demonstrate that both GRK3 and PKC contribute to Ser-346/7 phosphorylation following stimulation with CXCL12.

The pepducin ATI-2341 promotes phosphorylation of Ser346/7. Pepducins are a class of molecules that are derived from the intracellular loops (ICL) of GPCRs [Carr & Benovic, 2016]. ATI-2341 is a pepducin derived from ICL1 of CXCR4 that functions as a Gi-biased agonist to promote G protein-dependent signaling downstream of CXCR4 [Tchernychev et al., 2010; Quoyer et al., 2013]. Furthermore, ATI-2341 has been shown to promote the phosphorylation of Ser-324/5 that is dependent on PKC but not GRK6 [Quoyer et al., 2013] and also induce CXCR4 internalization [Tchernychev et al., 2010]. To determine if ATI-2341 can also promote the phosphorylation of Ser-346/7, HEK293 cells stably expressing CXCR4 were stimulated with ATI-2341 (3 μ M) and phosphorylation of Ser-346/7 was detected. Surprisingly, ATI-2341 induced more robust and faster phosphorylation of Ser-346/7 than CXCL12 (compare Fig. 2A with Fig. 1B). PKC inhibition reduced ATI-2341-induced phosphorylation of Ser-346/7 by >60% (Fig. 2B). This provides further evidence that PKC plays a central role in the

phosphorylation of Ser-346/7 at the distal portion of the CXCR4 C-terminal tail. Although Ser-346/7 does not fall within a putative consensus sequence for PKC, our data indicate that PKC plays an important role in CXCR4 phosphorylation at this site.

Serine 346/7 is not phosphorylated by casein kinase 1 (CK1), casein kinase 2 (CK2), or glycogen synthase kinase 3 (GSK3). Ser-346/7 falls within a putative consensus site for phosphorylation by CK1, CK2, and GSK3 [Pinna & Ruzzene, 1996; Tobin, 2002]. Therefore, we also evaluated if inhibition of CK1, CK2 and GSK3 affected phosphorylation of Ser-346/7. HEK293 cells stably expressing CXCR4 were pre-incubated with the kinase inhibitors Bis I (PKC inhibitor; 2.5 μ M), D4476 (CK1 inhibitor; 20 μ M), TBB (CK2 inhibitor; 20 μ M) or CT99021 (GSK3 inhibitor; 10 μ M) for 20 min, then either directly lysed (for basal phosphorylation) or stimulated with 50 nM CXCL12 for 5 min. We previously found that Ser-324/5 is phosphorylated by PKC [Busillo et al., 2010], which falls within a PKC consensus site, so blots were also probed for pS324/5. Inhibition of PKC effectively reduced basal and CXCL12-promoted phosphorylation of both Ser-346/7 and Ser-324/5 (Fig. 3A-B and D-G). In contrast, inhibition of CK1, CK2 or GSK3 had no effect on basal phosphorylation (Fig. 3A, D and E) or CXCL12-promoted phosphorylation (Fig. 3B, F and G) at either Ser-346/7 or Ser-324/5. While these results suggest that CK1, CK2 and GSK3 do not contribute to CXCR4 phosphorylation of Ser-324/5 or Ser-346/7, we did not demonstrate that the inhibitor treatments effectively inhibited the specific kinases. Thus, while we used inhibitor concentrations that have proven effective in previous studies [Pinna & Ruzzene, 1996; Tobin, 2002], we cannot completely rule out a potential role for these kinases in CXCR4 phosphorylation.

To further characterize the role PKC plays in phosphorylating Ser-346/7, we determined if directly activating PKC with phorbol 12-myristate 13-acetate (PMA) resulted in phosphorylation

of Ser-346/7. As shown in Fig. 3C, PMA resulted in robust phosphorylation of both Ser-346/7 and Ser-324/5 at 10 min. As expected, inhibition of PKC with Bis I effectively blocked PMA-promoted phosphorylation of Ser346/7 and Ser-324/5, while inhibition of CK1, CK2 or GSK3 had no effect (Fig. 3C, H and I). Together, these data provide further evidence for a role for PKC in phosphorylation of Ser-346/7 following activation of CXCR4.

Identification of PKC isoforms that phosphorylate Ser-346/7. The PKC family contains 10 isoforms that are classified as conventional, novel and atypical according to their regulatory domains [Reyland, 2009]. Therefore, we wanted to differentiate the PKC isoforms responsible for phosphorylation of Ser-346/7. Since Bis I broadly inhibits the conventional (PKC $\alpha/\beta/\gamma$) and novel (PKC $\delta/\epsilon/\theta/\eta$) PKC isoforms [Wu-Zang & Newton, 2013], we attempted to narrow down the isoform responsible for phosphorylation of Ser-346/7 by pretreating cells with Gö 6976 (to inhibit the conventional isoforms PKC α and β), CGX1037 (to inhibit the novel isoform PKC δ) and myristolated PKC ζ pseudosubstrate peptide (to inhibit the atypical isoform PCK ζ). We also assessed the effect of PI3 kinase (PI3K) inhibition with wortmannin. Gö 6976 reduced the basal phosphorylation of Ser-346/7 by 60% and Ser-324/5 by 70% and was comparable to the effects seen with Bis I (Fig. 4A-C). In contrast, inhibition of PKC δ , PKC ζ or PI3K had no effect on basal phosphorylation of either Ser-346/7 or Ser-324/5 (Fig. 4A-C).

Next, we examined the effect of these inhibitors on CXCL12- and ATI-2341-induced phosphorylation of Ser-346/7 and Ser-324/5. Inhibition of PKC α/β (Gö 6976) reduced CXCL12-promoted phosphorylation of Ser-346/7 (Fig. 5A and 5C) and Ser-324/5 (Fig. 5A and 5E) at 5 min; however, it was not as effective as Bis I (Fig. 5A, 5C, and 5E). Interestingly, whereas inhibition of PKC ζ did not affect the basal phosphorylation of CXCR4 (Fig. 4), CXCL12-promoted phosphorylation of Ser-346/7 and Ser-324/5 was significantly reduced by inhibition of

PKC ζ (Fig. 5A, 5C, and 5E). Moreover, the inhibition of PKC ζ reduced phosphorylation of Ser-324/5 to a similar extent as Bis I (Fig. 5A and 5E). Similar results were obtained when ATI-2341-promoted phosphorylation of CXCR4 at 5 min was examined (Fig. 5B, 5D, and 5F), although inhibition of PKC ζ was more effective at reducing phosphorylation of Ser-346/7 following treatment with ATI-2341 (Fig. 5B and 5D). Inhibition of PKC δ resulted in an ~20% reduction of ATI-2341- and CXCL12-induced phosphorylation of Ser346/7 but had no effect on phosphorylation of Ser324/5 (Fig. 5). The effective reduction in CXCR4 phosphorylation following stimulation with ATI-2341 is in line with the observation that ATI-2341 triggers PKC-dependent phosphorylation of CXCR4 [Quoyer et al., 2013].

Since the PKC α/β -specific inhibitor Gö 6976 was fairly effective in inhibiting CXCR4 phosphorylation (Fig. 5), we tried to further define which PKC isoform is involved in Ser-346/7 and Ser-324/5 phosphorylation using a knockdown approach. Endogenous PKC α and PKC β in HEK293 cells stably expressing CXCR4 were knocked down using ON-TARGETplus siRNAs (Fig. 6A). PKC α was reduced ~70% while PKC β was decreased >90%. While there was no significant effect of PKC β knockdown, there was some reduction of CXCL12- and ATI-2341-promoted phosphorylation of Ser-346/7 and Ser324/5 at 5 min in cells with PKC α knockdown, albeit only ~20% (Fig. 6B-G). The lack of a larger effect may reflect the incomplete knockdown of PKC α expression as well as potential contributions from other PKC isoforms. Thus, our data suggest that PKC α plays a role in agonist-promoted phosphorylation of CXCR4.

Phosphorylation of CXCR4 by GRK3 or PKC facilitates recruitment of β -arrestin2.

The recruitment of arrestins to agonist-activated and phosphorylated GPCRs is critical for receptor desensitization as well as to initiate arrestin-dependent signaling [DeWire et al., 2007; Moore et al., 2007]. Previous results suggested that β -arrestin2 was involved in receptor

desensitization, whereas β -arrestin1 was involved in signaling following CXCR4 activation [Busillo et al., 2010]. CXCL12 stimulation results in the recruitment of β -arrestin2 [Quoyer et al., 2013], which is lost when the C-terminal tail is truncated [McCormick et al., 2009]. Given the critical role the distal C-terminal tail plays in desensitization and internalization of CXCR4 [Orsini et al., 1999; Balabanian et al., 2008; McCormick et al., 2009], we hypothesized that the GRK- and PKC-mediated phosphorylation of Ser-346/7 might be involved in the recruitment of β -arrestin2. Therefore, we used BRET to investigate the interaction between CXCR4 and β -arrestin2 [Quoyer et al., 2013]. The distal C-terminal tail of CXCR4 contains five serine residues (Ser-346/7/8, Ser-351/2). Our mass spectrometry data suggested that there were two potential phosphorylation sites in these two serine clusters [Busillo et al., 2010]; therefore, we mutated all five residues to alanine (CXCR4-5AT). HEK293 cells were co-transfected with GFP10- β -arrestin2 and either wild-type or 5AT mutant CXCR4-RLucII to investigate the influence of PKC ζ inhibitor on the recruitment of β -arrestin2 to CXCR4 using BRET (Fig. 7A). In the absence of CXCL12, there was no change in the BRET signal for either wild-type or CXCR4-5AT and β -arrestin2 (Fig. 7B). Stimulation with CXCL12 induced the interaction between wild-type CXCR4 and β -arrestin2, which peaked at \sim 12 min after the addition of CXCL12 (Fig. 7B). While CXCL12 promoted a change in the BRET signal between CXCR4-5AT and β -arrestin2, the BRET signal was \sim 70% lower and the peak was slightly delayed compared to wild-type CXCR4 (Fig. 7B). Next, we knocked down GRK3 and determined the BRET signal between wild-type CXCR4 and β -arrestin2. Knockdown of GRK3 reduced the BRET signal by \sim 20% (Fig. 7C). In addition, broadly inhibiting PKC with Bis I reduced the BRET signal by \sim 15% (Fig. 7D). The combination of GRK3 knockdown and PKC inhibition did not have an additional effect (data not shown). Together, these data indicate that GRK3- and PKC-mediated phosphorylation

of Ser-346/7 are involved in β -arrestin2 recruitment. However, as the association between CXCR4 and β -arrestin2 was not completely lost when this cluster of serines was mutated to alanine, phosphorylation at other sites may also be involved in β -arrestin recruitment.

Given the significant effect inhibition of PKC ζ had on CXCR4 phosphorylation (Fig. 5), we also wanted to explore if PKC ζ -promoted phosphorylation of Ser-346/7 affected the recruitment of β -arrestin2. Interestingly, the PKC ζ inhibitor reduced the BRET signal in a dose-dependent manner and completely blocked β -arrestin2 recruitment at the highest dose (10 μ M) tested (Fig. 8A). In fact, inhibition of PKC ζ was more efficient at blocking β -arrestin2 recruitment than blocking the conventional and novel PKC isoforms with Bis I (compare Fig. 8A with Fig. 7D). To determine if this was a nonspecific effect of the pseudo-substrate peptide inhibitor for PKC ζ [Wu-Zhang et al., 2012], we also looked at the recruitment of β -arrestin2 to the β_2 -adrenergic receptor (β_2 AR), which is GRK-dependent [Violin et al., 2006]. While inhibition of PKC ζ blocked the association between CXCR4 and β -arrestin2, it had no effect on the recruitment of β -arrestin2 to the β_2 AR (Fig. 8B). In addition, different concentrations of PKC ζ inhibitor were also tested for inhibition of calcium flux activated by CXCL12 or ATI-2341. Fifty nM CXCL12 activated a modest calcium flux in CXCR4 stably expressing HEK293 cells and pre-incubation with the PKC ζ inhibitor effectively blocked calcium mobilization with an IC₅₀ of \sim 2 μ M (Fig. 8C). In comparison, 3 μ M ATI-2341 induced a more robust calcium flux, and pre-treatment of the cells with the PKC ζ inhibitor effectively inhibited calcium signaling with an IC₅₀ of \sim 0.5 μ M (Fig. 8D). In contrast, pre-treatment of HEK293 cells with the PKC ζ inhibitor at concentrations as high as 5 μ M had no effect on calcium flux activated by 100 μ M carbachol through endogenous M3 muscarinic acetylcholine receptors (Fig. 8E). These data demonstrate that the PKC ζ inhibitor can effectively and selectively block the function of CXCR4.

Next, we used an siRNA approach to knock down PKC ζ in HEK293 cells that stably express CXCR4 (Fig. 9A). In contrast to the PKC ζ inhibitor, knockdown of endogenous PKC ζ actually enhanced phosphorylation at Ser-346/7 and Ser-324/5, particularly at 15 min (Fig. 9B). PKC ζ knockdown also led to modestly enhanced recruitment of β -arrestin2 following stimulation with 20 nM CXCL12 (Fig. 9C). Thus, it appears that PKC ζ is not involved in CXCR4 phosphorylation and that the effects of the PKC ζ inhibitor on CXCR4 function might involve a direct effect on the receptor.

Together, these data show that the distal C-terminal tail of CXCR4 plays a critical role in β -arrestin2 recruitment following activation. Furthermore, the data suggest that GRK- and PKC-mediated phosphorylation of this region leads to the recruitment of β -arrestin2 to CXCR4 following activation.

Discussion

GPCRs play a very important role in many biological functions in various cells and tissues. Activated GPCRs are primarily regulated by three mechanisms: desensitization, internalization and degradation. Receptor desensitization is often initiated by phosphorylation and subsequent high-affinity binding of arrestin, which uncouples the receptor from its cognate G protein. Classically, it was thought that the GRKs were primarily responsible for phosphorylating activated GPCRs [Krupnick & Benovic, 1998]; however, it is becoming evident that the process of receptor phosphorylation is very complex and involves multiple kinase families [Premont & Gainetdinov, 2007; Tobin et al., 2008]. The complexity of receptor phosphorylation is further complicated by the fact that multiple kinases can phosphorylate a particular site and some kinases can phosphorylate multiple sites [Tobin et al., 2008]. For example, within the C-terminal tail of CXCR4, we have shown that Ser-324/5 can be phosphorylated by both GRK6 and PKC and that GRK6 phosphorylates two additional sites (Ser-330 and Ser-339) [Busillo et al., 2010]. In the present report, we extend our previous work characterizing site-specific phosphorylation of CXCR4 and provide evidence that phosphorylation of Ser-346/7 is mediated by GRK3 and PKC, in particular PKC α , and is involved in the recruitment of β -arrestin2, initiating the process of receptor desensitization.

The phosphorylation of CXCR4 mainly occurs in the serine/threonine rich C-terminal tail. To date, at least 7 phosphorylation sites have been identified through a variety of different techniques. We recently identified and confirmed several phosphorylation sites within the C-terminal tail of CXCR4 using a combination of mass spectrometry and phospho-specific antibodies. In addition, with the help of RNA interference and various kinase inhibitors, we identified the protein kinases that were responsible for site-specific phosphorylation of Ser-

324/5, Ser-330 and Ser-339 [Busillo et al., 2010]. Two of the phosphorylation sites detected by mass spectrometry were located between amino acids 346 and 352 of CXCR4, which contains two clusters of serine residues (Ser-346-348 and Ser-351/352). While we were unable to identify the exact residues phosphorylated or the kinases involved, we hypothesized that GRK2 and/or GRK3 could be responsible [Busillo et al., 2010]. Recently, it was demonstrated that Ser-346/7 is phosphorylated in response to CXCL12 and PMA. Moreover, CXCL12-induced phosphorylation of Ser-346/7 was mediated by GRK2/3, which appears to impact additional phosphorylation events within the C-terminal tail of CXCR4 [Mueller et al., 2013]. In the present study, we demonstrated that activation of CXCR4, with either CXCL12 or the peptidic agonist ATI-2341 [Quoyer et al., 2013], results in rapid phosphorylation of Ser-346/7. Consistent with previous results [Mueller et al., 2013], we found that phosphorylation at Ser-346/7 peaked slightly earlier than Ser-324/5 (Fig. 1B) [Busillo et al., 2010]. Furthermore, we also found that knockdown of GRK2, GRK3, or GRK6 reduced phosphorylation of Ser346/7 with GRK3 playing the major role (Fig. 1D). Interestingly, the majority of WHIM mutations described to date involve truncations of the last 10 to 15 amino acids of CXCR4 or have a selective loss of GRK3 [Diaz & Gulino, 2005; Balabanian et al., 2008]. These data suggest that the GRK3-mediated phosphorylation of Ser-346/7 is critical for regulating the desensitization of CXCR4. In agreement with this, knockdown of GRK3 (Fig. 7C) and mutation of the last five serine residues of CXCR4 (Ser346/7/8, Ser-351 and Ser-352) (Fig. 7B) significantly reduced the association between CXCR4 and β -arrestin2. However, as mutation of the last 5 serine residues did not completely abolish the recruitment of β -arrestin2, phosphorylation of other residues is likely involved in the process.

As indicated above, knockdown of GRK3 reduced phosphorylation of Ser-346/7 but did not eliminate it completely. As PMA also induced phosphorylation of these residues (Fig. 3C)

[Mueller et al., 2013], we investigated the role of PKC. We provide evidence for a role of PKC in phosphorylating Ser-346/7 (Fig. 1E) and recruiting β -arrestin2 (Fig. 7D). Following CXCR4 activation by CXCL12, both PKC and GRK3 are needed for robust phosphorylation of Ser346/7 (Fig. 1F). Additionally, ATI-2341, which allosterically activates CXCR4, results in the PKC-dependent phosphorylation of Ser-346/7 (Fig. 2A and 2B). This is consistent with ATI-2341 selectively activating G_i resulting in the PKC-dependent phosphorylation of Ser-324/5 [Quoyer et al., 2013]. In an attempt to narrow down the PKC isoforms involved in phosphorylation of Ser-346/7, we found that the conventional and/or novel isoforms of PKC are likely involved. Specifically, we showed that two PKC inhibitors, Bis I (conventional and novel PKC isoforms) and Gö 6976 (conventional PKC isoforms), reduced the basal phosphorylation of Ser-346/7 (Fig. 4A), whereas inhibition of PKC δ or PKC ζ had no effect. In contrast, phosphorylation of Ser-346/7 following stimulation with either CXCL12 (Fig. 5A and 5C) or ATI-2341 (Fig. 5B and 5D), was reduced by inhibition of the conventional PKC isoforms (Bis I and Gö 6976) and PKC ζ . This, in turn, reduced the recruitment of β -arrestin2 (Fig. 7D and 8A). The PKC ζ inhibitor (a PKC ζ pseudo-substrate) was much more effective at blocking arrestin recruitment to CXCR4 (Fig. 8A), while it had no effect on the recruitment of β -arrestin2 to the β_2 AR (Fig. 8B). Together, these data suggest that the conventional PKC isoforms regulate the basal phosphorylation state of CXCR4, whereas PKC ζ might be involved in phosphorylating the activated receptor and initiating arrestin recruitment. However, when we knocked down PKC ζ (Fig. 9A), the phosphorylation of both Ser-324/5 and Ser-346/7 were modestly increased (Fig. 9B), resulting in enhanced recruitment of β -arrestin2 (Fig. 9C). Given the non-specific actions of the PKC pseudo-substrates [Smith et al., 1990; Wu-Zhang & Newton, 2013] and the low affinity for PKC ζ in cells [Wu-Zhang et al., 2012], it is difficult to interpret the data using the PKC ζ

inhibitor. It's worth noting, however, that the PKC ζ pseudo-substrate is a highly basic peptide, and might be directly binding to and inhibiting CXCR4. Indeed, many of the known inhibitors of CXCR4, including AMD3100, IT1t, CVX15 and ALX-40C, all possess a positive charge and inhibit CXCR4 by interacting with the CXCL12 binding pocket [Wu et al., 2010]. In line with this hypothesis, we observed a dose-dependent inhibition of calcium mobilization downstream of CXCR4 activation but not downstream of the M3 muscarinic acetylcholine receptor (Fig. 8C-E) [Luo et al., 2008]. We also cannot rule out the possibility that the PKC ζ pseudo-substrate is inhibiting a number of different kinases [Wu-Zhang et al., 2012; Ling et al., 2002; Wu-Zhang & Newton, 2013], including the conventional PKC isoforms [Bogard & Tavalin, 2015].

Since the amino acid sequence around Ser-346/7 (TESESSSFHSS) does not contain a linear PKC consensus sequence for phosphorylation which requires a basic residue at -2 or +2, we searched for other kinases that have consensus sequences that would better fit and identified three kinases: CK1, CK2 and GSK3 β . These kinases are abundantly expressed and have previously been shown to phosphorylate GPCRs [Luo et al., 2008; Torrecilla et al., 2007; Lee et al., 2001; Kramer et al., 2000]. We showed that only inhibition of PKC significantly reduced both the basal and agonist-promoted phosphorylation of Ser-346/7 (Fig. 3A-C), ruling out CK1, CK2 and GSK3 β . Furthermore, we found that both PMA and ATI-2341 promoted phosphorylation of Ser-346/7. These data suggest that PKC is one of the main kinases responsible for the regulation of CXCR4 activity. While this region of CXCR4 does not contain a consensus site for PKC phosphorylation, one possibility is that the activation of CXCR4 causes the C-terminal tail to adopt a conformation that creates a structural PKC consensus motif, which has recently been demonstrated for both PKC and PKA phosphorylation of various substrates [Duarte et al., 2014]. Another possibility is that PKC-mediated phosphorylation of other residues

within the C-terminal tail of CXCR4 allows for subsequent phosphorylation of Ser-346/7.

Moreover, we cannot exclude the possibility that additional protein kinases downstream of PKC may be involved in the phosphorylation of CXCR4.

Although the GRK family has historically been considered the main kinase family to phosphorylate GPCRs, there is strong evidence to support a role for other protein kinases in mediating agonist-dependent phosphorylation of activated GPCRs. Advances in technology, such as tandem mass spectrometry and phospho-specific antibodies, have helped in identifying phosphorylation sites in many GPCRs [Tobin et al., 1997; Blaukat et al., 2001; Busillo et al., 2010; Nobles et al., 2011; Trester-Zedlitz et al., 2005]. Given that most receptors are phosphorylated on multiple residues, it is difficult to link a functional outcome with a specific phosphorylation site. This is further complicated by the fact that the pattern of phosphorylation (“phosphorylation barcode”) in one tissue may differ from the pattern elicited in another tissue. However, we have shown that there is overlap in kinase specificity for phosphorylating specific residues within CXCR4. This suggests that specific residues are critical for regulating the activity and function of CXCR4, and that redundancy exists between kinases to ensure that proper regulation is maintained in various tissues. This is of particular interest for CXCR4, given that the receptor plays a critical role in diverse physiological processes and is one of the most commonly expressed receptors found on tumor cells [Busillo & Benovic, 2007]. As we begin to better understand the kinetic and functional significance of site-specific phosphorylation, we will be able to better understand how this contributes to the dysregulation of CXCR4.

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Author Contributions

Participated in research design: Luo, Busillo and Benovic

Conducted experiments: Luo

Contributed new reagents or analytic tools: Stumm

Performed data analysis: Luo and Benovic

Wrote or contributed to the writing of the manuscript: Luo, Busillo, Stumm and Benovic

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***Footnotes**

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Figure Legends

Figure 1. GRKs and PKC Mediate Phosphorylation of CXCR4 at Ser-346/7 Following

Stimulation with CXCL12. A) Sequence of CXCR4 C-terminus with Ser-324/5 and Ser-346/7

highlighted in red. B) HEK293 cells stably expressing CXCR4 were incubated in serum free

medium for 4 hr, then stimulated with 50 nM CXCL12 for the indicated times. Cells were

washed twice with cold PBS and lysed. Phosphorylation of CXCR4 Ser-346/7 was detected by

Western-blot using a phospho-specific antibody (pS346/7). Shown is a representative Western

blot using pS346/7 and tubulin antibodies from at least three independent experiments. C) Shown

is a representative Western blot from at least three experiments where endogenous GRKs were

knocked down using ON-TARGETplus GRK siRNAs in HEK293 cells stably expressing

CXCR4 for 48 hr. D) HEK293 cells stably expressing CXCR4 were stimulated with CXCL12 (5

nM) following knockdown of endogenous GRK2, GRK3 or GRK6 and the phosphorylation of

CXCR4 at Ser-346/7 was detected by Western blot. Shown is a representative blot (upper panel)

and summary of the 5 min time point (lower panel) from three independent experiments

(** $p < 0.01$ as compared with control siRNA). AU, arbitrary units. E) HEK293 cells stably

expressing CXCR4 were incubated in serum free medium for 4 hr. Bis I (2.5 μ M) was added to

the cells 30 min prior to being stimulated with CXCL12. The phosphorylation of CXCR4 Ser-

346/7 was detected by Western blot. Shown is a representative blot (upper panel) and summary

(lower panel) from three independent experiments (* $p < 0.05$; ** $p < 0.01$ as compared with

control). AU, arbitrary units. F) GRK3 was knocked down in HEK293 cells stably expressing

CXCR4 while Bis I (2.5 μ M) was added 30 min prior to stimulation with CXCL12 (50 nM).

Shown is a representative blot (upper panel) and summary from three independent experiments

(# $p < 0.001$ as compared with control, ** $p < 0.01$ as compared with Bis I alone, + $p < 0.01$ as compared with siGRK3 alone). AU, arbitrary units.

Figure 2. The Pepducin ATI-2341 Induces Phosphorylation of CXCR4 at Ser-346/7 in a PKC-Dependent Manner. A) HEK293 cells stably expressing CXCR4 were starved with serum-free medium for 4 hr, then stimulated with 3 μM ATI-2341 for the indicated times. Whole cell lysates were probed with the pS346/7 antibody. Shown is a representative blot from three independent experiments. B) HEK293 cells stably expressing CXCR4 were pre-treated with Bis I (2.5 μM) for 30 min and then stimulated with ATI-2341 (3 μM). Phosphorylation of CXCR4 was detected with the pS346/7 antibody. Shown is a representative blot (upper panel) and summary (lower panel) from four independent experiments (* $p < 0.05$ as compared with control). AU, arbitrary units.

Figure 3. Phosphorylation of CXCR4 at Ser-346/7 and Ser-324/5 is Regulated by PKC and not CK1, CK2, or GSK3 β . HEK293 cells stably expressing CXCR4 were pre-incubated with Bis I (pan-PKC inhibitor; 2.5 μM), D4476 (CK1 inhibitor; 20 μM), TBB (CK2 inhibitor; 20 μM), or CT99021 (GSK3 inhibitor; 10 μM) for 20 min. Cells were either untreated (basal phosphorylation) or stimulated with CXCL12 (50 nM) for 5 min or PMA (200 nM) for 10 min. A) Shown is a representative blot from two independent experiments for basal or (B) three experiments for CXCL12-promoted phosphorylation of CXCR4. C) Shown is a representative blot from three independent experiments for PMA-promoted phosphorylation of CXCR4. D-I) Shown are summaries from 2-3 independent experiments. AU, arbitrary units.

Figure 4. Identification of the PKC Isoform that Regulates Basal Phosphorylation of

CXCR4 Ser-346/7 and Ser-324/5. HEK293 cells stably expressing CXCR4 were pre-treated with Bis I (pan-PKC inhibitor; 2.5 μ M), Gö 6976 (PKC α and PKC β inhibitor; 1 μ M), CGX1037 (PKC δ inhibitor; 2.5 μ M), PKC ζ pseudo-substrate (PKC ζ inhibitor; 10 μ M), or wortmannin (PI3K inhibitor; 100 nM) for 20 min. Cells were then lysed and whole cell lysates were probed with either pS346/7 or pS324/5 antibodies. A) Shown is a representative blot from three individual experiments. Quantification of pS346/7 (B) and pS324/5 (C) from three independent experiments (** $p < 0.01$, as compared with medium alone). AU, arbitrary units.

Figure 5. Phosphorylation of Ser346/7 and Ser324/5 is Mediated by PKC Following

Stimulation with either CXCL12 or ATI-2341. HEK293 cells stably expressing CXCR4 were pre-incubated with Bis I (2.5 μ M), Gö 6976 (1 μ M), CGX1037 (2.5 μ M), PKC ζ pseudo-substrate (10 μ M), or wortmannin (100 nM) for 20 min. Cells were then stimulated with 50 nM CXCL12 (A) or 3 μ M ATI-2341 (B) for 5 min and whole cell lysates were probed for either pS346/7 or pS324/5 by Western blot. Quantification of pS346/7 following either CXCL12 (C) (n=4) or ATI-2341 (D) (n=5) stimulation (* $p < 0.05$, ** $p < 0.01$, as compared with vehicle). AU, arbitrary units. Quantification of pS324/5 following either CXCL12 (E) (n=4) or ATI-2341 (F) (n=5) stimulation (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ as compared with vehicle). AU, arbitrary units. The measurement of CXCR4 phosphorylation at Ser-346/7 involved detection of all bands while Ser-324/5 involved detection of just the upper band.

Figure 6. Role of Conventional PKC in the Phosphorylation of Ser-346/7 and Ser-324/5. A)

HEK293 cells stably expressing CXCR4 were transfected with ON-TARGETplus PKC α , PKC β

or PKC α / β siRNA for 48 hr and endogenous PKC α and PKC β expression was examined by Western blotting (shown is a representative blot from four independent experiments). Following knockdown of PKC α and/or PKC β , HEK293 cells were incubated with or without 50 nM CXCL12 (B) or 3 μ M ATI-2341(E) for 5-15 min and whole cell lysates were probed for pS346/7 and pS324/5. Shown are representative Western blots from four individual experiments. Quantification of relative pS346/7 and pS324/5 levels following stimulation with CXCL12 (C and D) or ATI-2341 (F and G) are summarized (* $p < 0.05$ as compared with control). AU, arbitrary units.

Figure 7. Phosphorylation of CXCR4 by GRK3 or PKC Regulates the Interaction of

CXCR4 with β -arrestin2. A) Schematic of β -arrestin recruitment by CXCR4 in a BRET assay.

Upon agonist stimulation, GFP10- β -arrestin2 is recruited to CXCR4-RLucII and BRET signal is increased. B) HEK293 cells were co-transfected with either wild type or 5AT mutant CXCR4-RLucII with β -arrestin2-GFP10. The interaction between CXCR4 and β -arrestin2 was detected with or without stimulation of 100 nM CXCL12 in BRET assay after 48 hr co-transfection. Data shows a summary of seven individual experiments. C) HEK293 cells were transfected with ON-TARGETplus GRK3 siRNA; 24 hr later cells were co-transfected with CXCR4-RLucII and β -arrestin2-GFP10. Cells were stimulated with 100 nM CXCL12 after 48 hr co-transfection and interaction between CXCR4 and β -arrestin2 was detected in BRET assay. Data shows a summary of six individual experiments. D) HEK293 cells transiently co-transfected with CXCR4-RLucII and β -arrestin2-GFP10 were treated with or without 2.5 μ M Bis I for 20 min. Cells were then stimulated with 100 nM CXCL12 and interaction between CXCR4 and β -arrestin2 was detected. Data show a summary of three individual experiments.

Figure 8. Inhibition of CXCR4 Function by PKC ζ Myristoylated Pseudosubstrate Peptide

Inhibitor. A) HEK293 cells transiently co-transfected with CXCR4-RLucII and β -arrestin2 GFP10 were treated with different concentrations of PKC ζ inhibitor for 20 min, then cells were incubated with 100 nM CXCL12 and BRET signal was detected. Data shows a summary of five individual experiments. B) HEK293 cells transiently co-transfected with β_2 AR-RLucII and β -arrestin2-GFP10 were treated with or without 10 μ M PKC ζ inhibitor for 20 min. Cells were stimulated with 10 μ M isoproterenol and the interaction between β_2 AR and β -arrestin2 was detected. Data show a summary of four individual assays. C and D) Inhibition of calcium mobilization through CXCR4 induced by PKC ζ inhibitor. Stable CXCR4 HEK293 cells loaded with 2 μ M Fura2-AM were incubated with different concentrations of PKC ζ inhibitor for 1 min at 37 °C, then stimulated with either 50 nM CXCL12 (C) or 3 μ M ATI-2341 (D), and calcium flux was detected. This data is representative of at least three individual experiments. E) Effect of PKC ζ inhibitor on calcium mobilization through the M3 muscarinic acetylcholine receptor. A representative of at least three individual experiments using the stable CXCR4 HEK293 cells stimulated with 100 μ M carbachol with or without pre-incubation with PKC ζ inhibitor for comparison.

Figure 9. Knockdown of Endogenous PKC ζ Enhances Phosphorylation and β -arrestin2

Recruitment to CXCR4. A) HEK293 cells stably expressing CXCR4 were transfected with PKC ζ ON-TARGETplus siRNA or scrambled control for 48 hr and the expression of endogenous PKC ζ was probed using an anti-PKC ζ antibody. Shown is a representative Western blot from three independent experiments. B) Following knockdown of PKC ζ , HEK293 cells

stably expressing CXCR4 were incubated with or without CXCL12 (50 nM) for up to 15 min and whole cell lysates were probed for pS346/7 and pS324/5. Shown are representative Western blots from three individual experiments. C) Following knockdown of PKC ζ , HEK293 cells were co-transfected with CXCR4-RLucII and β -arrestin2-GFP10. Cells were stimulated with 20 nM CXCL12 and the relative interaction between CXCR4 and β -arrestin2 was determined by BRET. Data shown are a summary of six independent experiments.

A CXCR4 C-terminus:
 KFKTSAQHALTSVSRG**SSL**KILSKGKRGGHSSVSTES**ESS**FHSS
 308 324/5 346/7 352

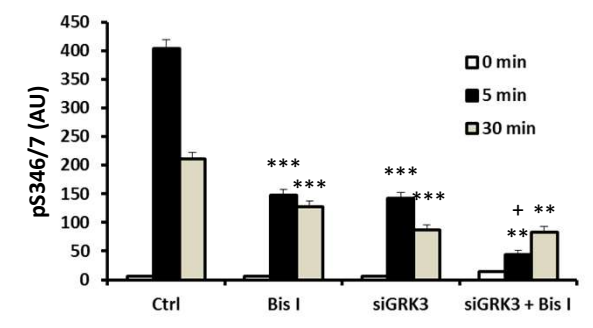
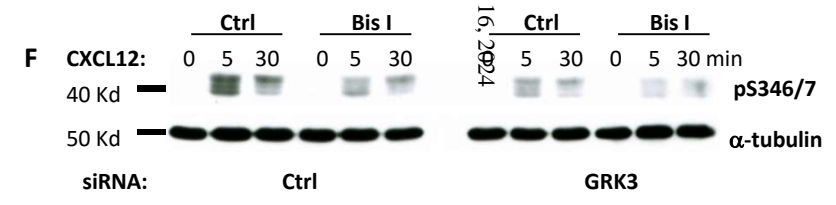
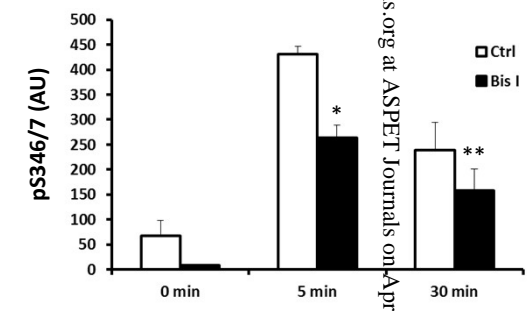
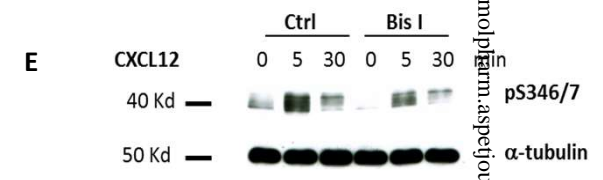
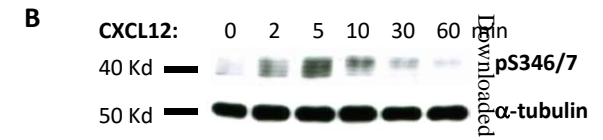
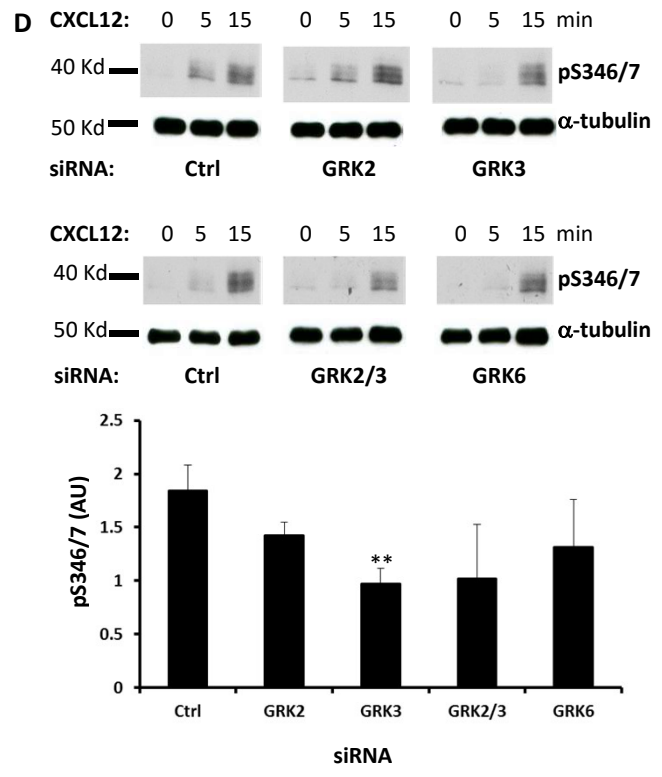
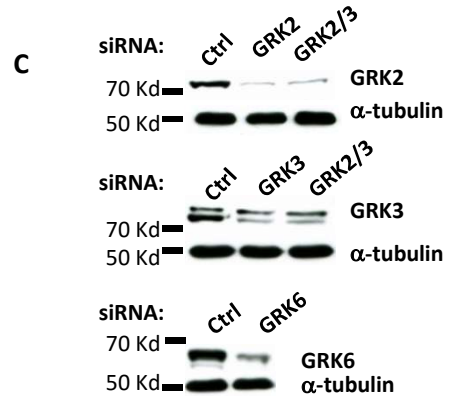


Fig. 1

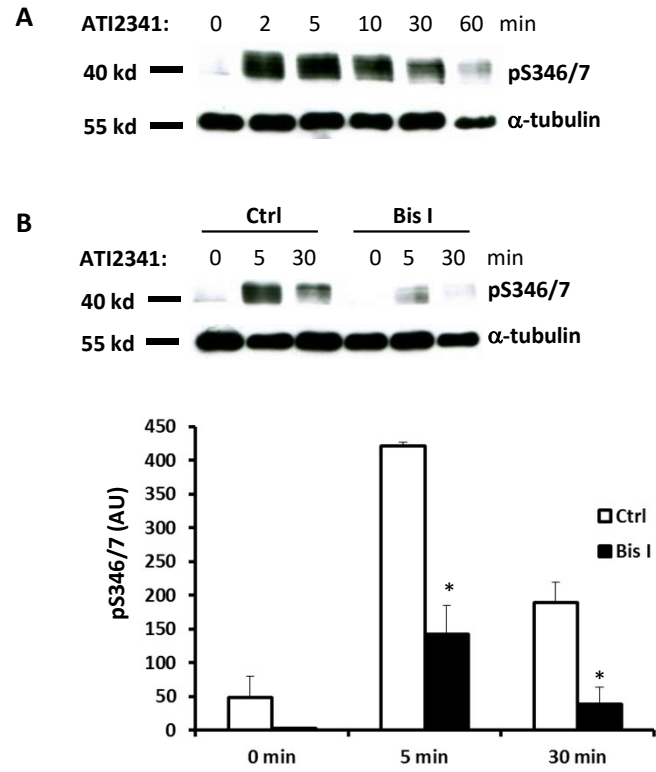


Fig. 2

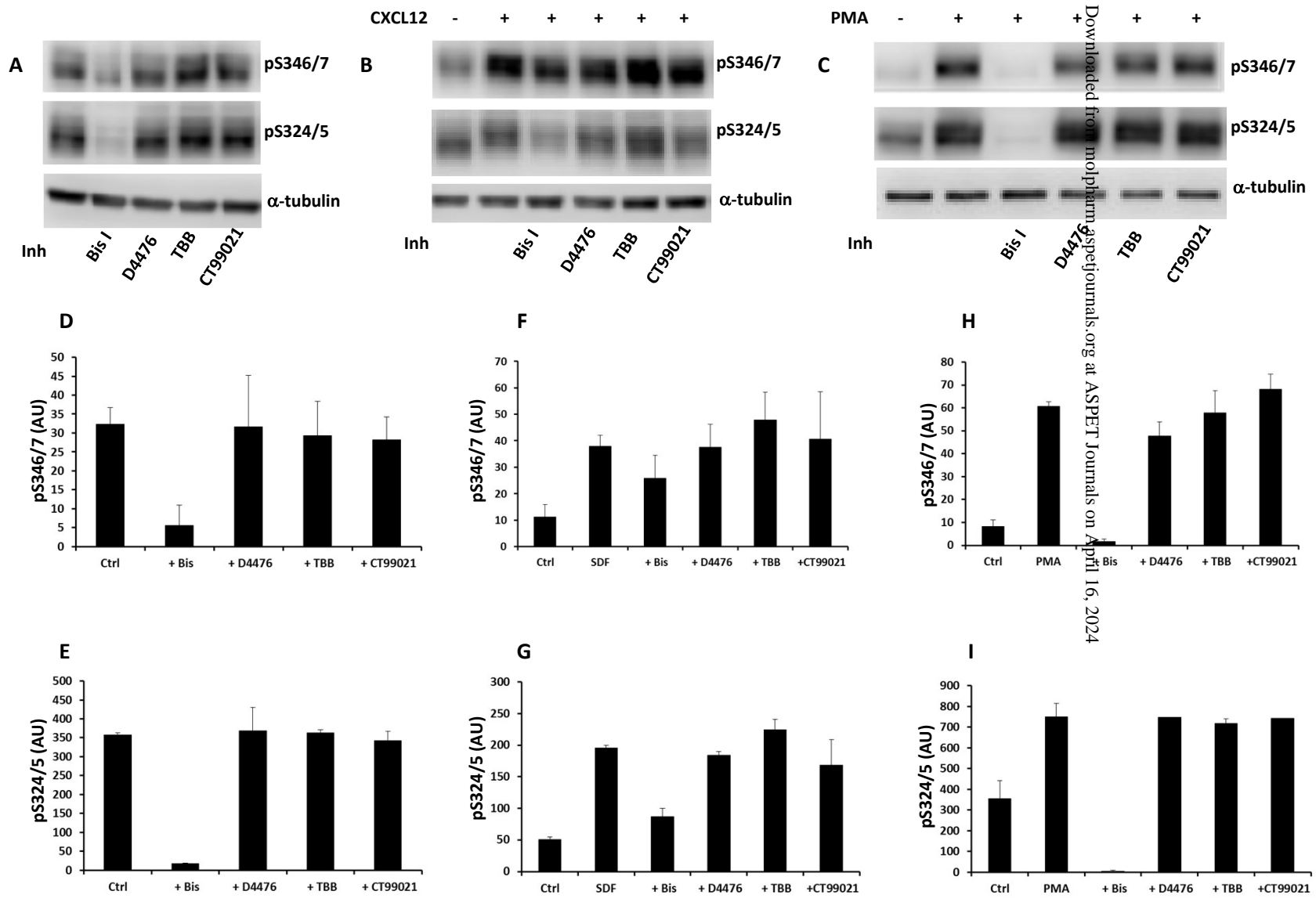


Fig. 3

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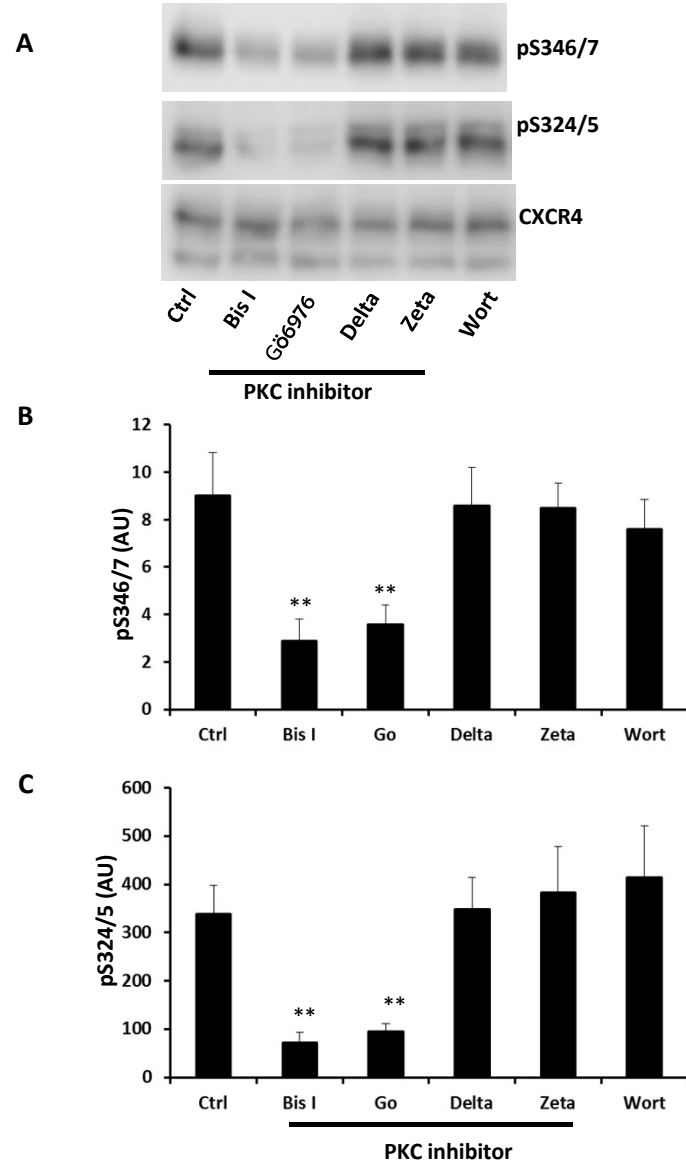


Fig. 4

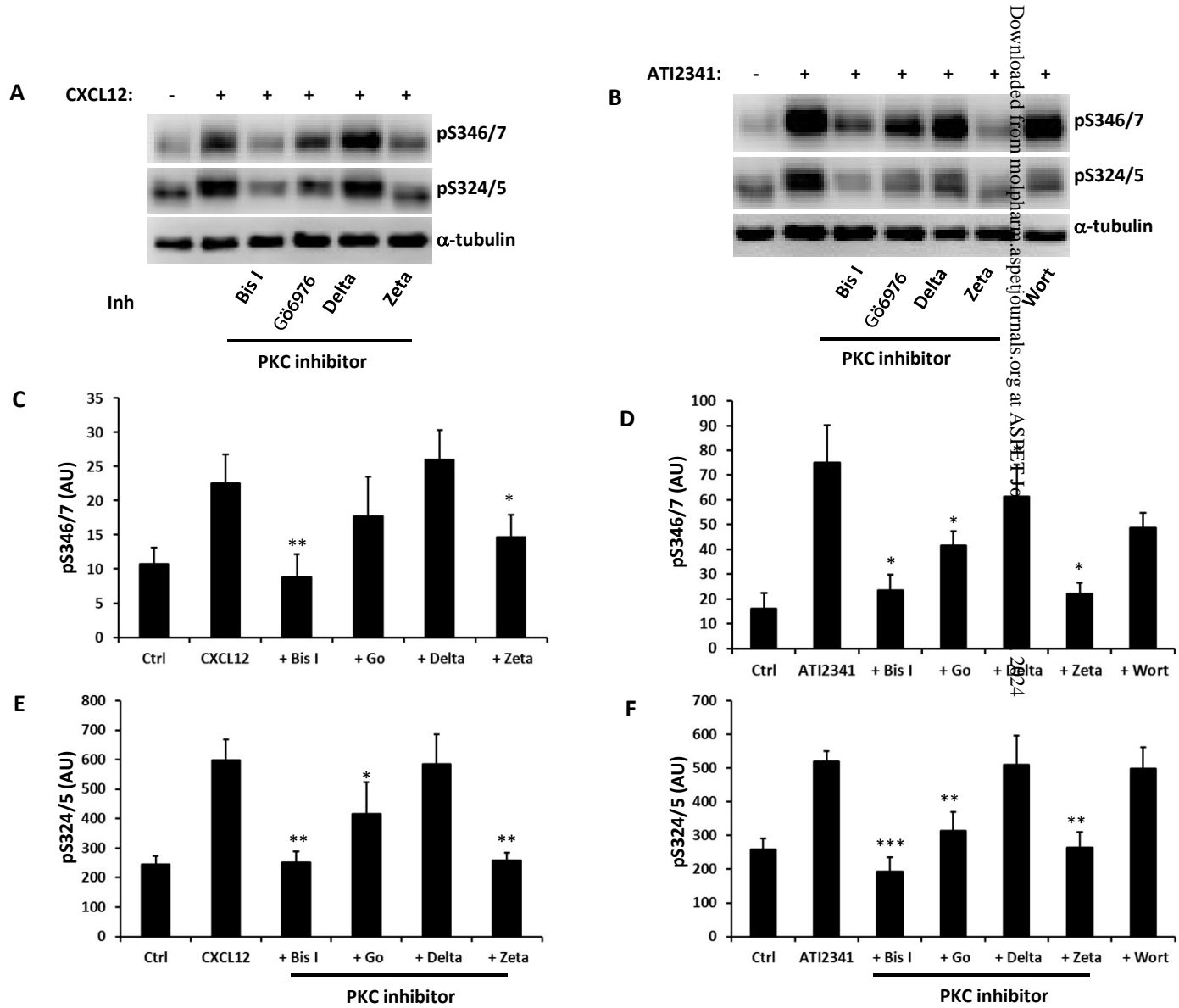


Fig. 5

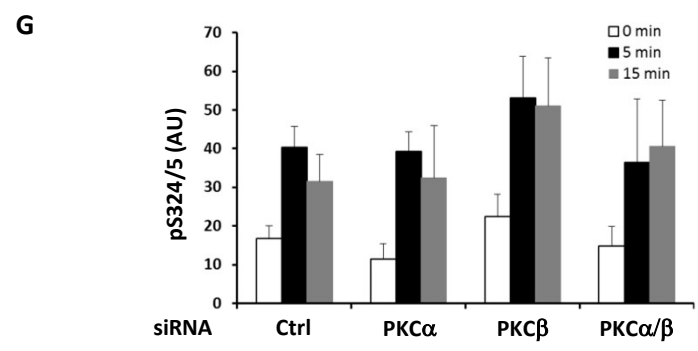
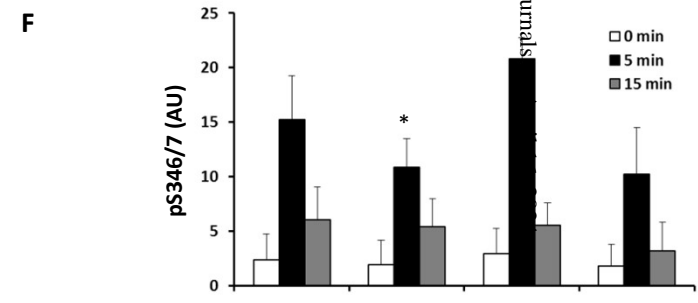
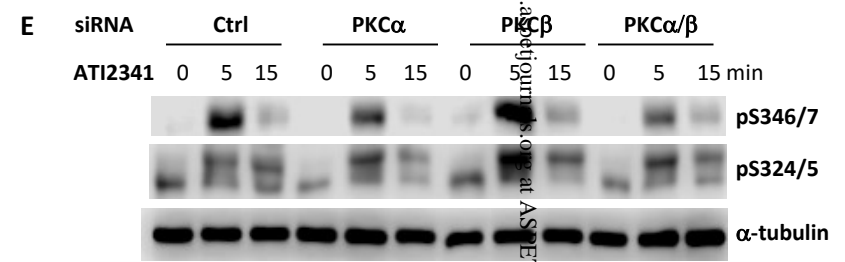
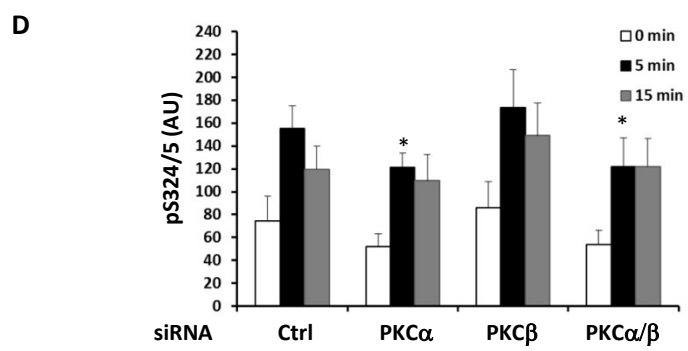
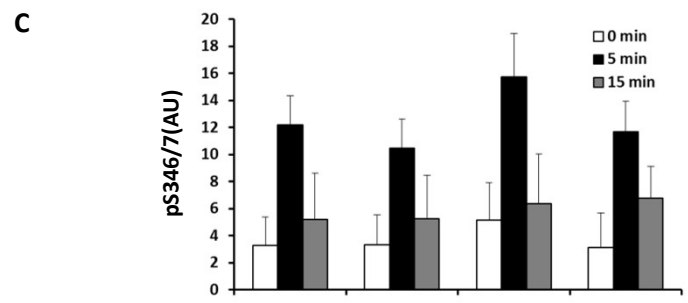
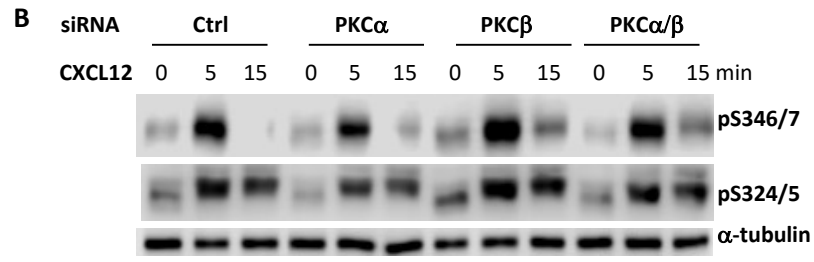
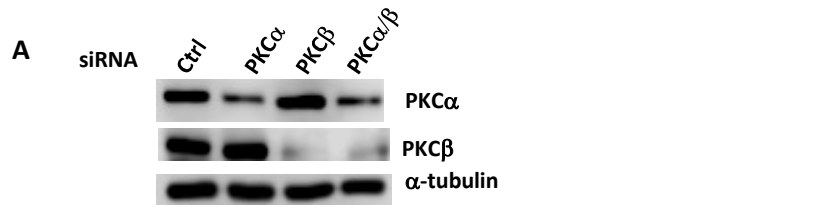
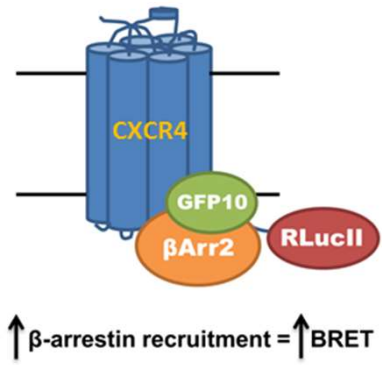


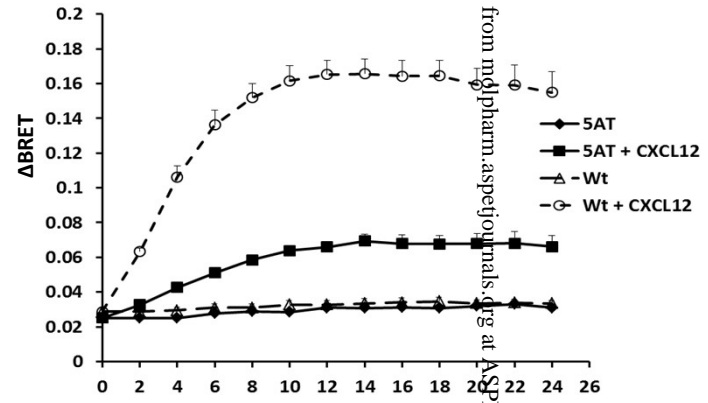
Fig. 6

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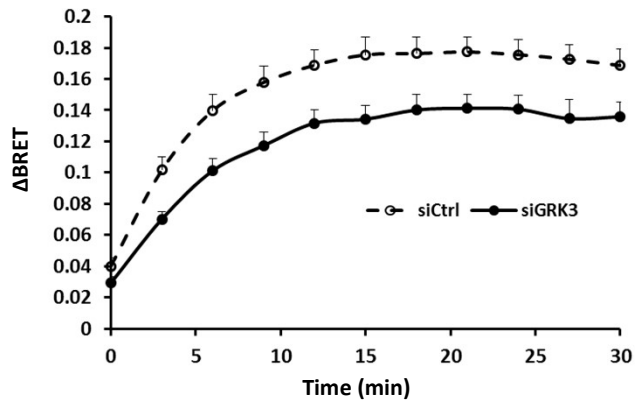
A



B



C



D

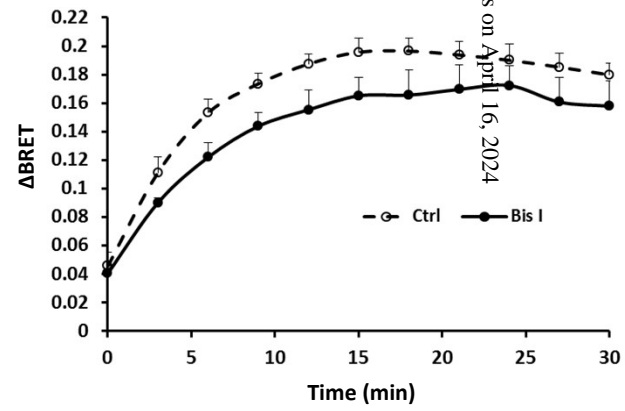


Fig. 7

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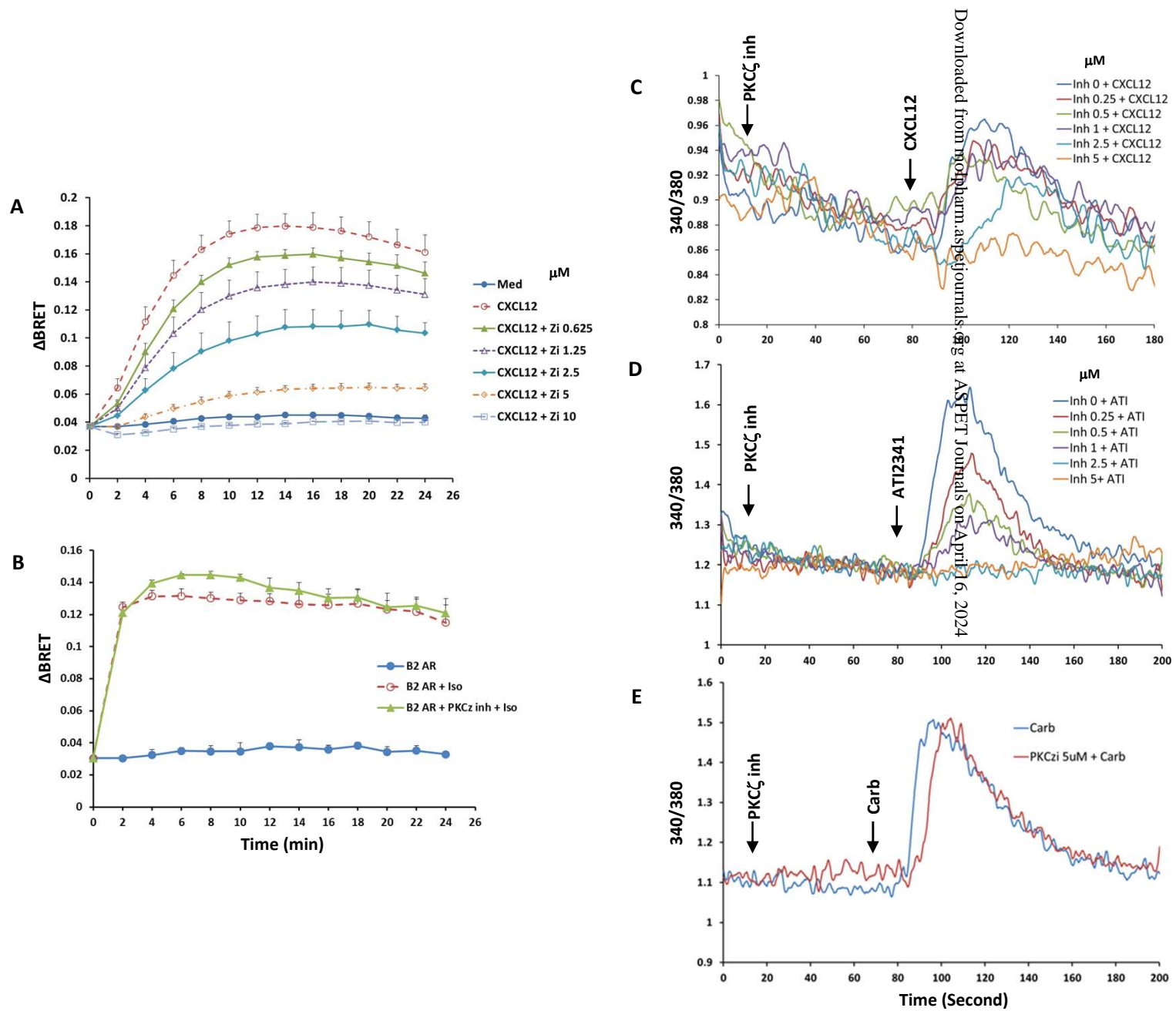


Fig. 8

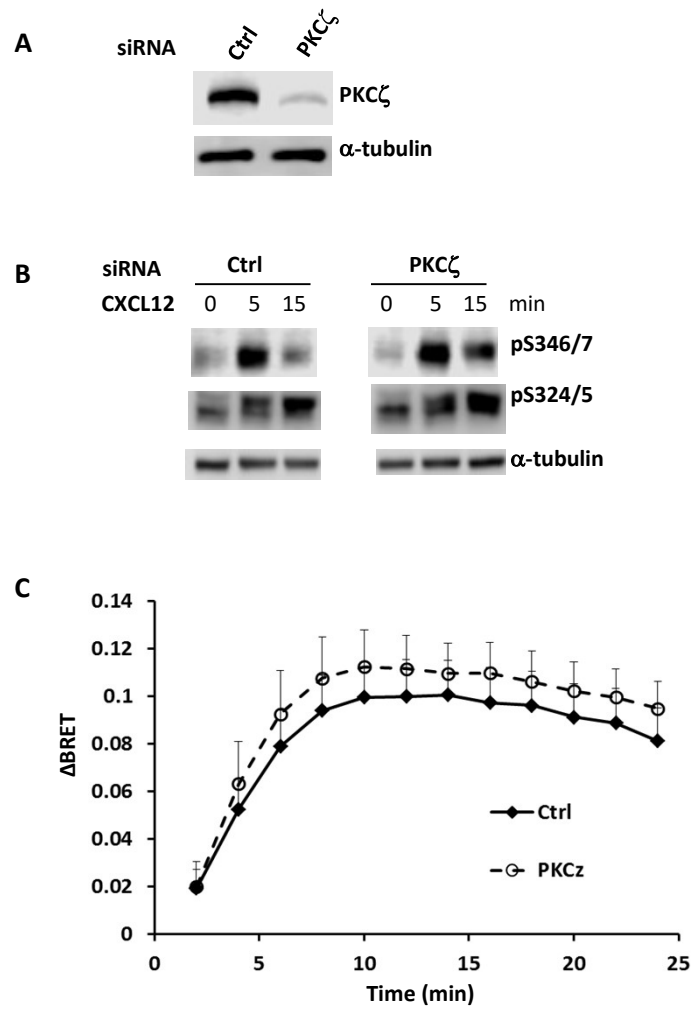


Fig. 9