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β-arrestin 2 Dependent AT_{1A} Receptor Mediated Pathway of Chemotaxis

Dacia L. Hunton, William G. Barnes, Jihee Kim, Xiu-Rong Ren, Jonathan D. Violin, Eric Reiter, Graeme Milligan, Dhavalkumar D. Patel, and Robert J. Lefkowitz

D.L.H. and R.J.L

Howard Hughes Medical Institute,

Duke University Medical Center,

Durham, NC 27710

W.G.B., J.K., X-R.R., J.V., E.R., and R.J.L

Departments of Biochemistry and Medicine,

Duke University Medical Center,

Durham, NC 27710

G.M.

Division of Biochemistry and Molecular Biology

Institute of Biomedical and Life Sciences

University of Glasgow,

Glasgow Scotland, United Kingdom

D.D.P.

Departments of Medicine and Microbiology and Immunology

Division of Rheumatology, Allergy and Immunology

Thurston Arthritis Research Center

University of North Carolina

Chapel Hill, NC 27599

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Corresponding Author:

Robert J. Lefkowitz

Howard Hughes Medical Institute

Departments of Medicine and Biochemistry

Box 3821

Duke University Medical Center

Durham, NC 27710

Tel: (919) 684-2974

Fax: (919) 684-8875

Email: lefko001@receptor-biol.duke.edu

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Abbreviations: $AT_{1A}R$: angiotensin receptor type 1A; Ang II: angiotensin II; HEK: human embryonic kidney; GPCR: G-protein coupled receptor; PKC: protein kinase C; EGF: epidermal growth factor; MAPK: mitogen-activated protein kinase; ERK: extracellular signal-regulated kinase; LPA: lysophosphatidic acid.

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ABSTRACT

Chemotaxis is a cellular response that directs cell migration toward a chemical gradient and is fundamental to a variety of cellular processes. The receptors for most known chemokines belong to the seven transmembrane spanning superfamily and signal through members of the $G_{\alpha i}$ family. β-arrestins, in addition to regulating desensitization, have emerged as potential mediators of G-protein independent signaling pathways and have been implicated in several chemotactic pathways. Here we report a system wherein chemotaxis is stimulated in a β-arrestin 2-dependent and apparently G-protein-independent manner. HEK293 cells with stable expression of the angiotensin II receptor type 1A ($AT_{1A}R$) undergo chemotaxis in response to Ang II. An Ang II peptide analogue $S^1I^4I^8$ Ang II that is unable to activate G-protein mediated responses induces chemotaxis in these cells that is unaffected by pertussis toxin-mediated suppression of $G_{\alpha i}$. Suppression of β-arrestin 2 expression using siRNA essentially eliminated $AT_{1A}R$ mediated chemotaxis induced by either Ang II or the $S^1I^4I^8$ Ang II peptide, while having no effect on EGF induced chemotaxis. It also abolished chemotaxis induced by LPA, which was completely sensitive to pertussis toxin. In contrast, reduction of $G_{\alpha q/11}$ through siRNA and inhibition of PKC, ERK1/2, or PI-3-Kinase did not diminish $AT_{1A}R$ mediated chemotaxis. Inhibiting p38 MAPK decreased $AT_{1A}R$ mediated chemotaxis and eliminated EGF mediated chemotaxis, suggesting that p38 plays a role in chemotaxis that is not specific to the $AT_{1A}R$ in this system. These data suggest that β-arrestin 2 can mediate chemotaxis through mechanisms which may be G-protein independent (Ang II receptors) or dependent (LPA receptors).

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Chemotaxis, the directed migration of cells toward a gradient of chemoattractants, is fundamental to a wide array of cellular processes including development, tissue homeostasis, wound healing, and immune responses (Bayes-Genis et al., 2000; Devreotes and Janetopoulos, 2003; Suzuki et al., 2003). This process, which is often mediated by signaling through seven transmembrane spanning receptors, is generally believed to be $G_{\alpha i}$ protein dependent. Recent evidence from our group and others has also implicated a role for β-arrestin 2 in the chemotactic process (Fong et al., 2002; Sun et al., 2002; Walker et al., 2003). However, the precise role of β-arrestin 2 in chemotaxis and its relation to G-protein activity remains unclear.

By binding to agonist-activated G-protein coupled receptors (GPCRs) such as the angiotensin II receptor type 1 (AT_1R), β-arrestins mediate homologous receptor desensitization and endocytosis via clathrin-coated pits. In addition, β-arrestins have recently emerged as potential mediators of G-protein independent signaling pathways by scaffolding components of the ERK MAPK cascade (Ahn et al., 2004b; Tohgo et al., 2003; Wei et al., 2003) and by acting as adaptors to recruit the tyrosine kinase Src and other proteins into signaling complexes with receptors (McDonald et al., 2000; McDonald and Lefkowitz, 2001).

AT_1R, a typical seven transmembrane spanning receptor, mediates most of the known physiological functions of angiotensin II (Ang II) including aldosterone secretion, vasoconstriction, and chemotaxis (de Gasparo et al., 2000; Sadoshima, 1998; Touyz and Schiffrin, 2000). In addition to its effects on cardiovascular pathology such as arterial hypertension, left ventricular hypertrophy and restenosis (Phillips and Kagiyama, 2002; Ruiz-Ortega et al., 2001; Suzuki et al., 2000), Ang II may initiate the inflammatory

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process (Suzuki et al., 2003). For example, Ang II contributes to the recruitment of inflammatory cells into tissue through the regulation of adhesion molecules and chemokines, and by directly activating chemotaxis (Phillips and Kagiyama, 2002; Riaz et al., 2004; Ruiz-Ortega et al., 2001; Suzuki et al., 2000) in a variety of cell types including monocytes (Ni et al., 2004), vascular smooth muscle cells (Meloche et al., 2000), neonatal cardiac fibroblasts (Graf et al., 2000), retinal pericytes (Nadal et al., 2002), T-cells (Weinstock et al., 1987) and neutrophils (Elferink and de Koster, 1997). A prototypical GPCR, AT_1R signaling is generally dependent on heterotrimeric G-proteins and is known to be primarily coupled to $G_{\alpha q/11}$ and in some circumstances to $G_{\alpha i}$ and $G_{\alpha o}$ (Berk, 2001; de Gasparo et al., 2000; Touyz and Schiffrin, 2000). However, recent studies using peptide analogues of Ang II and mutant AT_1R receptors that are impaired in the activation of G-proteins suggest that $AT_{1A}R$ may also mediate G-protein independent signaling cascades (Ahn et al., 2004b; Hansen et al., 2004; Holloway et al., 2002; Sadoshima, 2002; Wei et al., 2003). For example, mitogen-activated protein kinase (MAPK) and extracellular signal-related kinases 1 and 2 (ERK1/2) can be activated thru the AT_1R in a G-protein independent (Ahn et al., 2004b; Hines et al., 2003; Holloway et al., 2002; Seta et al., 2002; Wei et al., 2003) and a β-arrestin-dependent fashion (Ahn et al., 2003; Ahn et al., 2004b; Wei et al., 2003).

Both p38 and ERK1/2 have been implicated in chemotaxis in general and the ERK1 and ERK2 MAPKs are known to be required for angiotensin II-directed migration of vascular smooth muscle cells in particular (Xi et al., 1999). Accordingly, we hypothesized that the $AT_{1A}R$ system would be a good one in which to attempt to delineate the roles of β-arrestin 2 and G-proteins in chemotaxis. We used an Ang II

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peptide, $S^1I^4I^8$ Ang II, capable of activating ERK via β-arrestin 2 but not G-proteins (Holloway et al., 2002; Wei et al., 2003), as well as siRNAs directed against $G_{\alpha q/11}$ and β-arrestins, in a HEK293 cell model system to delineate the contributions of β-arrestin 2 and G-protein mediated signaling to Ang II induced chemotaxis.

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Materials and Methods

Materials. Tissue culture reagents and pertussis toxin (PTX) were purchased from Sigma (St. Louis, MO). Inhibitor compounds PD98059, RO-31-8425, SB203580, LY294002, wortmanin, and Y-27632 were purchased from Calbiochem (Darmstadt, Germany).

Cell Culture and Transfection. HEK293 cells were cultured in minimum Eagle's medium supplemented with 10% fetal bovine serum (Sigma). For stable transfection of the $AT_{1A}R$, HEK293 cells were transfected with DNA that contained a zeocin selectable marker using FuGENE (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. Stable clones were selected and maintained in the presence of zeocin (300 μg/ml). Whole cell binding determined $AT_{1A}R$ expression levels to be 1.6 +/- 0.2 pmol/mg protein.

Cells were split at least 24 hours prior to transfection and transfected with siRNA designed against β-arrestin 2, $G_{\alpha q/11}$, or control using the Gene Silencer transfection reagent (Gene Therapy Systems, San Diego, CA) as previously described (Ahn et al., 2003). The siRNA sequence targeting $G_{\alpha q/11}$ is 5' AAGATGTTCGTGGACCTGAAC 3', corresponding to the positions 931-951 relative to the start codon for both human $G_{\alpha q}$ and $G_{\alpha 11}$. All assays were performed 72 hours following transfection of siRNA.

Immunoblotting. Whole-cell lysates were separated by SDS/PAGE on 10% Tris-Glycine polyacrylamide gels (Invitrogen, Carlsbad, CA) and immunoblotted with a 1:3000 dilution of the rabbit polyclonal anti β-arrestin antibody A1CT (Attramadal et al., 1992) or a 1:650 dilution of the rabbit polyclonal anti $G_{\alpha q/11}$ antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Immunoblots were quantified by densitometry with a Fluor-S MultiImager (BioRad, Hercules, CA).

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Chemotaxis Assays. The assays were performed in transwell chambers of 24-well inserts with 8-micron pore membranes. Cells were serum starved overnight and assays were conducted in serum free media. Agonists were placed in the lower chamber and 10^{-5} cells were placed in the upper chamber and incubated for 5 hours at 37°C and 5% CO_2. Membranes were then stained with crystal violet and cells were removed from the upper chamber leaving only those cells that migrated through the membrane to the lower chamber. The membrane was then dried, excised, mounted on slides and quantified by densitometry with a Fluor-S MultiImager (BioRad). The chemotactic index was calculated by dividing values from membranes in the stimulated conditions by values from membranes in the control conditions. Values reported are the average of 3-7 experiments performed in duplicate**.**

Calcium Assays. Transfected or untransfected cells were split into glass-bottom dishes at least 12 hours prior to an experiment. Cells were loaded with 1 uM Fura-2 AM (Molecular Probes, Eugene, OR) as described in the manufacturer's instructions, and imaged in Hanks Balanced Salt Solution (Sigma) supplemented with 1.3 mM $CaCl_2$. Intracellular calcium was assayed by Fura-2 excitation ratio, determined by sequential acquisition of 340 nm and 380 nm wavelength excitation of green fluorescence. The excitation ratio was acquired every 5 seconds with a Zeiss Axiovert 200 M fluorescent microscope with filters (Chroma, Rockingham, VT) switched by filter wheels (Sutter, Novato, CA) and a MicroMax camera (Roper, Tucson, AZ) controlled by SlideBook software (Intelligent Imaging Innovations, Baltimore, MD). Stimulated calcium release was calculated as the change in excitation ratio from baseline integrated over 5 minutes of agonist stimulation.

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Results

$AT_{1A}R$ mediated chemotaxis has a G-protein independent component. To assess $AT_{1A}R$-dependent chemotaxis, we developed a stable HEK293 cell line expressing the $AT_{1A}R$ ($AT_{1A}R$-HEK293) and utilized a modified Boyden chamber transwell migration system. $AT_{1A}R$-HEK293 cells reproducibly migrated to Ang II in a dose-dependent manner (Fig 1A), demonstrating that Ang II can activate a chemotactic signaling pathway in these cells. The maximum chemotactic response was achieved at 1 nM Ang II with an average chemotactic index of 4 +/- 0.6 (n=4). Untransfected HEK293 cells were not responsive to Ang II in the transwell migration chemotactic assay (Fig 1A).

In order to assess the relative contributions of the β-arrestin compared to G-protein signaling pathways, we utilized a modified Ang II peptide $S^1I^4I^8$ Ang II. We have previously confirmed that this peptide does not effectively activate G-proteins, but is still capable of activating ERK (Wei et al., 2003) albeit at higher concentrations than Ang II, reflective of its lower affinity for $AT_{1A}R$. To further assess the ability of the $S^1I^4I^8$ Ang II peptide to promote $AT_{1A}R$ activation of G-proteins, we measured calcium increases using Fura-2. The maximum calcium increase induced by the $S^1I^4I^8$ Ang II peptide in $AT_{1A}R$-HEK293 cells was not significantly different from baseline in contrast with the robust calcium increases induced by Ang II (Fig 1B). However, the $S^1I^4I^8$ Ang II peptide was capable of inducing 80% the levels of chemotaxis stimulated by wild type Ang II in the $AT_{1A}R$-HEK293 cells, achieving a chemotactic index of 3.2 +/- 0.5 (n=3) (Fig 1C). HEK293 cells not expressing the $AT_{1A}R$ were not responsive to $S^1I^4I^8$ Ang II (Fig 1C). These data suggest that at least one mechanism of $AT_{1A}R$ mediated chemotaxis may not require G-protein activation following Ang II stimulation.

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Although it primarily stimulates $G_{\alpha q/11}$, the $AT_{1A}R$ is known to couple to $G_{\alpha i}$ in some systems. In many cell types, chemotaxis mediated by seven transmembrane receptors is sensitive to PTX, a selective inhibitor of $G_{\alpha i}/G_{\alpha o}$. To evaluate the contribution of $G_{\alpha i}$, $AT_{1A}R$-HEK293 cells were pretreated with 50 μM PTX for 1 hour prior to the chemotaxis assay. PTX decreased Ang II induced chemotaxis in $AT_{1A}R$-HEK293 cells by approximately 34% (Fig 2A), revealing a substantial (~66%) $G_{\alpha i}$ independent component of $AT_{1A}R$ mediated chemotaxis. Interestingly, these levels of chemotaxis are comparable to the levels of chemotaxis achieved with the $S^1I^4I^8$ Ang II peptide (Fig 1C). Notably, PTX-pretreatment had no effect on $S^1I^4I^8$ Ang II induced chemotaxis (Fig 2B), suggesting that $S^1I^4I^8$ Ang II activates a chemotactic pathway that does not require $G_{\alpha i}$. In contrast, LPA induced chemotaxis was completely prevented by the PTX-pretreatment (Fig 2C), consistent with previous reports (Gerrard et al., 1980; Maghazachi, 2003; Schenk et al., 2001; Stahle et al., 2003).

To investigate a potential role for $G_{\alpha q}$ in $AT_{1A}R$ mediated chemotaxis, we used siRNA against $G_{\alpha q}$ that also targets $G_{\alpha 11}$. Transfection of siRNA against $G_{\alpha q/11}$ reduced endogenous $G_{\alpha q/11}$ expression by an average of 92% +/- 3% compared to cells transfected with control siRNA as detected by Western blotting (Fig 3A). Suppression of $G_{\alpha q/11}$ effectively prevented Ang II stimulated calcium influx as assessed by Fura-2 (Fig 3B). Reduction of $G_{\alpha q/11}$ expression had no significant effect on either wild type Ang II (Fig 3C) or $S^1I^4I^8$ Ang II (Fig 3D) induced chemotaxis in $AT_{1A}R$-HEK293 cells. Thus, $AT_{1A}R$ mediated chemotaxis in this system is uncoupled from calcium influx and from $G_{\alpha q/11}$ signaling.

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$AT_{1A}R$ mediated chemotaxis requires β-arrestin 2. To assess the role of β-arrestin 2 in $AT_{1A}R$ mediated chemotaxis, $AT_{1A}R$-HEK293 cells were transfected with siRNA against β-arrestin 2 or control siRNA. Endogenous β-arrestin 2 expression was reduced by 85% +/- 2% as detected by Western blotting (Fig 4A). β-arrestin 2 knockdown with siRNA led to a profound reduction of both Ang II (Fig 4B) and $S^1I^4I^8$ Ang II (Fig 4C) induced chemotaxis, but had no impact on EGF (Fig 4D) stimulated chemotaxis in $AT_{1A}R$-HEK293 cells. LPA induced chemotaxis was also eliminated by β-arrestin 2 siRNA transfection (Fig 4D).

Effect of p38 MAPK, PKC, PI-3-Kinase, and ERK pathway inhibitors on $AT_{1A}R$ mediated chemotaxis. β-arrestin 2 expression has been previously shown to enhance both ERK and p38 MAPK activation, and reduction of β-arrestin 2 expression has been shown to impair both ERK and p38 MAPK activation for a variety of receptors including the $AT_{1A}R$ (Ahn et al., 2003; Ge et al., 2003; McDonald and Lefkowitz, 2001; Wei et al., 2003). Accordingly, specific inhibitors of these two kinases as well as PKC and PI-3-kinases were used in conjunction with the chemotaxis assay in order to determine if any of these down stream effectors plays a role in $AT_{1A}R$ mediated chemotaxis. $AT_{1A}R$-HEK293 cells were pretreated for 1 hour with the p38 MAPK inhibitor SB203580 (5μM), the ERK pathway inhibitor PD98059 (5 μM), the PKC inhibitor RO-31-8425 (1μM) which also blocks G-protein dependent ERK 1/2 activation in response to Ang II (Ahn et al., 2004b; Wei et al., 2003), or the PI-3-kinase inhibitors LY294003 (1 μM) and wortmanin (100 nM). The cells were then assayed for their chemotactic response to Ang II (Fig 5A) or $S^1I^4I^8$ Ang II (Fig 5B). The ERK pathway inhibitor PD98059, PKC inhibitor RO-31-8425 and PI-3-kinase inhibitors LY294002 and wortmanin showed no

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significant impairment of chemotaxis to either Ang II or $S^1I^4I^8$ Ang II. However, the p38 MAPK inhibitor significantly impaired $AT_{1A}R$ mediated chemotaxis to either Ang II ($P< 0.01$) or $S^1I^4I^8$ Ang II ($P<0.05$) and completely abolished EGF mediated chemotaxis (data not shown). Furthermore, while Ang II and SII were both capable of activating p38 MAPK, silencing of β-arrestin 2 had no effect on this activation (Fig 5C).

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Discussion

In order to investigate the molecular mechanisms for control of chemotaxis by Ang II, an important vasoactive peptide and modulator of the inflammatory process, we developed a model system in HEK293 cells with stable $AT_{1A}R$ expression. Transfected HEK293 cells have served as an important model system for studying chemotaxis mediated by a wide array of ligands (Limatola et al., 2003; Masuda et al., 1999; Neptune et al., 1999; Roland et al., 2003; Su et al., 1999; Ueda et al., 1997). $AT_{1A}R$-HEK293 cells underwent chemotaxis to Ang II in a dose-dependent manner with efficacy similar to that induced by LPA through endogenous receptors.

We found several lines of evidence that $AT_{1A}R$ mediated chemotaxis is largely G-protein independent: First, the Ang II peptide analogue $S^1I^4I^8$ Ang II, that is unable to activate G-protein mediated responses, induced chemotaxis in $AT_{1A}R$-HEK293 cells. Earlier reports illustrating the inability of $S^1I^4I^8$ Ang II to activate G-proteins utilized PI hydrolysis and $GTP\gamma S^{35}$ binding, which occur at higher ligand concentrations than chemotaxis, to determine G-protein coupling (Thomas et al., 2000; Wei et al., 2003). Here, we used a more sensitive calcium assay to assess G-protein pathway activation at concentrations that are appropriate for chemotaxis but which are below the PI hydrolysis threshold for detection. At concentrations of $S^1I^4I^8$ Ang II peptide that were effective at inducing chemotaxis, calcium increases were minimal as compared with the robust calcium increases induced by Ang II at concentrations relevant to chemotaxis. Second, we found that Ang II induced chemotaxis was largely resistant to PTX, and $S^1I^4I^8$ Ang II peptide induced chemotaxis was completely resistant to PTX, consistent with a mechanism for G-protein independent chemotaxis. Third, siRNA directed against $G_{\alpha q/11}$

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had no effect on Ang II or $S^1I^4I^8$ Ang II induced chemotaxis. Finally PKC and PI-3-kinase inhibition were also without effect.

While the actions of most known chemokines are mediated by GPCRs which are sensitive to PTX, a variety of growth factors also serve as chemotactic agents and are PTX insensitive (Bailly et al., 2000; Bayes-Genis et al., 2000; Bennett and Schultz, 1993; Bredin et al., 1999; Caric et al., 2001; Clunn et al., 1997; Cospedal et al., 1999; Puglianiello et al., 2000; Zhao et al., 2002). Our findings suggest the $AT_{1A}R$ is capable of mediating chemotaxis in a G-protein independent fashion. While a previous report found Ang II induced chemotaxis in neutrophils to be PTX sensitive (Elferink and de Koster, 1997) our findings suggest that in HEK293 cells only ~34% of the Ang II stimulated chemotaxis was mediated through $G_{\alpha i}/G_{\alpha o}$ proteins. Of course the possibility that G-proteins are involved to some extent can not be completely eliminated. For example, it has been reported that $G_{\alpha 12}/G_{\alpha 13}$ can couple to the $AT_{1A}R$ (Macrez-Lepretre et al., 1997) and activate Rho (Ushio-Fukai et al., 1999). However, while a Rho kinase inhibitor Y-27632 reduced $AT_{1A}R$ mediated chemotaxis by more than 65%, it also inhibited EGF mediated chemotaxis by more than 80% suggesting that Rho and Rho kinase may play a role in chemotaxis that is not specific to the $AT_{1A}R$ or to the activation of $G_{\alpha 12}/G_{\alpha 13}$ (data not shown). Therefore, taken together these data suggest that the $AT_{1A}R$ is able to mediate chemotaxis through a G-protein independent pathway.

β-arrestins have dual functions in regulating the signals emanating from GPCRs by simultaneously inactivating G-protein mediated signaling while also serving as potential mediators of G-protein independent signaling pathways by scaffolding components of the ERK MAPK cascade and other pathways (Ahn et al., 2004b; Tohgo et

al., 2003; Wei et al., 2003). MAPK ERK1/2 (Ge et al., 2003), p38 MAPK (Sun et al., 2002) and β-arrestin 2 (Fong et al., 2002; Ge et al., 2003; Sun et al., 2002; Walker et al., 2003) have all previously been implicated in a variety of chemotactic pathways. In this study, we found that reduction of β-arrestin 2 expression using siRNA essentially eliminated $AT_{1A}R$ mediated chemotaxis induced by either Ang II or the $S^1I^4I^8$ Ang II peptide. This effect showed receptor specificity as EGF induced chemotaxis was not impaired by the reduction of β-arrestin 2 expression, but LPA induced chemotaxis was completely blocked. However, unlike Ang II, LPA induced chemotaxis was quite sensitive to PTX. These results suggest that β-arrestin 2 may play distinct roles in chemotactic pathways stimulated by different receptors. Thus β-arrestin 2 is involved in both G-protein dependent (LPA receptors) and independent (Ang II receptors) chemotaxis.

We have previously proposed that β-arrestins might directly influence chemotaxis by their ability to serve as signaling adapters or scaffolds for molecules such as MAPKs (Fong et al., 2002). In this study, both Ang II and $S^1I^4I^8$ Ang II induced chemotaxis were sensitive to p38 MAP kinase inhibition, but not ERK1/2 inhibition. However, while both Ang II and $S^1I^4I^8$ Ang II were able to activate p38 MAPK, β-arrestin 2 could not be directly implicated in the p38 activation. It is possible that β-arrestin 1, which has recently been shown to also be activated by $S^1I^4I^8$ Ang II (Barnes et al., 2004), is responsible for the p38 MAPK activation. Alternatively, it may be that the 5 minute time point used in the present experiments was not appropriate to determine a β-arrestin 2 role, as recent evidence suggests that the relative contributions of G protein and β-arrestin dependent signaling pathways are highly dependent upon time, at least with regards to the

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activation of ERK1/2 (Ahn et al., 2004a). However, these results seem to indicate a role for p38 MAPK activation in the chemotaxis system studied here, though it appears not to be the locus of β-arrestin 2 involvement.

Many possibilities exist for how β-arrestins might mediate chemotaxis in a heterotrimeric G protein independent fashion. One possibility is that β-arrestins activate small GTPase signaling pathways which are important for cell motility. It has been shown that arrestins interact with ARNO (Claing et al., 2001) and Ral-GDS (Bhattacharya et al., 2002), the guanine nucleotide exchange factors for the small GTPases Arf6 and Ral, respectively. Furthermore, a recent report demonstrates that β-arrestin 1 mediates activation of the small GTPase RhoA by Ang II and $S^{1}I^{4}I^{8}$ Ang II (Barnes et al., 2004). RhoA has been shown to play important roles in cytoskeletal structure and cell movement. While RhoA activation was facilitated by the simultaneous activation of $G_{\alpha q/11}$ and β-arrestin 1, some RhoA activation was observed even in the absence of $G_{\alpha q/11}$ activation. This raises the distinct possibility that small GTPases that mediate chemotaxis may be activated by β-arrestins.

Taken all together, our data show that β-arrestin 2 may play crucial roles in mediating chemotaxis induced by both G protein dependent and independent mechanisms.

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Footnotes

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Reprint request should be sent to Robert J. Lefkowitz, Howard Hughes Medical Institute, Departments of Medicine and Biochemistry, Box 3821, Duke University Medical Center, Durham, NC 27710.

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Figure Legends

Fig. 1. $AT_{1A}R$ mediated chemotaxis and calcium release in response to Ang II and $S^1I^4I^8$ Ang II. Chemotactic responses and calcium increases were assessed in HEK293 and $AT_{1A}R$-HEK293 cells. Ang II induced dose dependent chemotaxis in $AT_{1A}R$-HEK293 cells with a maximum chemotactic index of 4 +/- 0.6 at 1 nM (A). Calcium responses were measured by fluorescence excitation ratio using Fura-2 as a calcium indicator. Values represent the average total calcium response over 5 minute agonist stimulation with Ang II or $S^1I^4I^8$ Ang II (SII) from three independent experiments**.** The $S^1I^4I^8$ Ang II peptide induced a maximum chemotactic index of 3.2 +/- 0.5 at 3 μM (B). Data are means +/- SEM of at least 3 independent experiments.

Fig. 2. Pertussis toxin sensitivity of chemotaxis. $AT_{1A}R$-HEK293 cells were pretreated with 50 μM pertussis toxin for 1 hour at 37 $^{\circ}$C and subjected to chemotactic assays in response to Ang II (A), $S^1I^4I^8$ Ang II (B), or LPA (C) at the concentrations indicated. Data are means +/- SEM of at least 5 independent experiments, * indicates ($P< 0.05$), and ** indicates ($P< 0.01$).

Fig. 3. Reduction of $G_{\alpha q/11}$ with siRNA fails to impair $AT_{1A}R$ mediated chemotaxis. $AT_{1A}R$-HEK293 cells were transfected with siRNA against $G_{\alpha q/11}$ or control siRNA and subjected to calcium and chemotaxis assays. Extracts of transfected cells were subjected to immunoblotting with an anti-$G_{\alpha q/11}$ antibody to check for $G_{\alpha q/11}$ content. Average reduction of $G_{\alpha q/11}$ was 92 +/- 3% (A). Calcium responses were measured by

fluorescence excitation ratio using Fura-2 as a calcium indicator. Values represent the average total calcium response over 5 minute agonist stimulation with $S^1I^4I^8$ Ang II or Ang II from 3 independent experiments (B). Transfected cells were subjected to chemotaxis assays in response to Ang II (C) or $S^1I^4I^8$ Ang II (D) at the concentrations indicated. Data are means +/- SEM of 3 independent experiments.

Fig. 4. Effect of β-arrestin 2 siRNA on Ang II and $S^1I^4I^8$ Ang II induced chemotaxis. $AT_{1A}R$-HEK293 cells were transfected with siRNA against β-arrestin 2 or control siRNA. Extracts of transfected cells were subjected to immunoblotting with an anti-β-arrestin antibody to check for β-arrestin content. Average reduction of β-arrestin 2 was 85% +/- 2% (A). The cells were then subjected to chemotaxis assays in response to Ang II (B), $S^1I^4I^8$ Ang II (C), EGF, or LPA (D). Data are means +/- SEM of 7 independent experiments, ** indicates ($P< 0.01$).

Fig. 5. Effect of protein kinase inhibitors on $AT_{1A}R$ mediated chemotaxis. $AT_{1A}R$-HEK293 were pretreated for 1 hr with the p38 MAPK inhibitor SB203580 (5μM), the ERK pathway inhibitor PD98059 (5 μM), the PKC inhibitor RO-31-8425 (1μM), or the PI-3-kinase inhibitors LY294003 (1 μM) and wortmanin (100 nM) and assayed for their chemotactic response to Ang II (A) or $S^1I^4I^8$ Ang II (B). (C) Ang II and $S^1I^4I^8$ Ang II activation of p38 MAPK with or without β-arrestin 2 depletion. Basal p38 activation was subtracted from all ligand-stimulated groups (Ang II activated p38 MAPK 3.8-fold over basal). Data are means +/- SEM of at least 3 independent experiments, * indicates ($P< 0.05$).

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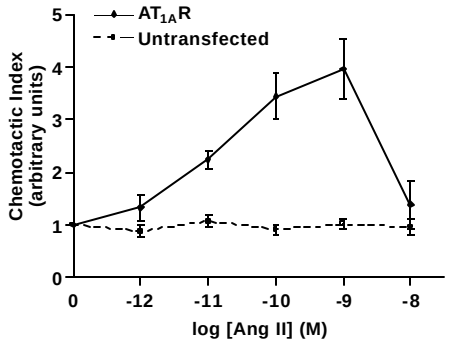
Fig 1

A



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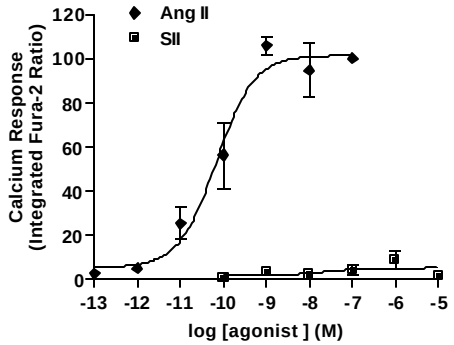

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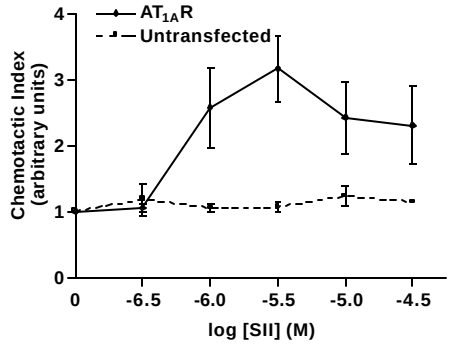

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Fig 2

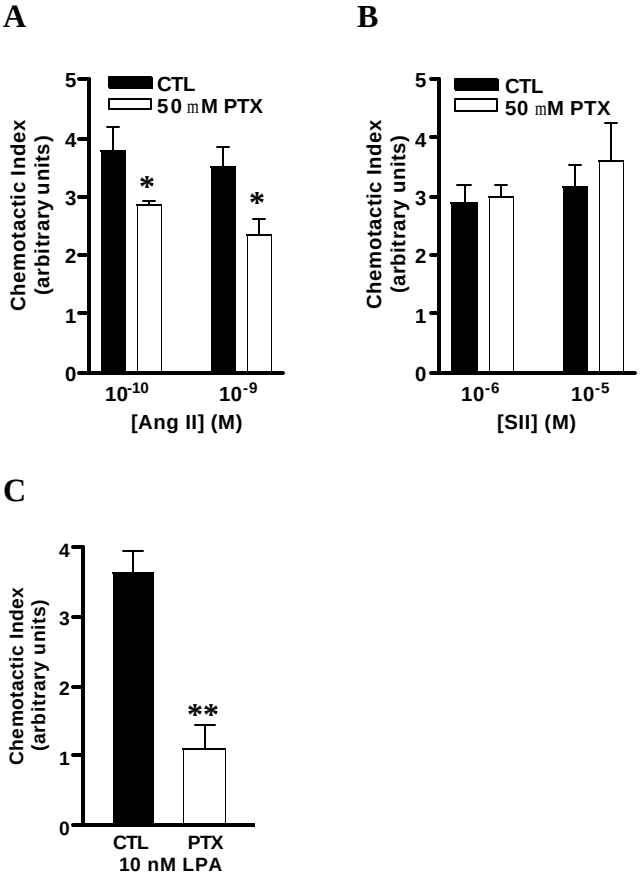

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Fig 3

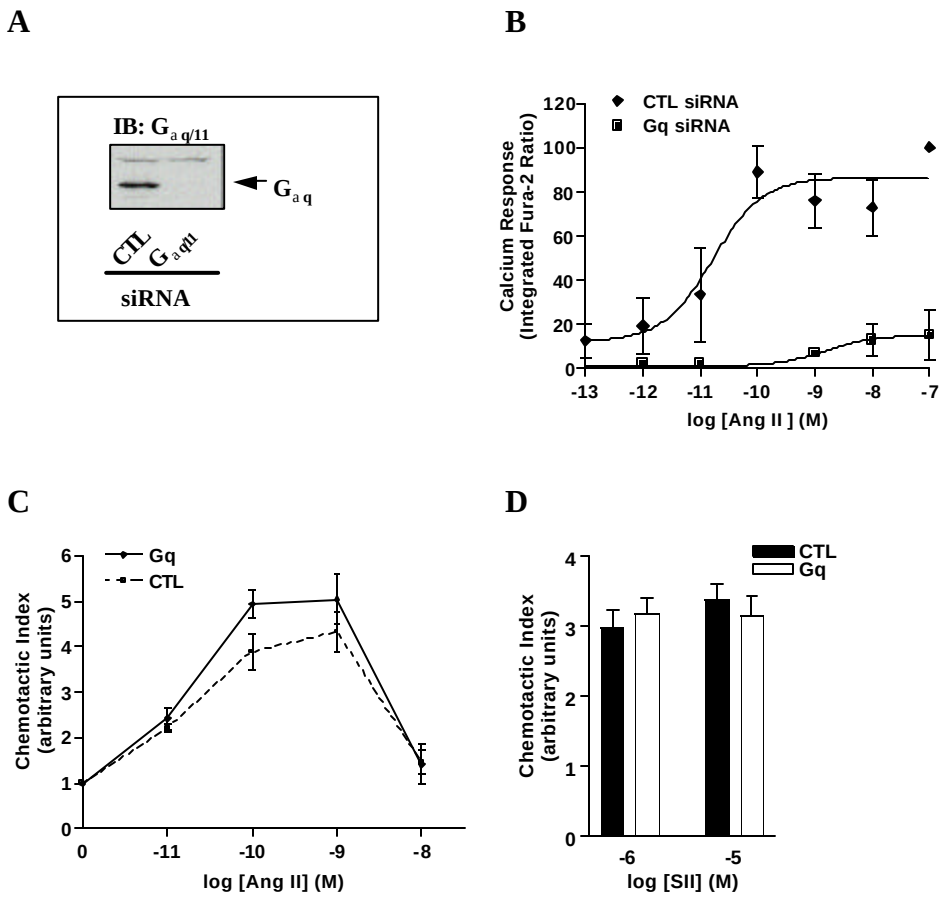

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Fig 4

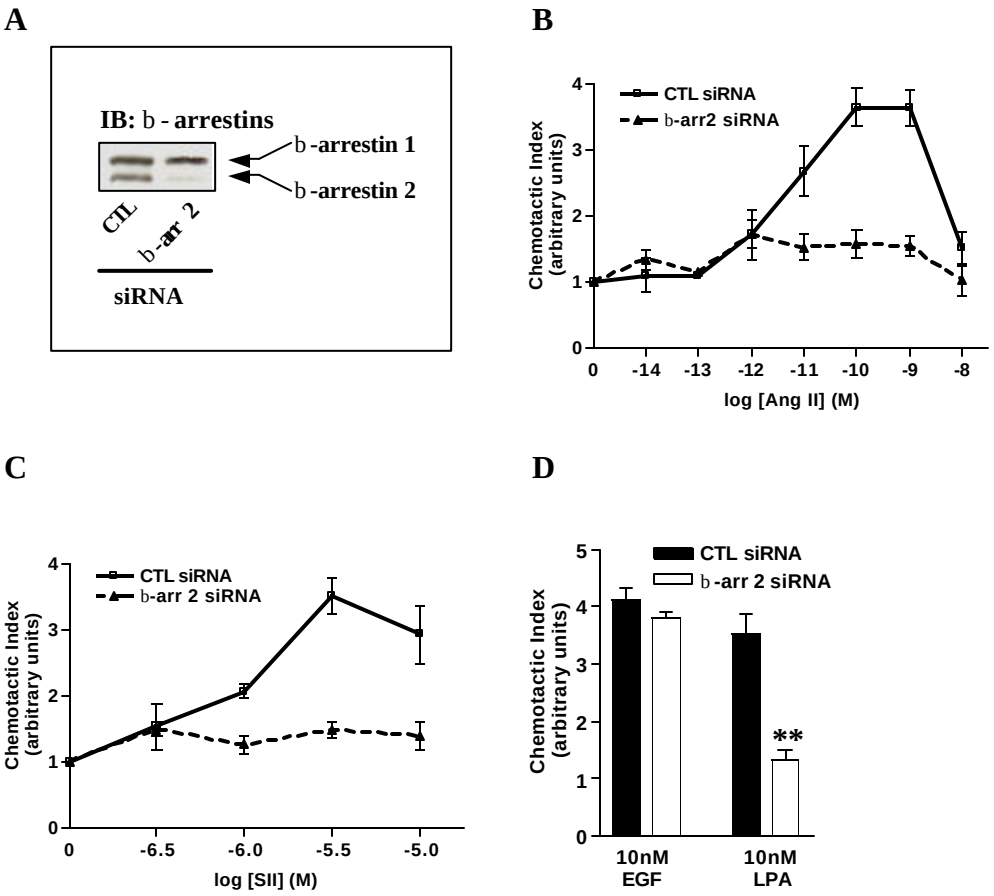

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Fig 5