Mol. Cells *28*, 25-30, July 31, 2009
DOI/10.1007/s10059-009-0104-1

Molecules and Cells

A Protein Tyrosine Phosphatase Inhibitor, Pervanadate, Inhibits Angiotensin II-Induced β-Arrestin Cleavage

Sei-Heon Jang, Si Ae Hwang, Mijin Kim, Sung-Hae Yun[1,3], Moon-Sook Kim[1], Sadashiva S. Karnik[2], and ChangWoo Lee*

β-Arrestins turn off G protein-mediated signals and initiate distinct G protein-independent signaling pathways. We previously demonstrated that angiotensin AT_1 receptor-bound β-arrestin 1 is cleaved after Phe^{388} upon angiotensin II stimulation. The mechanism and signaling pathway of angiotensin II-induced β-arrestin cleavage remain largely unknown. Here, we show that protein Tyr phosphatase activity is involved in the regulation of β-arrestin 1 cleavage. Tagging of green fluorescent protein (GFP) either to the N-terminus or C-terminus of β-arrestin 1 induced conformational changes and the cleavage of β-arrestin 1 without angiotensin AT_1 receptor activation. Orthovanadate and molybdate, inhibitors of protein Tyr phosphatase, attenuated the cleavage of C-terminal GFP-tagged β-arrestin 1 *in vitro*. The inhibitory effects of okadaic acid and pyrophosphate, which are inhibitors of protein Ser/Thr phosphatase, were less than those of protein Tyr phosphatase inhibitors. Cell-permeable pervanadate inhibited angiotensin II-induced cleavage of β-arrestin 1 in COS-1 cells. Our findings suggest that Tyr phosphorylation signaling is involved in the regulation of angiotensin II-induced β-arrestin cleavage.

INTRODUCTION

The angiotensin AT_1 receptor belongs to the G protein-coupled receptor family and transduces diverse signals in G protein- and β-arrestin-dependent pathways (Oro et al., 2007). Upon angiotensin II binding to the angiotensin AT_1 receptor, a conformational change in the receptor modulates G protein, Gq, and activates phospholipase C_β for IP_3 and Ca^{2+}-mediated vasoconstriction (Oro et al., 2007). β-Arrestin 1 and 2, ubiquitously expressed in mammalian tissues, are recruited from the cytoplasm to the activated angiotensin AT_1 receptor for clathrin-mediated endocytosis (Krupnick et al., 1997). In addition to the role in desensitization and endocytosis, β-arrestins form scaffolds for a number of signaling molecules in mitogen-activated protein kinase (MAPK) cascades and non-receptor Tyr kinases for G protein-independent signals (Lefkowitz and Shenoy, 2005). The β-arrestin 1 and 2 are comprised of N- and C-domains of equal size (Han et al., 2001), with the N-domain serving as a phosphate sensor recognizing the phosphorylated G protein-coupled receptor (Nobles et al., 2007) and the C-domain acting as adaptor binding sites for clathrin and AP-2 (Lin et al., 1997). Previous studies have shown that there are distinct inactive and active conformations in β-arrestin. In the inactive state, the polar core between the N- and C-domain of β-arrestin 1 is kept intact with the C-terminus buried in the structure (Han et al., 2001). However, once β-arrestin 1 is bound to the phosphorylated receptor, a conformational change in β-arrestin occurs and extrudes its C-terminal region towards the outside, thus enabling the binding of clathrin and AP-2 for clathrin-mediated endocytosis (Lefkowitz and Shenoy, 2005). β-Arrestin 1 and 2 have distinct active conformations when bound to the activated receptor (Nobles et al., 2007; Xiao et al., 2004).

In a previous study, we showed that a fraction of β-arrestin 1 bound to the angiotensin AT_1 receptor is cleaved upon receptor activation and it requires stable interaction between the angiotensin AT_1 receptor and β-arrestin (Lee et al., 2008). Angiotensin II and inverse agonist EXP3174 induced cleavage at distinct sites on β-arrestin 1; after Phe^{388} upon angiotensin II treatment and after Pro^{276} upon EXP3174 treatment, respectively. These results suggested ligand-induced selectivity in β-arrestin-mediated signaling. With distinct cleavage sites of β-arrestin 1 and 2 by angiotensin II, our data also demonstrated that the receptor-bound active conformations of β-arrestin 1 and 2 are different. The biological significance and the signaling pathway of ligand-induced β-arrestin cleavage, however, are largely unknown.

In this study, we sought to elucidate the regulatory mechanism of angiotensin II-mediated β-arrestin 1 cleavage in COS-1 cells expressing the angiotensin AT_1 receptor. Since protein kinase activation has been implicated in the activation of prote-

Department of Molecular Biology, Daegu University, Gyeongsan 712-714, Korea, [1]Daegu Science High School, Daegu 706-852, Korea, [2]Department of Molecular Cardiology, Lerner Research Institute, The Cleveland Clinic Foundation, Cleveland, OH 44195, USA, [3]Present address: Department of Biochemistry, College of Life Sciences and Biotechnology, Yonsei University, Seoul 120-749, Korea
*Correspondence: leec@daegu.ac.kr

Received March 8, 2009; revised May 20, 2009; accepted June 9, 2009; published online July 8, 2009

Keywords: β-arrestin, angiotensin AT_1 receptor, orthovanadate, pervanadate, protein tyrosine phosphatase

ases downstream of angiotensin AT_1 receptor (Eguchi et al., 2001), we hypothesized that β-arrestin proteolysis is regulated by cellular protein kinases or phosphatases. We used inhibitors of protein Tyr or Ser/Thr phosphatase to investigate the effect on β-arrestin proteolysis. Here, we show that cell-permeable pervanadate inhibits angiotensin II-induced cleavage of β-arrestin 1 in COS-1 cells. Our finding suggests that protein Tyr phosphatase activity is involved in the regulation of G protein-coupled receptor-engaged β-arrestin proteolysis.

MATERIALS AND METHODS

Materials

Angiotensin II was purchased from Bachem (USA). Lipofectamine 2000 was purchased from Invitrogen (USA). Monoclonal antibodies to myc and β-arrestin 2 (H-9) were purchased from Santa Cruz Biotechnology, Inc. (USA). Since the anti-β-arrestin 2 antibody (H-9) recognized both β-arrestin 1 and 2 in our previous studies (Lee et al., 2007; 2008), we used this antibody for the detection of transfected β-arrestin 1 in this study. Monoclonal antibody to GFP was purchased from Clontech (USA). Horseradish peroxidase (HRP)-labeled goat anti-mouse secondary antibody was purchased from Upstate (USA). COS-1 cells were purchased from American Type Culture Collection (USA). Western blot stripping buffer was purchased from Pierce (USA). All other reagents, unless stated otherwise, were from Sigma (USA).

Construction of myc-β-arrestin 1, GFP-β-arrestin 1, and β-arrestin 1-GFP plasmids

Three fusion constructs with myc or GFP-tagged β-arrestin 1 were generated by polymerase chain reaction by the following primers using *pfu* DNA polymerase. For Myc-β-arrestin 1, the forward primer was 5′-AACCGGATCCG**ATGGGCGACAAAGGGAC**-3′ (*Bam*HI site underlined, and the N-terminus of β-arrestin 1 in boldface type), and the reverse primer was 5′-AACCCTCGAGCTA**TCTGTCGTTGAGCCGCGGAG**-3′ (*Xho*I site underlined, the C-terminus of β-arrestin 1 in boldface type). For the N-terminal GFP-tagged GFP-β-Arrestin 1, the cDNA for cloning was subcloned into pEGFP-C1 vector (Clontech). The forward primer was 5′-AACCCTCGAGCC**ATGGGCGACAAAGGGAC**-3′ (*Xho*I site underlined, and the N-terminus of β-arrestin 1 in boldface type), and the reverse primer was 5′-AACCGGATCCCTA**TCTGTCGTTGAGCCGCG**-3′ (*Bam*HI site underlined, and the C-terminus of β-arrestin 1 in boldface type). The C-terminal GFP tagged β-Arrestin 1-GFP was generated using pEGFP-N3 vector. The forward primer was 5′-CCCCCTCGAGTCTACC**ATGGGCGACAAAGGGAC**-3′ (XhoI site underlined, and the N-terminus of β-arrestin 1 in boldface type), and the reverse primer was 5′-AACCGGATCC**TCTGTCGTTGAGCCCGCGGAGAGC**-3′ (*Bam*HI site underlined, and the C-terminus of β-arrestin 1 in boldface type). Accuracy of the fusion constructs in the expression vector was confirmed by DNA sequence analysis.

Cell culture and expression of the angiotensin AT_1 receptor and β-arrestin 1

The synthetic rat angiotensin AT_1 receptor gene, cloned in the shuttle expression vector pMT3, was used for expression. To express the angiotensin AT_1 receptor and β-arrestin 1, 60-65% confluent COS-1 cells were grown in 6-well plates and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. The cells were transfected with 2 μg of purified angiotensin AT_1 receptor and β-arrestin 1 cDNA using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions.

Western blotting

The following protocol was used for the detection of cleaved β-arrestin fragment. Transfected cells, cultured for 48 h, were treated with 1 μM angiotensin II or mock. Cells were washed with cold PBS and centrifuged at 13,000 rpm for 1 min at 4°C. Cell pellets were lysed in 100 μl of lysis buffer (25 mM HEPES, pH 7.2, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM PMSF, 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 10% glycerol) for 60 min. The Sigma protease inhibitor mixture (P2714) was added to the lysis buffer. Cell lysates were centrifuged at 13,000 rpm for 10 min at 4°C to remove cell debris. Protein concentration was measured using Bradford assay kit (Bio-Rad). The 20 μg of total protein was dissolved in Laemmli's sample buffer, boiled for 5 min at 95°C, and separated by SDS-PAGE with a 10% separation gel. Following electrophoresis, the proteins were transferred to nitrocellulose membranes and then blocked for 1 h at room temperature in 5% non-fat dry milk and 0.1% Tween-20 in PBS, pH 7.4. Incubation with primary antibody was carried out overnight at 4°C. Following washes with PBS, incubation with HRP-labeled secondary antibody was carried out for 1 h at room temperature. The detection was made with enhanced chemiluminescence (GE Healthcare) and the films were scanned for densitometry analysis using Fuji Multiguage V3.0 Software (Fuji Film). For the data shown in Fig. 3, the experimental procedures described above were followed except that the lysis buffer was prepared without any phosphatase inhibitors. Each phosphatase inhibitor component was added separately into lysis buffer to a final concentration of 25 mM β-glycerophosphate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM okadaic acid, 1 mM sodium orthovanadate, or 1 mM sodium molybdate. Data analysis was performed using GraphPad Prism 4 (GraphPad Software). Student's *t*-test was used for statistical analysis in Figs. 1 and 3.

Pervanadate preparation and treatment

Pervanadate was prepared using a 2 min incubation of 100 mM sodium orthovanadate and hydrogen peroxide in distilled water, followed by dilution to the appropriate concentration in serum-free DMEM. The solutions were used within 1 h of preparation.

RESULTS

β-Arrestin cleavage in COS-1 cells

In our previous pull-down assays, we demonstrated that β-arrestin 1 bound to the angiotensin AT_1 receptor is cleaved upon angiotensin II stimulation in rat aortic smooth muscle (A7r5) cells and COS-1 cells (Lee et al., 2008). To detect the cleavage of β-arrestin 1 without immunoprecipitation, samples were blotted with anti-β-arrestin antibody (H-9) after cell lysis, SDS-PAGE, and transfer to nitrocellulose membrane. As shown in Fig. 1A, the cleaved β-arrestin 1 was detected and band intensity for the cleaved fragment was increased upon angiotensin II treatment (1.34 ± 0.13 fold, $P < 0.05$).

To confirm the cleavage event, the N-terminal myc-tagged β-arrestin 1 was constructed and assayed for the cleavage with anti-myc or anti-β-arrestin antibody. Surprisingly, substantial amount of cleaved myc-β-arrestin 1 fusion protein was found in the absence of angiotensin AT_1 receptor in transiently transfected COS-1 cells. We speculate that the myc fusion to the N-terminus of β-arrestin 1 induces a conformational instability in β-arrestin. The cleavage of myc-β-arrestin 1 was enhanced following angiotensin II treatment (1.26 ± 0.11 fold, $P < 0.05$, blot-

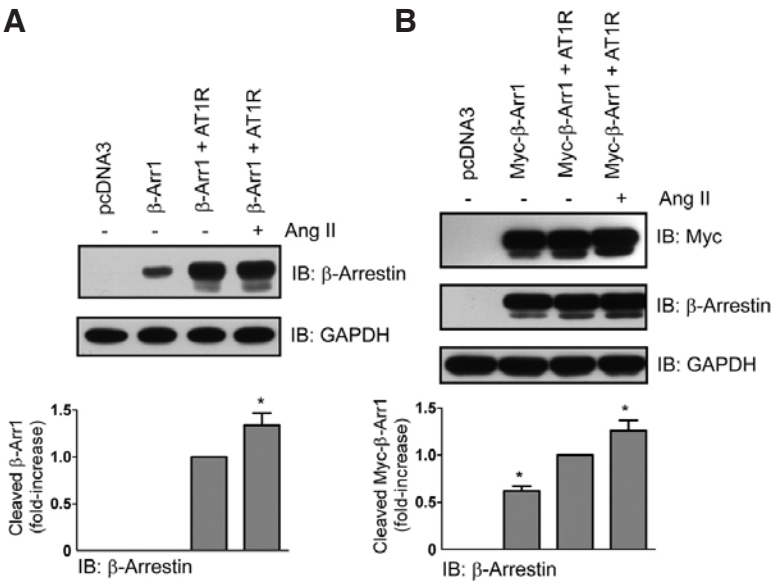

Fig. 1. β-Arrestin cleavage in COS-1 cells. (A) β-arrestin 1 was co-expressed with the angiotensin AT_1 receptor in COS-1 cells. The cleavage of β-arrestin upon angiotensin II stimulation was detected by anti-β-arrestin antibody (1:1,000). GAPDH was used as a loading control. (B) The N-terminal myc-tagged β-arrestin 1 was co-expressed with the angiotensin AT_1 receptor. The cleavage of β-arrestin was detected by anti-myc-antibody (1:1,000) (upper panel) or by anti-β-arrestin antibody (1:1,000) (middle panel). GAPDH was used as a loading control (bottom panel). Bar graph shows the band intensity of myc-β-arrestin 1 fusion protein blotted with anti-β-arrestin antibody shown in the middle panel. Experiments were repeated three times. A representative blot is shown. *IB*, immunoblot. *, $P < 0.05$.

ted with anti-β-arrestin antibody). In both Figs. 1A and 1B, fold-increase of the cleaved β-arrestin 1 fragment upon angiotensin II treatment were alike, suggesting involvement of a similar mechanism in the regulation of cleavage. Since the myc-tag is localized in the N-terminus of β-arrestin 1, the cleavage site is considered to be localized in the C-terminal region of β-arrestin 1 as was reported (Lee et al., 2008).

Effect of GFP fusion on conformational changes in β-arrestin 1

To investigate the effect of a fusion protein on conformational changes in β-arrestin 1 that could lead to subsequent cleavages, GFP fusion at the N-terminus or the C-terminus of β-arrestin 1 (82 kDa) was constructed, respectively. The expression of each GFP-tagged β-arrestin 1 construct alone in COS-1 cells induced cleavages in β-arrestin at distinct sites. The N-terminal GFP fusion protein was cleaved at three sites in β-arrestin 1 (see Fig. 2A). We assume that the cleavage at Phe^{388} in β-arrestin 1 is responsible for generating the ~78 kDa band, and the generation of the smaller band is due to cleavage at sites not defined yet in the N-terminal region of β-arrestin 1. The C-terminal GFP fusion showed a different pattern of cleavage (see Fig. 2B). However, it also included the anticipated cleavage in β-arrestin 1, Phe^{388} (~31 kDa band indicated by an arrow). When each of the two GFP-tagged β-arrestin 1 constructs were separately co-expressed with the angiotensin AT_1 receptor in COS-1 cells and stimulated by angiotensin II, there was no increase of cleaved β-arrestin bands for either N-terminal or C-terminal GFP-tagged β-arrestin 1 (Figs. 2A and 2B). This suggests that GFP fusion induces an intrinsic conformational change in β-arrestin, thus destabilizing its polar core and releasing the buried C-terminus of β-arrestin towards the outside. This finding of GFP-induced conformational change in β-arrestin as with myc-tag is consistent with previous findings that ligand induces a conformational change in ligand-receptor-arrestin ternary complex leading to the cleavage of visual arrestin (Azarian et al., 1995) and β-arrestin (Lee et al., 2008).

Effect of phosphatase inhibitors on the cleavage of β-arrestin 1-GFP fusion protein

We investigated whether inhibitors of protein Tyr or Ser/Thr phosphatase could inhibit the proteolysis of β-arrestin 1. We utilized the characteristic that, after cleavage, the C-terminal GFP-tagged β-arrestin 1 fusion protein yields more distinct band separation on Western blot than wild-type β-arrestin 1, resulting in the generation of a ~31 kDa fragment shown in Fig. 2B.

We examined the effect of Tyr or Ser/Thr phosphatase inhibitors on this cleavage. After preparing lysis buffer without any phosphatase inhibitors, we added β-glycerophosphate, sodium fluoride, sodium pyrophosphate, okadaic acid, sodium orthovanadate, or sodium molybdate into separate lysis buffers. To our surprise, sodium orthovanadate and sodium molybdate, both inhibitors of protein Tyr phosphatase, inhibited the cleavage of β-arrestin 1-GFP 43% (0.57 ± 0.10, $P < 0.05$) and 37% (0.63 ± 0.10, $P < 0.01$), respectively, as shown in Fig. 3, compared to when all phosphatase inhibitors were absent (fourth lane from left). Sodium pyrophosphate and okadaic acid, inhibitors of protein Ser/Thr phosphatase, inhibited the cleavage 35% (0.65 ± 0.05, $P < 0.01$) and 34% (0.66 ± 0.11, $P < 0.05$), respectively. However, sodium fluoride and β-glycerophosphate, which belong to Ser/Thr phosphatase inhibitors, had a lesser effect on the inhibition of β-arrestin 1 proteolysis, 9% (0.91 ± 0.10) and 21% (0.79 ± 0.19, $P < 0.05$), respectively. Sodium fluoride is also used as an inhibitor of acid phosphatase. When all phosphatase inhibitors were included in lysis buffer, the inhibitory effect was similar to the effect of sodium orthovanadate. Thus, our data suggests that protein Tyr phosphatase inhibitors, orthovanadate and molybdate, have larger inhibitory effects than protein Ser/Thr phosphatase inhibitors on the proteolysis of β-arrestin 1-GFP fusion protein.

Inhibition of angiotensin II-induced β-arrestin 1 cleavage by pervanadate

Although we have observed *in vitro* that inhibitors of both protein Tyr phosphatase and protein Ser/Thr phosphatase are effective in the inhibition of β-arrestin 1-GFP fusion protein cleavages, its cellular effect on angiotensin II-induced β-arrestin 1 cleavage is unknown. Hence, we preincubated COS-1 cells expressing the angiotensin AT_1 receptor and β-arrestin 1 with either cell-permeable pervanadate or okadaic acid for 30 min before angiotensin II treatment. Cells were stimulated with 1 μM

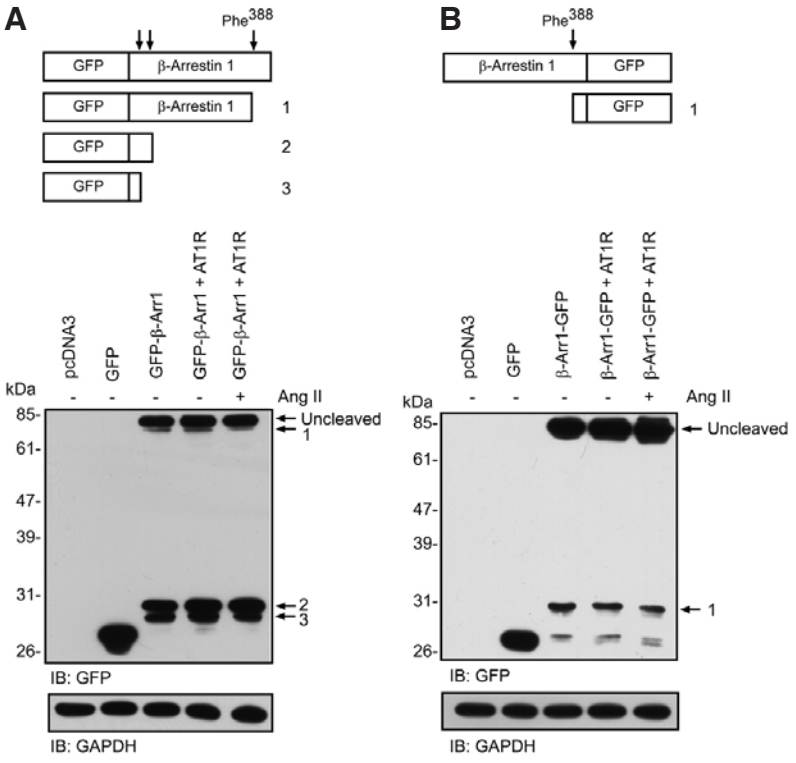

Fig. 2. Effect of GFP fusion on conformational changes in β-arrestin 1. (A) N-terminal GFP-tagged β-arrestin 1 was co-expressed with the angiotensin AT_1 receptor in COS-1 cells. Schematic diagrams depict the cleaved fragments shown in the Western blot. (B) C-terminal GFP-tagged β-arrestin 1 was co-expressed with the angiotensin AT_1 receptor in COS-1 cells. The C-terminal cleavage site, Phe^{388}, is labeled and marked with an arrow in (A) and (B). Experiments were repeated four times. A representative blot is shown. *IB*, immunoblot.

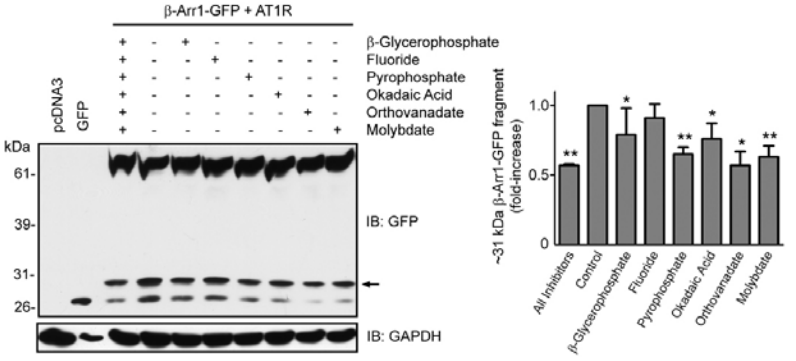

Fig. 3. Effect of phosphatase inhibitors on the cleavage of C-terminal GFP-tagged β-arrestin. β-Arrestin 1-GFP fusion protein was expressed in COS-1 cells in the absence of the angiotensin AT_1 receptor. Cell lysis buffer was prepared without any phosphatase inhibitors and each phosphatase inhibitor was added separately for a final concentration of 25 mM β-glycerophosphate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM okadaic acid, 1 mM sodium orthovanadate, or 1 mM sodium molybdate. The band intensity of the C-terminal cleaved β-arrestin 1-GFP (~31 kDa, indicated by an arrow) was expressed in the bar graph as a fold-increase over the band intensity in which phosphatase inhibitors were absent (fourth lane from left). The 20 μg of cell lysates were loaded on the SDS gel. For the GFP sample (second lane from left), a control was used, whereby 5 μg of cell lysate was loaded on the gel to adjust the band intensity to GFP bands that appeared in other lanes. The cleavage of β-arrestin was detected by anti-GFP-antibody (1:4,000). GAPDH was used as a loading control. Experiments were repeated five times. A representative blot is shown. *IB*, immunoblot. *, $P < 0.05$. **, $P < 0.01$.

angiotensin II for 1 h and harvested. During cell lysis phosphatase inhibitors described in "Materials and Methods" were included in the lysis buffer. Compared to angiotensin II-induced β-arrestin 1 cleavage shown in Fig. 1, the pretreatment of 200 μM and 500 μM pervanadate prevented the cleavage of angiotensin II-induced β-arrestin 1 in COS-1 cells (see Fig. 4A). Pervanadate itself did not interfere with the expression of β-arrestin 1 as shown in Fig. 4B. Contrary to the effect of pervanadate, pretreatment of COS-1 cells with cell-permeable okadaic acid did not prevent the proteolysis of β-arrestin 1. This data supports our hypothesis that protein kinase activation, especially through Tyr phosphorylation, is implicated in the activation of proteases downstream of angiotensin AT_1 receptor signaling pathway leading to the cleavage of β-arrestin.

Orthovanadate and molybdate concentration-dependent inhibition of β-arrestin 1-GFP cleavage

Next, we examined concentration-dependent inhibition of β-arrestin 1-GFP cleavage by protein Tyr phosphatase inhibitors using the ~31 kDa β-arrestin 1-GFP fragment. The attenuation of β-arrestin 1 cleavage correlated with increasing concentrations of orthovanadate and molybdate, 0.01 to 1 mM, as shown in Fig. 5. It was shown that protein tyrosine phosphatase activities in cytosolic fraction of cultured rat hepatocytes were inhibited with the IC_{50} values of 0.03-0.05 mM (Pugazhenthi et al., 1996). Our data indicates that the cleavage of β-arrestin 1 is inhibited by protein tyrosine phosphatase inhibitors in a dose-dependent manner and the IC_{50} values are between 0.1 to 0.5 mM for orthovanadate (see Fig. 5A) and close to 0.1 mM for

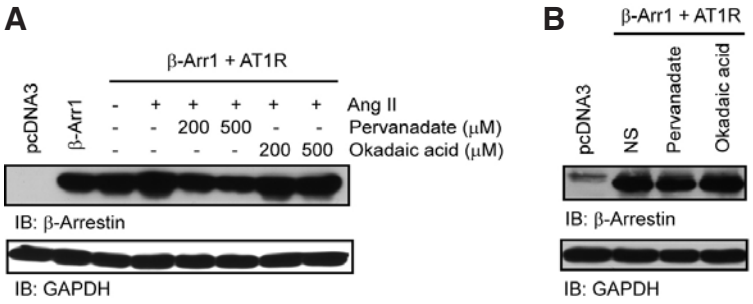

Fig. 4. Inhibition of angiotensin II-induced β-arrestin cleavage in COS-1 cells by pervanadate. Cells were pretreated with pervanadate or okadaic acid for 30 min, before angiotensin II treatment. The cell lysis buffer contains phosphatase inhibitors described in "Materials and Methods" (10 mM sodium pyrophosphate, 10 mM sodium fluoride, and 1 mM sodium orthovanadate). Experiments were repeated three times. A representative blot is shown. *NS*, nonstimulated; *IB*, immunoblot.

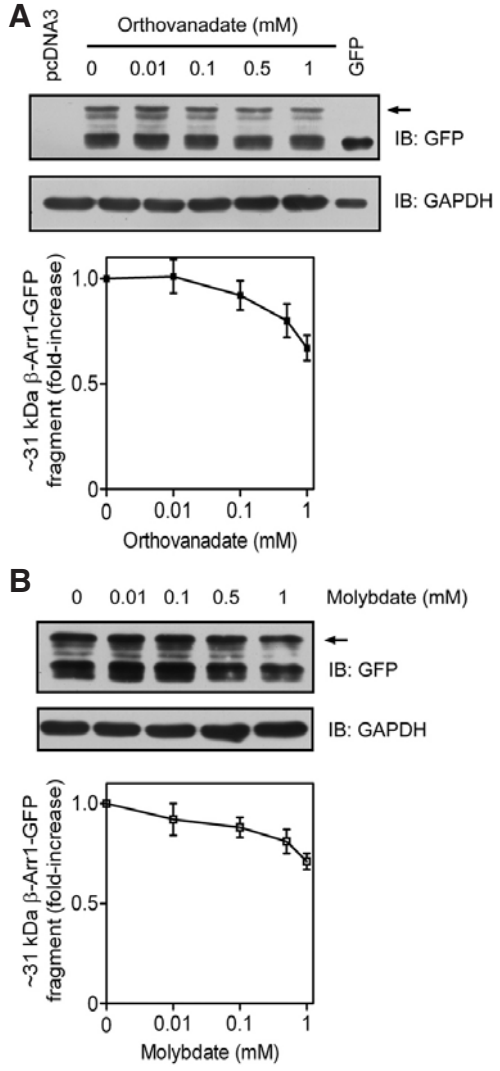

Fig. 5. Orthovanadate and molybdate concentration-dependent inhibition of β-arrestin 1-GFP cleavage. Cell lysis buffer was prepared with increasing concentrations of orthovanadate or molybdate in addition to PMSF and the protease inhibitor mixture. (A) Inhibition by orthovanadate; the band indicated by an arrow in Fig. 5A was plotted in the graph as fold-increase (■). (B) Inhibition by molybdate; the band indicated by an arrow in Fig. 5B was plotted in the graph as fold-increase (□). The 20 μg of cell lysates were loaded on the SDS gel. For the GFP sample, 5 μg of cell lysates was used as a control for the cleaved GFP fragment. The cleavage of β-arrestin was detected by anti-GFP-antibody (1:4,000). GAPDH was used as a loading control. *IB*, immunoblot.

molybdate (see Fig. 5B). Since we measured the band intensity of cleaved β-arrestin 1-GFP fragment rather than the enzymatic activities of protein tyrosine phosphatase, we speculate that the IC_{50} values of protein tyrosine phosphatase will be lower than what we measured in this study.

DISCUSSION

The experiments presented in this study show that protein Tyr phosphatase activity is involved in the regulation of β-arrestin cleavage. Although the biological significance of angiotensin II-induced β-arrestin cleavage is unclear, it raises the possibility that once β-arrestin is cleaved, β-arrestin-mediated internalization of angiotensin AT_1 receptor may be attenuated. The angiotensin AT_1 receptor mediates diverse hormonal signals, leading to vasoconstriction and water-electrolyte balance through G protein-dependent and β-arrestin-dependent signaling pathways (Oro et al., 2007).

The ligand-receptor induced conformational changes in β-arrestin disrupt the intramolecular interaction of the basic N-domain and acidic C-domain, which allows binding of a hydrophobic portion of arrestin distal to Arg^{175} and subsequent release of the β-arrestin C-terminus (Vishnivetskiy et al., 1999). Our previous report shows that a specific conformation of ligand-receptor-β-arrestin ternary complex is required for the site-specific cleavage of β-arrestin 1 and the cleavage is independent of IP_3 and Ca^{2+}-mediated signaling pathway (Lee et al., 2008). It was shown that the N-domain of β-arrestin is protected from *in vitro* tryptic digestion upon binding to phosphorylated receptor C-terminal region (Nobles et al., 2007), but N-terminal GFP fusion of β-arrestin 1 in our study destabilized the structure of β-arrestin, thus allowing cleavages at additional sites in the N-terminal region of β-arrestin 1 (see Fig. 2A). The N-terminal myc-tag generated a cleavage in the C-terminal region of β-arrestin 1, which perhaps is caused by less steric hindrance in the N-domain by the small sized myc-tag. Although GFP fusion to β-arrestin has been shown not to interfere with the normal functioning of β-arrestin such as translocation and endocytosis (Barak et al., 1997), our data indicates that GFP fusion to the N-terminus or the C-terminus of β-arrestin 1 induces distinct conformational changes in β-arrestin 1 and subsequent proteolysis by protease(s). The appearance of cleaved β-arrestin bands in the absence of angiotensin AT_1 receptor activation suggests that conformational changes in β-arrestin by GFP fusion occur intrinsically. Our data suggests that using C-terminal GFP-tagged β-arrestin in imaging studies might show the movement of GFP tethered with the cleaved short fragment (4 kDa) as well as full-length β-arrestin. The two additional cleavage sites in the N-terminal region of β-arrestin shown in GFP-β-arrestin fusion protein need to be determined to elucidate whether the same protease or another protease with different specificity is involved in the cleavage of β-arrestin.

Phosphorylation plays a key role in determining whether cell signaling will increase or decrease the activity of a protein. Several kinases and phosphatases have been shown to interact with the angiotensin AT_1 receptor including Janus kinase, c-Src, SHP-1 and SHP-2 (Marrero et al., 1996; 1998). Since adding phosphatase inhibitors in cell culture medium or lysis buffer alter the general phosphorylation status of a cell or cell lysates, our work is limited in elucidating the specific kinase or phosphatase involved in the cleavage of β-arrestin. However, our findings show that Tyr-mediated angiotensin AT_1 receptor signaling, independent of IP_3 and Ca^{2+}-mediated signaling pathway, is involved in this process, and that inhibitors of protein Tyr phosphatase such as orthovanadate and molybdate need to be included in lysis buffer to prevent the cleavage of β-arrestin during lysis and further experimental steps. The attenuation mechanism of β-arrestin 1-GFP cleavage by inhibitors of protein Ser/Thr phosphatases *in vitro* is uncertain, but it was reported that there are protein Tyr phosphatases regulated by Ser phosphorylation (Brautigan and Pinault, 1993; Garton and Tonks, 1994; Strack et al., 2002).

In conclusion, we observed that protein Tyr phosphatase activity is involved in the regulation of angiotensin II-induced β-arrestin cleavage. Our findings suggest that Tyr-mediated angiotensin AT_1 receptor signaling, independent of IP_3 and Ca^{2+}-mediated signaling pathway, leads to the proteolysis of receptor-bound β-arrestins. Future studies need to be focused on elucidating the biological significance of ligand-induced β-arrestin cleavage and the kinases, phosphatases, and proteases responsible for the cleavage.

ACKNOWLEDGMENTS

This work was supported by a research grant (2005) from Daegu University (to S.-H.J.) and the National Institutes of Health Grant RO1 HL57470 (to S.S.K.). M.K. is the recipient of a Korea Science and Engineering Foundation S&T Graduate Scholarship. We thank Dr. Chang-Soo Hong for valuable discussions.

REFERENCES

Azarian, S.M., King, A.J., Hallett, M.A., and Williams, D.S. (1995). Selective proteolysis of arrestin by calpain. Molecular characteristics and its effect on rhodopsin dephosphorylation. J. Biol. Chem. *270*, 24375-24384.

Barak, L.S., Ferguson, S.S., Zhang, J., and Caron, M.G. (1997). A β-arrestin/green fluorescent protein biosensor for detecting G protein-coupled receptor activation. J. Biol. Chem. *272*, 27497-27500.

Brautigan, D.L., and Pinault, F.M. (1993). Serine phosphorylation of protein tyrosine phosphatase (PTP1B) in HeLa cells in response to analogues of cAMP or diacylglycerol plus okadaic acid. Mol. Cell. Biochem. *127-128*, 121-129.

Eguchi, S., Dempsey, P.J., Frank, G.D., Motley, E.D., and Inagami, T. (2001). Activation of MAPKs by angiotensin II in vascular smooth muscle cells. Metalloprotease-dependent EGF receptor activation is required for activation of ERK and p38 MAPK but not for JNK. J. Biol. Chem. *276*, 7957-7962.

Garton, A.J., and Tonks, N.K. (1994). PTP-PEST: a protein tyrosine phosphatase regulated by serine phosphorylation. EMBO J. *13*, 3763-3771.

Han, M., Gurevich, V.V., Vishnivetskiy, S.A., Sigler, P.B., and Schubert, C. (2001). Crystal structure of β-arrestin at 1.9 A: possible mechanism of receptor binding and membrane translocation. Structure (Camb) *9*, 869-880.

Krupnick, J.G., Goodman, O.B., Jr., Keen, J.H., and Benovic, J.L. (1997). Arrestin/clathrin interaction. Localization of the clathrin binding domain of nonvisual arrestins to the carboxy terminus. J. Biol. Chem. *272*, 15011-15016.

Lee, C., Hwang, S.A., Jang, S.H., Chung, H.S., Bhat, M.B., and Karnik, S.S. (2007). Manifold active-state conformations in GPCRs: agonist-activated constitutively active mutant AT1 receptor preferentially couples to Gq compared to the wild-type AT1 receptor. FEBS Lett. *581*, 2517-2522.

Lee, C., Bhatt, S., Shukla, A., Desnoyer, R.W., Yadav, S.P., Kim, M., Jang, S.H., and Karnik, S.S. (2008). Site-specific cleavage of G protein-coupled receptor-engaged β-arrestin: Influence of the AT1 receptor conformation on scissile site selection. J. Biol. Chem. *283*, 21612-21620.

Lefkowitz, R.J., and Shenoy, S.K. (2005). Transduction of receptor signals by β-arrestins. Science *308*, 512-517.

Lin, F.T., Krueger, K.M., Kendall, H.E., Daaka, Y., Fredericks, Z.L., Pitcher, J.A., and Lefkowitz, R.J. (1997). Clathrin-mediated endocytosis of the β-adrenergic receptor is regulated by phosphorylation/dephosphorylation of β-arrestin1. J. Biol. Chem. *272*, 31051-31057.

Marrero, M.B., Paxton, W.G., Schieffer, B., Ling, B.N., and Bernstein, K.E. (1996). Angiotensin II signalling events mediated by tyrosine phosphorylation. Cell. Signal. *8*, 21-26.

Marrero, M.B., Venema, V.J., Ju, H., Eaton, D.C., and Venema, R.C. (1998). Regulation of angiotensin II-induced JAK2 tyrosine phosphorylation: roles of SHP-1 and SHP-2. Am. J. Physiol. *275*, C1216-1223.

Nobles, K.N., Guan, Z., Xiao, K., Oas, T.G., and Lefkowitz, R.J. (2007). The active conformation of β-arrestin1: direct evidence for the phosphate sensor in the N-domain and conformational differences in the active states of β-arrestins 1 and 2. J. Biol. Chem. *282*, 21370-21381.

Oro, C., Qian, H., and Thomas, W.G. (2007). Type 1 angiotensin receptor pharmacology: signaling beyond G proteins. Pharmacol. Ther. *113*, 210-226.

Pugazhenthi, S., Tanha, F., Dahl, B., and Khandelwal, R.L. (1996). Inhibition of a Src homology 2 domain containing protein tyrosine phosphatase by vanadate in the primary culture of hepatocytes. Arch. Biochem. Biophys. *335*, 273-282.

Strack, V., Krutzfeldt, J., Kellerer, M., Ullrich, A., Lammers, R., and Haring, H.U. (2002). The Protein-tyrosine-phosphatase SHP2 is phosphorylated on serine residues 576 and 591 by protein kinase C isoforms α, β1, β2, and η. Biochemistry *41*, 603-608.

Vishnivetskiy, S.A., Paz, C.L., Schubert, C., Hirsch, J.A., Sigler, P.B., and Gurevich, V.V. (1999). How does arrestin respond to the phosphorylated state of rhodopsin? J. Biol. Chem. *274*, 11451-11454.

Xiao, K., Shenoy, S.K., Nobles, K., and Lefkowitz, R.J. (2004). Activation-dependent conformational changes in β-arrestin 2. J. Biol. Chem. *279*, 55744-55753.