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26. We monitored disulfide content in substrates by SDS-PAGE analysis after modification with 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS), a thiol-modifying reagent that increases a protein's molecular weight by ~500 daltons per free thiol (*3*).
27. Microsomes were prepared as described and resuspended in Buffer 88 (*24*) containing 20 mM DTT for 1 hour, washed with Buffer 88 to remove DTT, and resuspended in Buffer 88 in the presence or absence of 200 μM FAD. At the indicated times, aliquots were quenched with trichloroacetic acid (TCA) to 10% (w/v). TCA precipitates were resuspended in a solution of 1% SDS, 50 mM tris-Cl (pH 7.5),1 mM phenylmethylsulfonyl fluoride, and 20 mM AMS, incubated at room temperature for 15 min and at 37°C for 10 min, and boiled for 2 min before Endo H treatment and SDS-PAGE analysis.
28. Oxidative refolding was initiated by addition of reduced RNase A (*18*) to the indicated concentration of purified Ero1p (*15*), bacterially expressed PDI (*15*), and/or FAD (100 μM) in a buffer containing 18 mM cytidine 2′,3′-cyclic monophosphate (cCMP), 0.1 M tris-acetate (pH 8.0), 65 mM NaCl, 2 mM EDTA, and 0.005% digitonin. RNase A activity (hydrolysis of cCMP) was assayed by monitoring the rate of change of absorbance at 296 nm at 25°C (*18*). The disulfide content of RNase A was monitored in a similar buffer, but without cCMP. Samples were analyzed at the indicated times by the addition of SDS-PAGE buffer and 10 mM AMS, incubation for 30 min at room temperature, followed by nonreducing SDS-PAGE.
29. Reduced wild-type PDI or PDI $(CxxA)_2$ (1.7 μM) was added to Ero1p (1.3 μM) in a buffer containing 50 mM Hepes (pH 7.5), 100 mM NaCl, 2 mM EDTA, and 0.05% digitonin. After 10 min at room temperature, free sulfhydryls were quenched by addition of SDS-PAGE buffer and 10 mM *N*-ethylmaleimide for 1 hour. The sample was then divided in two and subjected to SDS-PAGE under reducing or nonreducing conditions.
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β-Arrestin 2: A Receptor-Regulated MAPK Scaffold for the Activation of JNK3

Patricia H. McDonald,[1]* Chi-Wing Chow,[2]* William E. Miller,[1]* Stéphane A. Laporte,[3] Michael E. Field,[1] Fang-Tsyr Lin,[1] Roger J. Davis,[2] Robert J. Lefkowitz[1]†

β-Arrestins, originally discovered in the context of heterotrimeric guanine nucleotide binding protein–coupled receptor (GPCR) desensitization, also function in internalization and signaling of these receptors. We identified c-Jun amino-terminal kinase 3 (JNK3) as a binding partner of β-arrestin 2 using a yeast two-hybrid screen and by coimmunoprecipitation from mouse brain extracts or cotransfected COS-7 cells. The upstream JNK activators apoptosis signal–regulating kinase 1 (ASK1) and mitogen-activated protein kinase (MAPK) kinase 4 were also found in complex with β-arrestin 2. Cellular transfection of β-arrestin 2 caused cytosolic retention of JNK3 and enhanced JNK3 phosphorylation stimulated by ASK1. Moreover, stimulation of the angiotensin II type 1A receptor activated JNK3 and triggered the colocalization of β-arrestin 2 and active JNK3 to intracellular vesicles. Thus, β-arrestin 2 acts as a scaffold protein, which brings the spatial distribution and activity of this MAPK module under the control of a GPCR.

β-Arrestins bind to β_2–adrenergic receptors (β_2ARs) after the receptors have been phosphorylated by guanine nucleotide binding protein (G protein)–coupled receptor kinases (GRKs), thereby interdicting further signal transduction to heterotrimeric G proteins (*1*). Once viewed as involved exclusively in receptor desensitization, β-arrestins are now known to act as adaptors to facilitate clathrin-mediated endocytosis of certain members of the GPCR family (*2*, *3*). They also recruit activated c-Src into complexes with the β_2AR, which appears to be involved in activation of extracellular signal-regulated kinases (ERK1 and ERK2) (*4*–*6*).

In mammalian cells, at least three groups of mitogen-activated protein kinases (MAPKs) have been identified: the ERKs (1 and 2), the p38 protein kinases (α, β, γ, and δ), and the c-Jun NH_2-terminal kinases (JNKs; also referred to as stress-activated protein kinases or SAPKs) (*7*). The JNKs are encoded by at least three genes (JNK1, -2, and -3), and the transcripts of each of these genes are alternatively spliced to create mRNAs that encode 46- and 54-kD JNK isoforms (*8*). Moreover, JNK activity is increased in settings of cell stress, mitogenesis, differentiation, morphogenesis, and apoptosis (*7*, *9*).

JNK is activated by dual phosphorylation on threonine and tyrosine residues catalyzed by a MAPK kinase (such as MKK4 or MKK7), which, in turn, is phosphorylated and activated by a MAPKK kinase (such as MEKK1, MLK, or ASK1). The functional integrity of each MAP kinase cascade is thought to be established by specific molecular interactions both between the kinases and cytoplasmic scaffolds or anchor proteins (*10*).

MAPK pathways are activated by GPCRs, including m1 and m2 muscarinic and angiotensin II type 1A (AT1A) receptors (*11*, *12*) and by growth factor receptors with intrinsic tyrosine kinase activity. Here we report the identification of β-arrestin 2 as a binding partner of JNK3 and describe a GPCR-regulated MAPK module wherein β-arrestin 2 functions as a scaffold protein.

To identify novel binding partners of β-arrestin 2, we used the yeast two-hybrid system based on a GAL4–β-arrestin 2 fusion protein to screen a rat brain cDNA library (*13*). Three positive clones were obtained, two of which encoded fusion proteins of the GAL4 activation domain with a COOH-terminal portion of JNK2 and one that encoded a COOH-terminal portion of the JNK3 isoform (*14*). All three clones encoded p54 splice variants of JNK. These interactions were confirmed by cotransforming yeast strain pJ69-4A with pAS2-1–β-arrestin 2 and the JNK clones (ct-JNK2 and ct-JNK3) obtained from the yeast two-hybrid screen. pAS2-1–β-arrestin 1 also interacted with both ct-JNK clones in the same assay. However, yeast transformed with pAS2-1–β-arrestin 1 and either ct-JNK2 or ct-JNK3 grew more poorly than those transformed with pAS2-1–β-arrestin 2 and either ct-JNK clone. The β-arrestins did not interact in the yeast two-hybrid system with the GAL4 activation domain encoded by pGAD10, nor did the ct-JNK clones interact with the GAL4 DNA binding domains encoded by pAS2-1 or with pAS2-1–Lamin, suggesting that the interactions of β-arrestins with the ct-JNKs are specific (*14*).

To determine whether the endogenous proteins interact, β-arrestin was immunoprecipitated from mouse brain extract with a β-arres-

[1]Howard Hughes Medical Institute and Departments of Medicine, Cardiology, and Biochemistry, Duke University Medical Center, Box 3821, Durham, NC 27710, USA. [2]Howard Hughes Medical Institute and Program in Molecular Medicine, Department of Biochemistry and Molecular Biology, University of Massachusetts Medical School, Worcester, MA 01605, USA. [3]Department of Cell Biology, Duke University Medical Center, Box 3287, Durham, NC 27710, USA.

*These authors contributed equally to this work.
†To whom correspondence should be addressed.

tin–specific antibody covalently crosslinked to Reactigel beads (*15*) or preimmune serum as a control (Fig. 1A). The p54 isoform of JNK3 was more abundant in the β-arrestin immunoprecipitates than in the preimmune serum immunoprecipitates. These data demonstrate a specific interaction between endogenous JNK3 and β-arrestin.

To further analyze the β-arrestin–JNK interaction in cells, FLAG epitope–tagged β-arrestin 1 (*16*) or β-arrestin 2 (*3*, *4*) was co-expressed with full-length hemaglutinin (HA)-tagged JNK1, -2, or -3 (*17*) in COS-7 cells (*15*). The HA-JNKs were immunoprecipitated with HA affinity beads, and the immunoprecipitates were resolved by SDS–polyacrylamide gel electrophoresis (SDS-PAGE). Only β-arrestin 2 coimmunoprecipitated with the JNK3 isoform (Fig. 1B). Reciprocal assays demonstrated that JNK3 also coimmunoprecipitated with β-arrestin 2 (*18*).

To assess the effect of β-arrestin on activation of the JNK pathway, COS-7 cells were transiently transfected (*15*) with either HA-JNK1 or HA-JNK3 alone or with HA-ASK1 (*17*) in the absence or presence of FLAG–β-arrestin 1 or FLAG–β-arrestin 2. Proteins from cell lysates were immunoprecipitated with HA affinity beads, and JNK activity was determined by in-gel kinase assays with c-Jun as the substrate (*19*). Co-expression of ASK1 with JNK1 produced robust phosphorylation of c-Jun. Thus, overexpression of ASK1 provided sufficient constitutive ASK1 activity to allow the MAPK cascade leading to JNK1 activation to proceed. The addition of FLAG–β-arrestin 1 or FLAG–β-arrestin 2 had no apparent effect on the ASK1-activated JNK1 phosphorylation of c-Jun. In contrast, JNK3 only phosphorylated c-Jun when co-expressed with both ASK1 and β-arrestin 2 (Fig. 2).

The β-arrestin 2–dependent ASK1-mediated activation of JNK3 was confirmed by immunoblot analysis with an antibody specific to phosphorylated JNK. COS-7 cells were transiently transfected with HA-JNK3 and with various amounts of HA-ASK1 in the absence or presence of FLAG–β-arrestin 1 or FLAG–β-arrestin 2. Co-expression of ASK1 and JNK3 produced a small increase in JNK3 phosphorylation. In contrast, co-expression of ASK1 and JNK3 with FLAG–β-arrestin 2 (but not FLAG–β-arrestin 1) led to a substantial increase (typically 20- to 30-fold) in JNK3 activation (*14*). Moreover, in-gel kinase assays indicate that the β-arrestin 2–JNK3 complex formed endogenously in mouse brain also contains activated JNK3 (*18*).

In cells overexpressing only FLAG–β-arrestin 1 or FLAG–β-arrestin 2, β-arrestin distribution is predominantly cytoplasmic (*20*). In cells overexpressing green fluorescent protein (GFP)–JNK3 (*21*) only, JNK3 was equally distributed between the cytosol and the nucleus (*18*). Remarkably, in cells co-expressing GFP-JNK3 and FLAG–β-arrestin 2, JNK3 was excluded from the nucleus (Fig. 3A). As is typical of transient transfections, most cells expressed both GFP-JNK3 and FLAG–β-arrestin 2, but some cells expressed only one of the proteins. In cells expressing GFP-JNK3 only (indicated by the arrow), JNK3 remained evenly distributed throughout the cell (Fig. 3A). In cells co-expressing GFP-JNK3 and FLAG–β-arrestin 1, JNK3 distribution was unaffected (Fig. 3A). Activated JNK normally accumulates in the nucleus (*22*). However, in cells co-expressing β-arrestin 2, JNK3, and ASK1 (which leads to JNK activation), activated JNK3 remained excluded from the nucleus (Fig. 3B). GFP-JNK3, like the HA-JNK3, was phosphorylated by ASK1 in a β-arrestin 2–dependent manner (*18*).

The effect of β-arrestin 2 on JNK3 phosphorylation suggests that β-arrestin 2 may serve as a scaffold protein for the JNK3 cascade. Therefore, a MAPKKK and a MAPKK might be expected to interact with β-arrestin 2 to complete the MAPK module. We expressed HA-ASK1 and FLAG–β-arrestin 2 in COS-7 cells, immunoprecipitated β-arrestin 2, and immunoblotted the precipitates with antibody to HA-ASK1. ASK1 was found in a complex with β-arrestin 2 (Fig. 4A). When FLAG–β-arrestin 2 was expressed with either glutathione *S*-transferase (GST)–MKK4 or GST-MKK7 (Fig. 4, B and C), weak binding of MKK4 to β-arrestin 2 was observed. This binding was enhanced in the presence of JNK3 or ASK1 (but not JNK1). MKK7 was not associated with β-arrestin 2 under the conditions tested. These data suggest that ASK1 binds directly to β-arrestin 2, and MKK4 interacts indirectly with β-arrestin 2 via ASK1 or JNK3, thus forming a complete MAPK module.

Analysis of deletion mutants of β-arrestin 2 demonstrated that the NH_2-terminus was required for optimal interaction with ASK1 and that the COOH-terminus was required for optimal interaction with JNK3 (*14*). Furthermore, the stimulatory effect of β-arrestin 2 on JNK3 activation mediated by ASK1 was reduced by the deletion of the ASK1 or JNK3 binding domains (*14*).

JNK is activated in response to several GPCRs (*11*, *12*). To determine whether β-arrestin 2 had any effect on GPCR-mediated activation of JNK3, COS-7 cells were transfected with AT1A receptor (*23*) and HA-JNK3 in the presence or absence of FLAG–β-arrestin 2. Cells were stimulated with an-

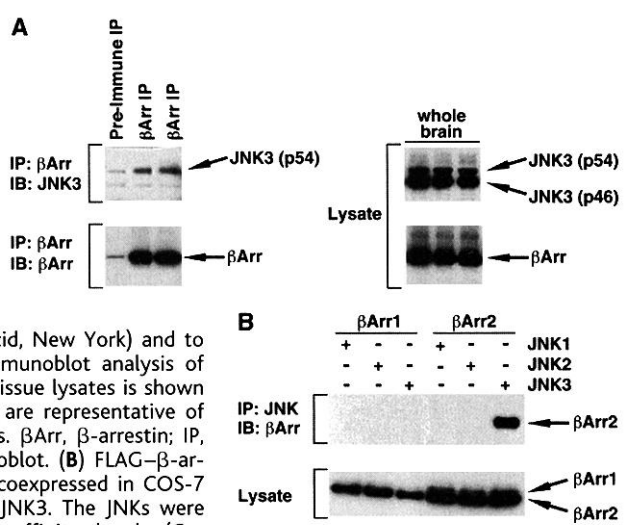

Fig. 1. Co-immunoprecipitation of β-arrestin and JNK3 from mouse brain extracts and transfected COS-7 cells. (**A**) β-Arrestin was immunoprecipitated from mouse brain lysates with an antibody to β-arrestin and immunoprecipitates were resolved by SDS-PAGE. The presence of JNK3 and β-arrestin in the immunoprecipitates was detected with antibodies to JNK3 (UBI, Lake Placid, New York) and to β-arrestin [(A), left panel]. Immunoblot analysis of JNK3 and β-arrestin in whole tissue lysates is shown [(A), right panel]. Data shown are representative of three independent experiments. βArr, β-arrestin; IP, immunoprecipitate; IB, immunoblot. (**B**) FLAG–β-arrestin 1 or –β-arrestin 2 was coexpressed in COS-7 cells with HA-JNK1, JNK2, or JNK3. The JNKs were immunoprecipitated using HA-affinity beads (Covance), and the presence of β-arrestins in the IP was detected using rabbit polyclonal antibody to FLAG (Santa Cruz Biotechnology, Santa Cruz, California) (upper panel). The amount of β-arrestin in the whole cell lysates was examined by immunoblot analysis (lower panel). Data shown are representative of three independent experiments. βATT-1, β-arrestin 1; βArr 2, β-arrestin-2.

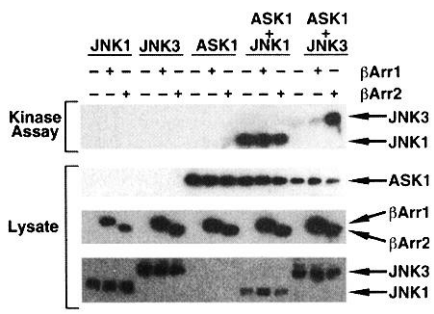

Fig. 2. Enhanced activation of JNK3 by ASK1 in cells expressing β-Arrestin 2. HA-JNK1 and -JNK3 were immunoprecipitated from cells expressing either JNK1 or JNK3 alone or with ASK1 in the presence or absence of β-arrestin 1 or β-arrestin 2. Immunoprecipitates were resolved by SDS-PAGE, and protein kinase activity was assessed with an in-gel kinase assay (*10*) using c-Jun as the substrate (top panel). Whole cell extracts were analyzed for expression of ASK1, β-arrestins, and JNK by immunoblot analysis (lower panels). Data shown are representative of four independent experiments.

giotensin II peptide (Ang II), and the activation of JNK3 was assessed by blotting cell lysates with an antibody to phosphorylated JNK. After agonist stimulation, cells expressing the AT1A receptor showed only a small increase in JNK3 activation over the time course studied (Fig. 5A). However, stimulation of the AT1A receptor in cells also expressing FLAG–β-arrestin 2 caused greater activation of JNK3, peaking at ~60 min (Fig. 5A). As ASK1 was not transfected in these cells, agonist stimulation of the AT1A receptor presumably leads to activation of an endogenous MAPKKK. This result provides evidence for a role of β-arrestin 2 in the agonist-dependent activation of JNK3.

To determine the effect of agonist stimulation on scaffold complex formation, cells were stimulated with Ang II for various amounts of time and β-arrestin 2 was immunoprecipitated. JNK3, MKK4, and ASK1 were all detected in the β-arrestin 2 immunoprecipitates, and the levels of binding were unaffected by agonist (*18*). This suggests that the β-arrestin 2–MAPK complex is formed constitutively and that the effect of receptor stimulation is to regulate the subcellular distribution of the complex and its activity state, probably via activation of a MAPKKK such as ASK1.

A hallmark of β-arrestins is that their spatial distribution within the cell is regulated by stimulation of GPCRs (*23*). In HEK 293 cells co-expressing GFP-JNK3 and FLAG–β-arrestin 2, both proteins were located in the cytoplasm in unstimulated cells (Fig. 3A). Therefore, we examined the effects of Ang II stimulation on the subcellular distribution of the JNK3 associated with β-arrestin 2 in cells transiently expressing AT1A receptors. After treatment of cells with Ang II (20 min), a portion of β-arrestin 2 was found on endosomal vesicles, indicative of agonist-dependent translocation of β-arrestin 2 to the AT1A receptor (*23*). However, after Ang II stimulation, a portion of the JNK3 translocated to the nucleus (Fig. 5B). As assessed by staining with phospho-specific JNK antibody, the nuclear JNK3 was inactive. Active JNK3 colocalized with β-arrestin 2 on the endosomal vesicles (Fig. 5B). In agonist-treated cells expressing AT1A receptors and JNK3 only, no activation of JNK3 was observed and JNK3 distribution was unaffected (*18*). Although the steady-state amounts of the JNK3–β-arrestin 2 complex determined by coimmunoprecipitation were not altered by Ang II stimulation (*18*), the relocalization of subcellular JNK3 in response to Ang II, as assessed by confocal microscopy, probably reflects the dynamic nature of the JNK3–β-arrestin 2 interaction.

To determine whether the activation and localization of JNK3 on intracellular vesicles was an Ang II–specific β-arrestin 2–dependent effect, we treated cells expressing either GFP-JNK3 only or GFP-JNK3 with β-arrestin 2 with 400 mM sorbitol, which activates JNK (*24*). After the sorbitol treatment (30 min), perinuclear- and nuclear-activated JNK3 was observed in both sets of cells. These data demonstrate that active JNK3 can be detected in the nucleus and is unaffected by β-arrestin 2 under these experimental conditions (*18*).

In budding yeast, Ste5 is a scaffold protein that forms a multicomponent complex with the Fus3 (Kss1) MAPK, Ste7 MAPKK, and Ste11 MAPKKK to facilitate the specific and efficient activation of the mating pheromone pathway (*10*). Also in yeast, Pbs2, which itself is a MAPKK, has been proposed as a possible scaffold protein in the HOG

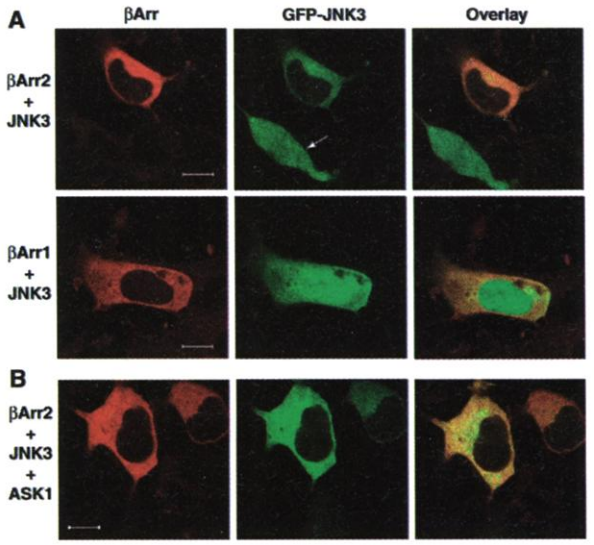

Fig. 3. Subcellular distribution of transfected JNK3 and β-arrestin. (**A**) Retention of JNK3 in the cytoplasm of cells overexpressing β-arrestin 2 but not β-arrestin 1. HEK-293 cells expressing GFP-JNK3 and either FLAG–β-arrestin 1 or FLAG–β-arrestin 2 were fixed in 4% paraformaldehyde in 1× phosphate buffered saline (PBS) for 20 min at room temperature and were permeabilized in 0.2% Triton X-100 in PBS for 5 min. FLAG–β-arrestins were detected with M2 FLAG monoclonal antibody (Sigma) and a Texas Red–conjugated secondary antibody. Confocal microscopy was performed on a Zeiss LSM-510 laser scanning microscope using a Zeiss 100×/1.4 numerical aperture oil immersion lens. Images were collected using sequential line excitation filters at 488 and 568 nm, and emission filter sets at 505 to 550 nm for GFP detection (green) and 585 nm for Texas Red detection (red). Single optical sections of FLAG–β-arrestin 2, GFP-JNK3 and overlay images are shown. Scale bars in β-arrestin images represent 10 μm. Arrow indicates a cell overexpressing GFP-JNK3 only. (**B**) Retention of ASK1 stimulated JNK3 in the cytoplasm of cells overexpressing β-arrestin 2. Cells expressing GFP-JNK3, FLAG–β-arrestin 2, and HA-ASK1 were treated in the same manner. Data shown are representative of four independent experiments.

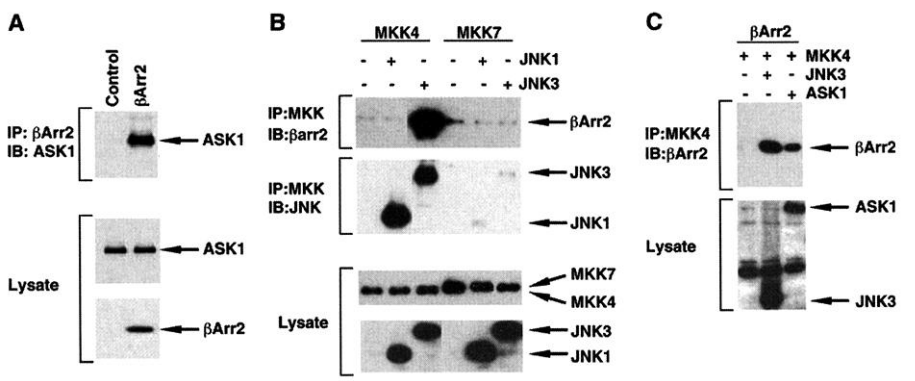

Fig. 4. Selective interaction of β-arrestin 2 with the MAPKKK ASK1 and the MAPKK MKK4. (**A**) Interaction of ASK1 and β-arrestin 2. FLAG–β-arrestin 2 was immunoprecipitated from cells expressing HA-tagged ASK1, and the presence of ASK1 in the IPs was detected with an antibody to HA (top panel). The amount of ASK1 and β-arrestin 2 in the cell lysates was examined by protein immunoblot analysis (lower panels). (**B**) Enhanced interaction of MKK4 with β-arrestin 2 in the presence of JNK3. MKK4 or MKK7 was expressed in cells as a GST fusion protein together with β-arrestin 2 in the presence or absence of either HA-tagged JNK1 or JNK3. The MKKs were immunoprecipitated with GST affinity beads; β-arrestin 2 in the IPs was detected with the M2 antibody (top panel). JNK1 and JNK3 were detected with an antibody to the HA epitope (second panel). Whole cell extracts were analyzed for the expression of the MKKs and JNKs by immunoblot analysis (lower panels). (**C**) Enhanced interaction of MKK4 with β-arrestin 2 in the presence of ASK1. MKK4 was immunoprecipitated from cells expressing β-arrestin 2 in the presence or absence of either ASK1 or JNK3. β-arrestin 2 was detected with the M2 antibody (top panel). Expression of ASK1 and JNK3 was assessed in whole cell extracts by immunoblot analysis (lower panel). Data shown are representative of four independent experiments.

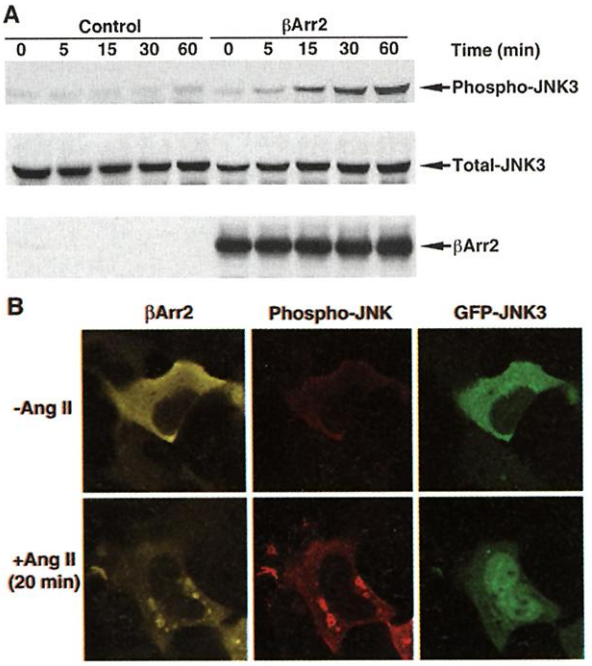

Fig. 5. JNK3 activation and colocalization of activated JNK3 with β-arrestin 2 on intracellular vesicles after agonist stimulation of the AT1A receptor. (**A**) Cells were transiently transfected with AT1A receptor, HA-JNK3, and FLAG–β-arrestin 2. Forty-eight hours post-transfection, cells were stimulated with 1 μM Ang II for the indicated length of time. The presence of active JNK in the cell lysates was detected by immunoblot analysis with an antibody specific to phosphorylated JNK (top panel). Expression of JNK3 and β-arrestin 2 was assessed in cell lysates by immunoblot (lower panels). Data shown are representative of three independent experiments. (**B**) HEK-293 cells were transiently transfected with AT1A receptor, FLAG–β-arrestin 2, and GFP-JNK3. Forty-eight hours post-transfection, cells were either left unstimulated (−Ang II) or stimulated with 1 μM Ang II (+Ang II, 20 min) and were then fixed in 4% paraformaldehyde in PBS for 20 min at room temperature. Cells were permeabilized in 0.2% Triton X-100 in PBS for 5 min. FLAG–β-arrestin 2 was detected using an M2 antibody and an allophycocyanin-conjugated secondary antibody (Molecular Probes, Eugene, Oregon), and phosphorylated JNK was detected using the phospho-specific JNK antibody (Promega, Madison, Wisconsin) and a Texas Red–conjugated secondary antibody. Fluorescent confocal images were collected using sequential line excitation filters at 488, 568, and 633 nm and emmision filter sets at 505 to 550 nm for GFP-JNK detection (green), 585 nm for phospho-JNK detection (red), and 650 nm for β-arrestin 2 detection (yellow). Data shown are representative of four independent experiments.

(high-osmolarity glycerol response) signal transduction pathway (*10*). An intriguing similarity between Ste5 and β-arrestin 2 is that both are recruited to the plasma membrane as a consequence of agonist stimulation of a GPCR (*25*). In mammalian cells, a group of JNK-interacting proteins (JIP1, -2 and -3) have been identified as scaffolding proteins for specific JNK signaling modules (*26*, *27*).

Like members of the JIP family, β-arrestin 2 associates with all three kinase components of a single MAPK cascade, thus enhancing signaling efficiency. However, unlike JIPs, the β-arrestin 2–MAPK module is regulated by agonist stimulation of GPCRs. It is likely that individual JNK isoforms exhibit distinct patterns of regulation. For example, as demonstrated here, JNK3 activity appears to be specifically enhanced by β-arrestin 2, whereas JNK1 activity is unaffected. Thus, formation of similar complexes may prevent inappropriate cross talk between the various MAPK pathways.

JNK1 is activated in response to several GPCRs (*11*, *12*). The association of JNK3 with β-arrestin 2 provides a mechanism whereby β-arrestin 2 might localize JNK3 to specific subcellular compartments and/or target JNK3 to specific substrates in response to GPCR agonists. The results reported here add to a growing list of functions subserved by β-arrestins in regulating signaling through heptahelical receptors. By acting as a scaffold for a specific MAPK pathway, β-arrestin 2 provides a mechanism for bringing this signaling module under the control of such receptors. Other evidence (*5*, *6*, *16*, *28*) suggests that β-arrestins may also play roles in organizing pathways leading from GPCRs to activation of the ERKs.

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13. C. T. Chien, P. L. Bartel, R. Sternglanz, S. Fields, *Proc. Natl. Acad. Sci. U.S.A* **88**, 9578 (1991). The yeast two-hybrid screen was performed using the yeast strain PJ69-4A and a pGAD10 rat brain cDNA library (MATCHMAKER GAL4 two-hybrid user manual; Clontech, Palo Alto, CA). Full-length β-arrestin 2 was subcloned into pAS2-1 and was expressed as a GAL4 DNA binding domain fusion protein. Approximately 5×10^6 clones of a rat brain cDNA library were screened, and three independent clones, two coding for JNK2 and one coding for JNK3, were isolated.
14. Web figures 1 through 3 are available at *Science* Online at www.sciencemag.org/cgi/content/full/290/5496/1574/DC1.
15. For coimmunoprecipitation of endogenous proteins from whole brain extract, brain tissue was solubilized in lysis buffer (LB) [50 mM Hepes (pH 7.4), 0.5% NP40, 250 mM NaCl, 2 mM EDTA, 10% (v/v) glycerol, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, and leupeptin (10μg/ml)]. β-Arrestin was immunoprecipitated by incubation for 4 hours at 4°C with antibody specific for β-arrestin (A1CT antibody, generated in our laboratory), covalently cross-linked to Reactigel beads (Pierce, Rockford, IL), Immunoprecipitated proteins were analyzed by SDS-PAGE and detected by immunoblot analysis. For cellular experiments, plasmid DNA was transfected into COS-7 and HEK-293 cells by the lipofectamine (Life Technologies, Rockville, MD) and Fugene (Roche, Indianapolis, IN) methods, respectively. For GPCR activation assays, cells were stimulated with Ang II (1μM) (Sigma). For coimmunoprecipitation studies, cells were harvested 48 hours post-transfection and solubilized in LB. Epitope-tagged proteins were immunoprecipitated by incubation for 4 hours at 4°C with FLAG (Sigma) or HA (Covance, Princeton, NJ) affinity beads. Immunoprecipitated proteins were analyzed by SDS-PAGE and detected by immunoblot analysis. Protein kinase activity was measured by the in-gel kinase method with substrate polymerized in the gel [c-Jun (0.25mg/ml)].
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