Constitutive ERK1/2 Activation by a Chimeric Neurokinin 1 Receptor-β-Arrestin1 Fusion Protein

PROBING THE COMPOSITION AND FUNCTION OF THE G PROTEIN-COUPLED RECEPTOR “SIGNALSONE”*

Received for publication, November 28, 2005, and in revised form, April 26, 2006 Published, JBC Papers in Press, May 2, 2006, DOI 10.1074/jbc.M512643200

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The β-arrestins, a small family of G protein-coupled receptor (GPCR)-binding proteins involved in receptor desensitization, have been shown to bind extracellular signal-regulated kinases 1 and 2 (ERK1/2) and function as scaffolds for GPCR-stimulated ERK1/2 activation. To better understand the mechanism of β-arrestin-mediated ERK1/2 activation, we compared ERK1/2 activation by the wild-type neurokinin 1 (NK1) receptor with a chimeric NK1 receptor having β-arrestin1 fused to the receptor C terminus (NK1-βArr1). The NK1 receptor couples to both Go and Gq/11, resides on the plasma membrane, and mediates rapid ERK1/2 activation and nuclear translocation in response to neurokinin A. In contrast, NK1-βArr1 is a G protein-uncoupled “constitutively desensitized” receptor that resides almost entirely in an intracellular endosomal compartment. Despite its inability to respond to neurokinin A, we found that NK1-βArr1 expression caused robust constitutive activation of cytosolic ERK1/2 and that endogenous Raf, MEK1/2, and ERK1/2 coprecipitated in a complex with NK1-βArr1. While agonist-dependent ERK1/2 activation by the NK1 receptor was independent of protein kinase A (PKA) or PKC activity, NK1-βArr1-mediated ERK1/2 activation was completely inhibited when basal PKA and PKC activity were blocked. In addition, the rate of ERK1/2 dephosphorylation was slowed in NK1-βArr1-expressing cells, suggesting that β-arrestin-bound ERK1/2 is protected from mitogen-activated protein kinase phosphatase activity. These data suggest that β-arrestin binding to GPCRs nucleates the formation of a stable “signalsome” that functions as a passive scaffold for the ERK1/2 cascade while confining ERK1/2 activity to an extranuclear compartment.

Seven membrane-spanning G protein-coupled receptors (GPCRs)² constitute the largest superfamily of membrane receptors and transmit signals in response to an extraordinary array of extracellular stimuli. GPCRs function as ligand-activated guanine nucleotide exchange factors for heterotrimeric guanine nucleotide binding (G) proteins. GPCR-catalyzed guanine nucleotide exchange on the Gα subunit of heterotrimeric G proteins leads to dissociation of GTP-bound Gα subunits from Gβγ heterodimers, each of which in turn positively or negatively regulates the activity of enzymatic effectors, such as adenyl cyclases, phospholipase Cβ isoforms, and ion channels.

This classic paradigm of GPCR signaling is sufficient to account for most of the rapid cellular responses to GPCR activation. However, GPCRs engage in numerous other protein-protein interactions that modulate the character and duration of G protein-mediated signaling or in some cases confer additional G protein-independent signaling capacity (1–3). Recently, the arrestins, a family of four GPCR-binding proteins originally discovered through their role in GPCR desensitization and sequestration, have emerged as important adaptors linking activated GPCRs to a number of cellular signaling systems. Arrestins are recruited to agonist-occupied GPCRs that have been phosphorylated on C-terminal Ser/Thr residues by G protein-coupled receptor kinases. Arrestin binding precludes receptor-G protein coupling, accounting for the phenomenon of homologous desensitization. In addition, the C termini of the two non-visual arrestins, β-arrestin 1 and 2, contain binding motifs for clathrin and the β2-adaptin subunit of the AP-2 complex that allow β-arrestins to target GPCRs for clathrin-mediated endocytosis (4, 5).

Apart from their role in GPCR desensitization and sequestration, emerging data suggest that β-arrestins function as signal transducers in their own right. A number of catalytically active proteins bind β-arrestins and translocate with them to agonist-occupied GPCRs, among them Src family tyrosine kinases, components of the extracellular signal-regulated kinase 1 and 2 (ERK1/2) and c-Jun N-terminal Kinase 3 mitogen-activated protein (MAP) kinase cascades, the E3 ubiquitin ligase, Mdm2, and the cAMP phosphodiesterase, PDE4D. The

G protein, guanine nucleotide-binding protein; GFP, green fluorescent protein; HA, influenza virus hemagglutinin; MAP, mitogen-activated protein; NKA, neurokinin A; PKA, protein kinase A; PKC, protein kinase C; PMA, phorbol myristate acetate; VASP, vasodilator- and A kinase-stimulated phosphoprotein; E3, ubiquitin-protein isopeptide ligase; NK1, neurokinin 1; MEK, MAPK/ERK kinase; CMV, cytomegalovirus; MKP, MAP kinase phosphatase.

* This work was supported by National Institutes of Health Grant DK55524 (to L. M. L.), the South Carolina Center for Biomedical Research Excellence in Cardiovascular Diseases (to D. K. L.), and the Department of Veterans Affairs. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: GPCR, G protein-coupled receptor; EGF, epidermal growth factor; ERK1/2, extracellular signal-regulated kinases 1 and 2;
agonist-induced assembly of these receptor-β-arrestin-effector complexes confers discrete enzymatic properties upon the receptor that appear to expand the repertoire of GPCR signaling (5–8).

One of the most studied β-arrestin-dependent signals is activation of the ERK1/2 MAP kinase cascade.GPCRs employ several mechanisms to activate ERK1/2, including activation of classic G protein-regulated effectors such as protein kinase (PKA) and PKC, ectodomain shedding-dependent transactivation of epidermal growth factor (EGF) receptors, and signals transmitted via β-arrestins (9–11). Depending on receptor and cell type, one mechanism may predominate or multiple mechanisms may act concurrently. Interestingly, β-arrestin-dependent ERK1/2 activation appears to be G protein-independent, because it can be demonstrated under conditions where β-arrestin recruitment is induced in the absence of detectable G protein activation (12–14). β-Arrestin-mediated ERK1/2 activation is of slower onset and more prolonged duration than G protein-mediated signaling (15, 16) and in the case of receptors that form stable GPCR-β-arrestin complexes, such as the pro tease-activated receptor PAR1, angiotensin AT1a and vaso pressin V2 receptors, the active ERK1/2 is largely confined to the cytosol (17–20). The mechanism of GPCR-stimulated ERK1/2 activation also affects its function. Transactivation of EGF receptors, a GPCR-mediated process that does not require β-arrestin (14), plays an important role in cell proliferation and differentiation in a number of settings (21, 22). In contrast, β-arrestin-dependent ERK1/2 activation may be involved in cytoskeletal rearrangement during chemotaxis (23, 24) and appears to support a distinct pattern of ERK1/2-dependent gene transcription (14). Collectively, these data indicate that G protein-dependent and β-arrestin-dependent ERK1/2 activation are mechanistically distinct, produce ERK1/2 activation with different time courses and in different subcellular locations, and probably dictate different functional consequences of ERK1/2 activation. Little is known, however, about how agonist-promoted recruitment of β-arrestin to a GPCR leads to ERK1/2 activation, except that it can occur in the absence of heterotrimeric G protein activation.

In one sense, a β-arrestin-bound GPCR might be thought of as a “signalosome nucleus” upon which a multiprotein signaling complex is assembled (5–8). Efforts to experimentally characterize the composition and function of this putative “signalosome” with respect to its role in ERK1/2 activation are faced with two experimental limitations. The first is that, because native GPCRs can employ multiple simultaneous or sequential mechanisms to activate ERK1/2, it is difficult to establish a model system in which β-arrestin-dependent ERK1/2 activation accounts for the majority of the observed ERK1/2 signal. The second is that it is difficult to isolate stoichiometric GPCR-β-arrestin complexes from detergent lysates for the purpose of identifying endogenous components of the GPCR signalosome. To circumvent these obstacles and provide an initial means of characterizing the composition and function of the GPCR-arrestin signalosome, we have compared the mechanism of ERK1/2 activation by wild-type human tachykinin NK1 receptors to that used by a “constitutively desensitized” NK1 receptor-β-arrestin1 chimera (NK1-βArr1) that generates robust constitutive ERK1/2 activity in the absence of increased G protein signaling. Our data suggest that β-arrestin-bound GPCRs increase cellular ERK1/2 activity primarily by functioning as passive scaffolds for the Raf-MEK-ERK kinase cascade that increase the efficiency of ERK1/2 phosphorylation while slowing the rate of ERK1/2 dephosphorylation.

**EXPERIMENTAL PROCEDURES**

**Materials**—FuGENE 6 was purchased from Roche Diagnostics (Indianapolis, IN). Neurokinin A (NKA) was from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA). Tyrosinestin AG1478, PD98059, phorbol myristate acetate (PMA), 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2), and Ro-31-8220 were from Calbiochem-EMD Biosciences Inc. (San Diego, CA). Monoclonal M2 anti-FLAG affinity agarose was from Sigma. HA-11 affinity matrix was from Covance Inc. (Berkeley, CA). Rabbit polyclonal anti-HA and anti-FLAG IgG were from Affinity BioReagents (Golden, CO). Rabbit polyclonal phosphorylation state-specific anti-ERK1/2, phosphorylation state-specific anti-MEK1/2, and anti-MEK1/2 IgG were from Cell Signaling Technology Inc. (Beverly, MA). Rabbit polyclonal anti-ERK1/2, anti-HA, anti-B-Raf, and anti-ubiquitin IgG were from Upstate Biotechnology Inc. (Waltham, MA). Rabbit polyclonal anti-cRaf-1 IgG was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Horseradish peroxidase-conjugated donkey anti-rabbit IgG was from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). Alexa Fluor 488 and Alexa Fluor 543 goat anti-rabbit IgG and ToPro3 were from Molecular Probes (Carlsbad, CA). Rabbit polyclonal anti-β-arrestin was a gift from Robert J. Lefkowitz (Duke University, Durham, NC).

**cDNA Constructs**—The pNSIneo expression plasmid encoding FLAG epitope-tagged NK1-βArr1 was a gift from T. Schwartz (University of Copenhagen, Copenhagen, Denmark) as was prepared as described (25). The cDNA encodes an N-terminal FLAG epitope followed by the human tachykinin (NK1) receptor without its stop codon fused in-frame to full-length bovine β-arrestin1. The pcDNA3.1 expression plasmid encoding HA epitope-tagged wild-type NK1 receptor was a gift from Robert J. Lefkowitz. The pEGFP-N1 expression plasmid encoding green fluorescent protein (GFP)-tagged ERK2 was provided by N. Bunnett (University of California, San Francisco, CA). The pCMV-FLAG-VASP expression plasmid encoding FLAG epitope-tagged vasodilator- and A kinase-stimulated phosphoprotein (VASP) was from M. Uhler (University of Michigan, Ann Arbor, MI).

**Cell Culture and Transfection**—HEK-293 cells were obtained from the American Type Culture Collection and were maintained in Ham’s F-12 medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. Transient transfection of HEK-293 cells was performed in 100-mm dishes using FuGENE 6 according to the manufacturer’s instructions with 3–6 μg of total plasmid DNA per dish and 3 μl of FuGENE per microgram of DNA. Prior to each experiment, transfected cells were seeded into multiwell plates as appropriate, and monolayers were incubated for 24 h in serum-free growth medium supplemented with 10 mM HEPES (pH 7.4), 0.1% bovine serum albumin, and penicillin/streptomycin.
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Immunoprecipitation and Immunoblotting—To assay ERK1/2, GFP-ERK2, and MEK1/2 phosphorylation in whole cell lysates, serum-starved cell monolayers in 12-well plates were stimulated at 37 °C as described in the figure legends and lysed directly with 1× Laemmli sample buffer. Sonicated samples were resolved by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Phosphorylated ERK1/2 and MEK1/2 were detected by immunoblotting using rabbit polyclonal anti-phospho-ERK1/2 IgG and anti-phospho-MEK1/2 IgG, respectively, with horseradish peroxidase-conjugated goat anti-rabbit IgG as secondary antibody. Total ERK1/2, measured immunoblotting with polyclonal anti-ERK1/2 IgG. Immune complexes were visualized on x-ray film by enzyme-linked chemiluminescence and quantified using a Fluor-S Multimag.

To assay endogenous PKA activity cells were cotransfected with wild-type NK1 receptor or NK1-β-arrestin1 and FLAG-VASP. VASP is a specific PKA substrate that undergoes a prominent electrophoretic mobility shift on SDS-PAGE when phosphorylated. FLAG-VASP in whole cell lysates was visualized by immunoblotting using rabbit polyclonal anti-FLAG IgG with horseradish peroxidase-conjugated goat anti-rabbit IgG as secondary antibody. The effect of receptor expression and stimulation on endogenous PKA activity was quantified by measuring the fraction of total FLAG-VASP in each lane that was present in the slow migrating upper band.

For detection of NK1-β-arrestin1 and wild-type NK1 receptor-associated proteins, 10-cm plates of appropriately transfected HEK-293 cells were stimulated as described, then solubilized in 1.0 ml of lysis buffer (50 mM Tris-Base (pH 7.5), 0.5% deoxycholic acid, 1% Triton X-100, 10 mM EDTA, 0.5 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 1 μg/ml pepstatin A, 100 μM benzamidine) containing 10 mM N-ethylmaleimide. Lysates were clarified by centrifugation, and immunoprecipitation was performed using 20 μl of 50% slurry of M2 anti-FLAG affinity agarose or HA-11 anti-HA affinity matrix, with constant agitation overnight at 4 °C. Immune complexes were washed three times with lysis buffer and boiled in Laemmli sample buffer prior to SDS-PAGE. Immunoprecipitated receptors and endogenous receptor-associated proteins were detected by immunoblotting with polyclonal IgG against the FLAG or HA epitopes, ubiquitin, β-arrestin1/2, c-Raf-1, B-Raf, MEK1/2, ERK1/2, or phospho-ERK1/2 as appropriate.

Quantitative Real-time PCR—The expression of Egr1 mRNA was measured by quantitative real-time PCR using an iCycler iQ™ Multicolor Real-time Detection System. Total RNA was isolated from cells with the High Pure RNA Isolation Kit (Roche Applied Science), after which cDNA was synthesized from 0.1 μg RNA using the iScript™ cDNA Synthesis Kit (Bio-Rad). cDNAs amplified through one cycle by PCR using random hexamer DNA primers were used as templates for quantitative real-time PCR. Specific primer sets were designed to span intron-exon borders to distinguish amplified cDNA from genomic DNA. The primers used for Egr1 amplification were: sense, 5’-GAGGATGCTGCTGACATTAG-3’ EGR1; antisense, 5’-TACCGTCAAGCAGATTTAC-3’. The expression level of Egr1 from each sample was normalized to the mRNA expression level of a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase.

Confocal Fluorescence Microscopy—For visualization of HA-NK1 or FLAG-NK1-βArr1 receptors in intact cells, transfected HEK-293 cells grown on collagen-coated glass bottom chamber slides were incubated for 1 h with a 1:100 dilution of rabbit polyclonal anti-FLAG or anti-HA IgG in serum-free medium prior to fixation for 10 min with 4% paraformaldehyde in phosphate-buffered saline. After washing with phosphate-buffered saline, cells were incubated with a 1:200 dilution of AlexaFluor 488 goat anti-rabbit IgG in blocking buffer (2% bovine serum albumin in phosphate-buffered saline) for 1 h at room temperature, washed again, and examined. For visualization under permeabilized conditions, cells were fixed with 4% paraformaldehyde and treated with 0.1% Triton X-100 in phosphate-buffered saline for 5 min. Receptors were stained overnight at 4 °C using a 1:100 dilution of either anti-FLAG or anti-HA IgG followed by a 1:250 dilution of Alexa Fluor 488 goat anti-rabbit IgG for 1 h at room temperature. For visualization of endogenous phospho-ERK1/2, transfected cells were stimulated as described, formalin-fixed, and permeabilized. Phospho-ERK1/2 was stained overnight using a 1:1000 dilution of rabbit polyclonal anti-phospho-ERK1/2. Monolayers were then incubated with a 1:250 dilution of Alexa Fluor 488 goat anti-rabbit IgG for 1 h at room temperature prior to final washing and examination. For visualization of the nuclear translocation of ERK2, cells coexpressing wild-type NK1 or NK1-βArr1 receptors and GFP-ERK2 were stimulated as described and formalin-fixed, and random fields were examined. In each sample, ToPro3 was used as a nuclear counterstain to identify those cells in the field in which the confocal imaging plane accurately transected the nucleus. To quantify changes in the nuclear distribution of GFP-ERK2, National Institutes of Health ImageJ software was employed to determine the ratio of nuclear to cytosolic GFP fluorescence in at least 20 individual cells under each condition in each experiment. Data were normalized such that the mean nucleus:cytosol ratio in wild-type NK1 receptor-expressing cells under nonstimulated conditions was arbitrarily defined as 1.0. Confocal microscopy was performed on a Leica TCS SP2 AOBS laser scanning microscope using dual channel excitation (495 nm Alexa Fluor 488 and GFP; 642 nm ToPro3) and emission (519 nm Alexa Fluor 488 and GFP; 657 nm ToPro3) filter sets.

RESULTS

Expression of a Constitutively Desensitized Chimeric NK1-β-arrestin1 Receptor Causes Sustained Activation of a Cytosolic Pool of ERK1/2—Fig. 1A schematically depicts the membrane topography of the HA-tagged human tachykinin NK1 receptor and the FLAG-tagged NK1-βArr1 chimera. NK1-βArr1 consists of the human NK1 receptor with full-length bovine β-arrestin1 (amino acids 1–418) fused in-frame immediately following the receptor C terminus. As shown in Fig. 1B, NK1 receptor in anti-HA immunoprecipitates from transiently transfected HEK-293 cells appeared as multiple bands of >50–55 kDa apparent molecular mass on anti-HA immunoblots, probably due to variable receptor glycosylation. In anti-FLAG immunoprecipitates, NK1-βArr1 appeared as a group of
100-kDa bands that immuno-blotted for both FLAG epitope and β-arrestin. Interestingly, both NK1 and NK1-βArr1 immunoprecipitates contained a small amount of endogenous β-arrestin, due either to binding to intracellular receptor domains or to dimerization between endogenous β-arrestin and the β-arrestin1 moiety of the NK1-βArr1 chimera (25–28).

The ligand-binding, G protein-coupling, and intracellular trafficking properties of both the NK1 receptor and NK1-βArr1 have been characterized in detail (29–32). The NK1 receptor binds two endogenous peptide ligands, substance P and NKA, and stimulates robust cAMP production and phosphatidylinositol hydrolysis through coupling to Gs and Gq/11, respectively. The NK1-βArr1 chimera also binds to substance P and NKA with high affinity but exhibits markedly impaired G protein coupling due to the proximity of the intrinsic β-arrestin moiety to the intracellular receptor domains involved in G protein activation.

FIGURE 2. Constitutive ERK1/2 phosphorylation in HEK-293 cells expressing NK1-βArr1. A, serum-deprived cells expressing wild-type NK1 receptors were preincubated for 30 min in the presence or absence of PD98059 (1 μM) prior to stimulation for 5 min with NKA (1 μM) or PMA (100 nM). Phospho-ERK1/2 and total ERK1/2 in whole cell lysate samples were determined by immunoblotting as described. B, results of identical experiments performed on cells expressing NK1-βArr1. In each panel, a representative phospho-ERK1/2 immunoblot is shown above a bar graph representing mean ± S.E. for three independent experiments. The change in ERK1/2 phosphorylation was quantified as the -fold increase above basal ERK1/2 phosphorylation measured in PD98059-treated cells in the absence of NKA.
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protein activation. In cells expressing NK1-βArr1, agonist exposure fails to elicit a detectable cAMP response, and production of inositol phosphates is <3% that of the wild-type receptor (29). Fig. 1C depicts the cellular distribution of NK1 receptor and NK1-βArr1 expressed in HEK-293 cells as determined by confocal immunofluorescence microscopy. Nonpermeabilized cells expressing NK1 receptor exhibited strong surface staining for HA-epitope, consistent receptor expression on the plasma membrane. In contrast, nonpermeabilized NK1-βArr1-expressing cells exhibited minimal cell-surface FLAG-epitope staining. Both receptors showed diffuse staining following membrane permeabilization. This agrees with published work demonstrating that NK1-βArr1 undergoes constitutive β-arrestin-dependent endocytosis and resides primarily resides within recycling endosomes (29). Thus, the fusion of β-arrestin1 to the NK1 receptor causes it to behave as a constitutively desensitized receptor, uncoupled from heterotrimeric G proteins and sequestered off the plasma membrane in an intracellular compartment.

Fig. 2 depicts the effect of NK1 receptor and NK1-βArr1 expression on ERK1/2 phosphorylation. As shown in Fig. 2A, 5-min exposure of NK1 receptor-expressing cells to NKA increased endogenous ERK1/2 phosphorylation to an extent comparable to that observed following treatment with a maximally efficacious concentration of PMA, which stimulates PKC-dependent ERK1/2 activation (33). Fig. 2B shows the results of the identical experiment performed on cells expressing NK1-βArr1. In these cells, basal ERK1/2 phosphorylation was markedly elevated and was not further increased either by exposure to either NKA or PMA. In both NK1 receptor- and NK1-βArr1-expressing cells, 30-min incubation with the MEK1/2 inhibitor PD98059 reduced ERK1/2 phosphorylation to a similar minimally detectable level. Although the wild-type NK1 receptor behaved as a typical membrane-localized GPCR with respect to agonist responsiveness, NK1-βArr1 expression alone was sufficient to produce near maximal ERK1/2 phosphorylation, as evidenced by the failure of PMA to generate a further increase. This constitutive activation of the ERK1/2 cascade contrasts dramatically with the constitutive desensitization of G protein signaling and intracellular sequestration of the chimera. The failure of NK1-βArr1 to respond to NKA probably reflects a combination of factors; the high level of basal ERK1/2 phosphorylation elicited by the chimera, the ability of the intrinsic β-arrestin moiety to preclude G protein coupling, and the fact the NK1-βArr1 is sequestered intracellularly, where it is not accessible to peptide ligands.

To determine whether NK1 receptor and NK1-βArr1 differentially affected the cellular distribution of active ERK1/2, we examined the distribution of endogenous phospho-ERK1/2 by confocal immunofluorescence microscopy. As shown in Fig. 3A, HEK-293 cells expressing wild-type NK1 receptors exhibited minimal phospho-ERK1/2 staining in the absence of agonist. Exposure to NKA for 5 min increased phospho-ERK1/2 staining, primarily within the cytosol, and a modest increase of nuclear staining was apparent. Treatment with PMA produced a similar pattern of cytosolic and nuclear phospho-ERK1/2 staining. Fig. 3B shows the distribution of endogenous phospho-ERK1/2 in cells expressing NK1-βArr1. Consistent with the whole cell phospho-ERK1/2 immunoblots, NK1-βArr1 expression was sufficient to increase ERK1/2 phosphorylation, and neither the intensity nor distribution of phospho-ERK1/2 changed upon the addition of NKA. In contrast to cells expressing the wild-type receptor, no detectable nuclear phospho-ERK1/2 was observed. Treatment with PMA, however, did appear to increase nuclear staining beyond that in untreated cells despite having little effect on the overall intensity of phospho-ERK1/2 staining.

To quantitatively assess the effect of NK1 receptor and NK1-βArr1 signaling on the nuclear translocation of ERK1/2, we employed cells coexpressing GFP-ERK2. GFP-ERK2 has previously been shown to undergo GPCR-stimulated phosphorylation and nuclear translocation like endogenous ERK1/2 (17, 34) and to bind β-arrestin in coprecipitation assays (18). As shown in Fig. 4A, the pattern of GFP-ERK2 phosphorylation paralleled that of endogenous ERK1/2 in HEK-293 cells expressing NK1 receptor or NK1-βArr1. Fig. 4B shows representative low mag-
nification fields of HEK-293 cells expressing GFP-ERK2 and NK1 receptor or NK1-β/H9252/Ar1 under varying conditions of stimulation. Fig. 4 presents the mean nucleus:cytosol ratio of GFP fluorescence for untreated and stimulated NK1 receptor- and NK1-β/H9252/Ar1-expressing cells. As evidenced by the relative lack of nuclear GFP fluorescence and decrease in cytosolic fluorescence, it is a measure of relative change, not a measure of the fraction of GFP-ERK2 in the nucleus. Data were therefore normalized such that the Nuc/Cyto ratio in untreated cells expressing NK1 receptor was arbitrarily assigned a value of 1.0 and the Nuc/Cyto ratios under other conditions were normalized to that baseline. Data shown represent the mean ± S.E. values from measurements of 60 cells in fields from each of three separate experiments. D, cells expressing wild-type NK1 or NK1-β/H9252/Ar1 were treated for 90 min with vehicle (NS) or NKA (1 μM) in the absence or presence of PD98059 (1 μM), and whole cell mRNA was isolated as described. Expression of Egr1 mRNA was determined by quantitative real-time PCR. Data shown represent the mean ± S.E. values from three separate experiments.

To test whether ERK1/2 activated by the NK1 receptor and NK1-β/H9252/Ar1 was capable of stimulating gene transcription, we assayed ERK1/2-dependent expression of mRNA encoding
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**FIGURE 5.** Coprecipitation of the kinases of the ERK1/2 pathway with NK1 and NK1-βArr1. Serum-deprived HEK-293 cells expressing wild-type HA-NK1 receptor or FLAG-NK1-βArr1 were incubated with or without NKA (1 μM) for 5 min prior to solubilization in lysis buffer. Anti-HA and anti-FLAG immunoprecipitates were resolved by SDS-PAGE, and parallel filters were probed with rabbit polyclonal IgG against the FLAG or HA epitopes, ubiquitin, β-arrestin1/2, cRaf-1, B-Raf, MEK1/2, ERK1/2, or phospho-ERK1/2 as indicated (left panels). Immunoblots of whole cell lysates depicting expression of endogenous β-arrestin1/2, cRaf-1, B-Raf, MEK1/2, and ERK1/2 are shown for comparison (right panels). Shown are representative immunoblots from one of three separate experiments that produced similar results.

**FIGURE 6.** Effect of tyrphostin AG1478 and GM6001 on NK1 receptor- and NK1-βArr1-stimulated ERK1/2 phosphorylation. A, serum-deprived HEK-293 cells expressing wild-type NK1 were preincubated for 30 min in the presence or absence of AG1478 (100 nM) or GM6001 (10 μM) prior to stimulation for 5 min with NKA (1 μM). Phospho-ERK1/2 in whole cell lysate samples was determined by immunoblotting as described. B, results of identical experiments performed on cells expressing NK1-βArr1. In each panel, representative phospho-ERK1/2 immunoblots are shown above a bar graph representing mean ± S.E. for three independent experiments. *p < 0.05.

**Egr1,** a zinc finger transcription factor induced as part of the immediate-early gene expression program. As shown in Fig. 4D, 90-min exposure to NKA produced a >10-fold increase in Egr1 mRNA abundance in NK1 receptor-expressing cells. The response was predominantly ERK1/2-dependent as indicated by its sensitivity to PD98059. In contrast, NK1-βArr1 expression had no effect on Egr1 transcription despite its ability to generate robust constitutive ERK1/2 activation. These results are consistent with the hypothesis that ERK1/2 activated by NK1-βArr1 is largely confined within the cytosol and unable to elicit a transcriptional response.

**NK1-β-arrestin1 Forms a Stable Multiprotein Complex with the Components of the Raf-MEK-ERK Cascade**—The finding that NK1-βArr1 expression was sufficient to activate cytosolic arrestin complex (38, 39). When probed for ERK1/2 pathway components, we found that both of the endogenous Raf isoforms expressed in HEK-293 cells, cRaf-1 and B-Raf, were detected in NK1-βArr1 immunoprecipitates, along with MEK1/2 and ERK1/2. As expected, none of the protein-protein interactions were affected by NKA treatment (data not shown). Consistent with the confocal immunofluorescence studies showing colocalization of activated ERK1/2 with internalized GPCR-arrestin complexes in early endosomes (18, 39), we detected a strong phospho-ERK1/2 signal in NK1-βArr1 immunoprecipitates.

Parallel immunoprecipitates of HA-tagged NK1 receptor showed that the native receptor engages in a similar pattern of protein-protein interactions but in an agonist-dependent man-
ERK1/2 activation observed in cells expressing NK1-β-arrestin1, but not NKA-stimulated ERK1/2 activation by the Wild-type NK1 Receptor, Is Dependent on Basal PKC and PKA Activity—Depending on receptor and cell type, multiple signals can contribute to GPCR-mediated ERK1/2 activation (9–11). We therefore employed a panel of pharmacologic inhibitors to determine similarities and differences in the mechanism of ERK1/2 activation employed by each receptor. Fig. 6 compares the effects of the EGF receptor kinase inhibitor, tyrphostin AG1478, and the broad-spectrum matrix metalloprotease inhibitor, GM6001, on ERK1/2 activation in cells expressing the wild type NK1 or NK1-β-arrestin1 receptors. As shown in Fig. 6A, NKA-stimulated ERK1/2 activation by the wild-type receptor was strongly inhibited by both AG1478 and GM6001, suggesting that, under conditions where the receptor, but not β-arrestin, is overexpressed, the signal arises almost entirely from EGF receptor transactivation occurring as a result of metalloprotease-dependent release of an EGF receptor ligand. In contrast, the constitutive ERK1/2 activation observed in cells expressing NK1-β-arrestin1 was insensitive to either inhibitor.

Wild-type NK1 receptors couple to both Gs-adenyl cyclase and Gq/11-phospholipase Cβ, leading to activation of PKA and PKC, respectively (31, 32). To determine whether these second messenger-dependent protein kinases were involved in NK1 receptor-mediated ERK1/2 activation, we tested whether the NKA response was sensitive to either the PKA inhibitor H-89 or the PKC inhibitor Ro-31-8220. To assess the efficacy of PKA inhibition, experiments were performed in cells expressing FLAG-tagged VASP. VASP is a PKA substrate that undergoes a prominent electrophoretic mobility shift on SDS-PAGE when phosphorylated, permitting endogenous PKA activity to be measured as the fraction of FLAG-VASP in the slower migrating phosphorylated state in anti-FLAG immunoblots of whole cell lysates (40). As shown in Fig. 7A, isoproterenol stimulation of endogenous β2-adrenergic receptors in FLAG-VASP-expressing HEK-293 cells caused ~50% of the protein to become phosphorylated, an effect that was completely blocked when cells were preincubated with H-89. PKC activation with PMA did not cause VASP phosphorylation. Fig. 7B depicts the effects of NKA stimulation on PKA activity in cells expressing both NK1 receptor and FLAG-VASP. As expected, 5-min stimulation with NKA increased FLAG-VASP phosphorylation to an extent similar to that seen with isoproterenol, and the effect was completely blocked by H-89 but not Ro-31-8220. Fig. 7C shows the effect H-89 and Ro-31-8220 on NKA-stimulated MEK1/2 and ERK1/2 phosphorylation in the same cell lysates. In these experiments, Ro-31-8220 was employed at a concentration sufficient to completely inhibit PMA-stimulated ERK1/2 phosphorylation (data not shown). Despite complete blockade of PKA and PKC activity, H-89 and Ro-31-8220 did not significantly effect NKA-stimulated MEK1/2 and ERK1/2 phosphorylation either when used alone or in combination. Thus, the major signaling pathway linking wild-type NK1 receptors to rapid activation of the ERK1/2 cascade involves neither PKA nor PKC.
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Fig. 8 shows the results of analogous experiments performed with the NK1-βAr1 chimera. As shown in Fig. 8A, cells expressing NK1-βAr1 did not exhibit increased FLAG-VASP phosphorylation either basally or after NKA treatment when compared with HEK-293 cells expressing only FLAG-VASP. This is consistent with earlier reports that NK1-βAr1 is effectively uncoupled from Gs (29). Although there was no detectable increase in PKA activity in cells expressing NK1-βAr1, H-89 did significantly reduce basal PKA activity. Fig. 8B depicts the effects of H-89 and Ro-31-8220 on MEK1/2 and ERK1/2 phosphorylation in these lysates. Surprisingly, both inhibitors produced a marked reduction of MEK1/2 and ERK1/2 phosphorylation, and when used in combination the phosphorylation was reduced to undetectable levels.

These data demonstrate that rapid activation of ERK1/2 by the wild-type NK1 receptor and constitutive ERK1/2 activation in NK1-βAr1-expressing cells are mechanistically distinct processes. Acute NK1 receptor-mediated ERK1/2 activation primarily reflects EGF receptor transactivation and does not require PKA or PKC activity. Such dissociation of transactivation-dependent ERK1/2 activation from second messenger-dependent protein kinase signaling has been described with other GPCRs (9, 11). In contrast, the pattern of inhibitor sensitivity seen for ERK1/2 activation in NK1-βAr1-expressing cells appears paradoxical. The NK1-βAr1 chimera is uncoupled from G proteins and constitutively activates ERK1/2 in the absence of agonist. Its expression does not increase basal PKA activity, yet its ability to cause ERK1/2 phosphorylation requires basal PKA and PKC activity, as demonstrated by the inhibitory effects of H-89 and Ro-31-8220 on MEK1/2 and ERK1/2 phosphorylation. These data suggest that NK1-βAr1 promotes ERK1/2 activation by increasing the efficiency with which a low basal level of PKA/PKC activity is translated through Raf into the sequential phosphorylation of MEK1/2 and ERK1/2. Of note, PMA treatment did increase ERK1/2 phosphorylation in H-89-treated NK1-βAr1-expressing cells, indicating that PKC signaling is intact and that the inability to detect a PMA response in the absence of inhibitors is due to the high level of ERK1/2 phosphorylation generated by NK1-βAr1 using basal PKA/PKC activity (data not shown).

ERK1/2 Activated by NK1-β-arrestin1 Is Less Susceptible to Dephosphorylation than ERK1/2 Activated by the Wild-type NK1 Receptor—Another mechanism whereby NK1-βAr1 might increase in ERK1/2 phosphorylation is by affecting the rate of ERK1/2 dephosphorylation by MAP kinase phosphatases (MKPs). The classic MKPs are a family of ten dual specificity threonine/tyrosine phosphatases that differ in tissue distribution, MAP kinase specificity, and subcellular distribution (41). Some MKPs are exclusively nuclear, others localize to the cytosol or shuttle between nucleus and cytosol. All share an N-terminal MAP kinase docking site. Because the majority of the phospho-ERK1/2 in NK1-βAr1-expressing cells was confined to the cytosol, it should be protected from dephosphorylation by nuclear MKPs. In addition, β-arrestin might compete with MKPs for binding to phospho-ERK1/2, thereby slowing the rate of dephosphorylation by cytosolic MKPs. To determine whether NK1-βAr1 expression affected ERK1/2 dephosphorylation, we measured the rate at which the phospho-ERK1/2 signal decayed following addition of PD98059. As shown in Fig. 9, addition of PD98059 to cells expressing NK1 receptor led to a complete loss of the phospho-ERK1/2 signal within 4 min. In cells expressing NK1-βAr1, the initial rate of signal loss was slower. In addition, only ~50% of the total ERK1/2 pool appeared to be susceptible to rapid dephosphorylation. The residual phospho-ERK1/2 signal persisted for at least 10 min, although by 30 min the ERK1/2 pool was completely dephosphorylated. This suggests that NK1-βAr1-bound ERK1/2 is resistant to dephosphorylation, but that the complex either dissociates...
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FIGURE 9. Effect of NK1 and NK1-βArr1 on the rate of dephosphorylation of endogenous ERK1/2. Serum-deprived HEK-293 cells expressing wild-type NK1 or NK1-βArr1 were incubated for 5 min in the presence of absence of NKA (1 μM), after which the medium was exchanged for medium containing PD98059 (1 μM) plus NKA. Phospho-ERK1/2 was determined by immunoblotting whole cell lysate samples obtained at the indicated times after the addition of PD98059. Data were normalized such that the level of ERK1/2 phosphorylation measured prior to adding PD98059 was defined as 100%. Depicted are representative phospho-ERK1/2 immunoblots (A) shown above a graph representing mean ± S.E. for three independent experiments (B).

slowly or that cytosolic MKPs eventually gain access to β-arrestin1-bound phospho-ERK1/2.

DISCUSSION

Despite convincing data that β-arrestin-dependent ERK1/2 activation is a mechanistically distinct process, relatively little is known about how β-arrestin binding activates ERK1/2 independent of G protein coupling. Our results suggest that NK1-βArr1 expression has three effects that promote sustained ERK1/2 phosphorylation. First, the binding of cRaf-1, B-Raf, MEK1/2, and ERK1/2 to the signalsome complex enhances efficiency within the phosphoresponse cascade. Second, the recruitment of β-arrestin-bound Raf to a membrane surface appears to be necessary for Raf activation. Overexpression of β-arrestin1 alone does not activate ERK1/2 despite its ability to bind c-Raf1, MEK1/2, and ERK1/2 (18). In contrast, translocation of β-arrestin2 to the plasma membrane can activate ERK1/2 independent of GPCR binding (42). Similarly, membrane targeting of c-Raf1 by the addition of a C-terminal prenylation (CAAX) motif increases Raf activity in the absence of upstream Ras-activating stimuli (43). In conjunction with our data, these findings suggest that the critical event in β-arrestin-dependent ERK1/2 activation is the targeting of β-arrestin-bound Raf to the membrane and that the major role of the GPCR is simply to provide an agonist-regulated docking site for β-arrestin. Third, NK1-βArr1 expression slows the rate of ERK1/2 dephosphorylation, allowing for the accumulation of a cytosolic pool of active ERK1/2. Nuclear ERK1/2 is dephosphorylated more rapidly than it is returned to the cytosol, causing inactive ERK1/2 to be sequestered in the nucleus where it cannot be immediately reactivated (44). Because NK1-βArr1-bound phospho-ERK1/2 is retained in the cytosol, it is may be protected from both cytosolic and nuclear MKPs, favoring the buildup of active kinase.

Although our data suggest that membrane localization of β-arrestin-bound Raf is necessary for G protein-independent ERK1/2 activation, it does not appear to be sufficient. Although NK1-βArr1 expression does not activate PKA or PKC, NK1-βArr1-dependent ERK1/2 activation is dependent on the basal cellular activity of those kinases. This suggests that ERK1/2 activation results from an increase in the efficiency with which the membrane-associated NK1-βArr1 scaffold converts basal PKA/PKC-dependent Raf activation into robust ERK1/2 activation, rather than from stimulation of a unique signaling pathway upstream of Raf. It has been shown that PKC stimulation by phorbol esters activates cRaf-1, perhaps due to direct phosphorylation (33). The effects of PKA on Raf activity are more complex. Although PKC phosphorylation inhibits cRaf-1 (45), it activates B-Raf, either directly or by modulating the activity of the low molecular weight G protein, Rap1 (46, 47). Both cRaf-1 and B-Raf probably contribute to ERK1/2 activation in NK1-βArr1-expressing cells, because we were able to detect both in NK1-βArr1 immunoprecipitates.

In the case of a native GPCR, where G protein-mediated signals leading to Ras and Raf activation are initiated prior to β-arrestin recruitment, the dominant mechanism of ERK1/2 activation at any point in time probably reflects a dynamic equilibrium between active G protein-coupled and β-arrestin-bound desensitized receptors. β-Arrestins act as the switch between signaling modes, because β-arrestin binding is sufficient both to terminate receptor-G protein coupling and to initiate β-arrestin-dependent ERK1/2 activation (5, 6). Predictably, the extent to which β-arrestin-dependent signaling contributes to overall cellular ERK1/2 activity varies depending on the GPCR, the cellular context in which it is expressed, and the duration of the stimulus. Some GPCRs, such as protease-activated receptor 2, apparently utilize predominantly β-arrestin-dependent signals to activate ERK1/2 (17). The angiotensin AT1a receptor activates ERK1/2 through both G protein-dependent and β-arrestin-dependent pathways in approximately equal measures (12, 16), whereas the β-arrestin-dependent signaling represents a minor component of β2-adrenergic receptor-mediated ERK1/2 activation (20). In many cases, the initial phase of GPCR-stimulated ERK1/2 activation is G protein-dependent, whereas ERK1/2 activation at longer time points is β-arrestin-dependent. In β-arrestin1/2 null murine embryo fibroblasts, lysophosphatidic acid receptors generate persistent G protein-dependent ERK1/2 activation primarily by transactivating EGFR receptors. Restoring β-arrestin2 expression to the null background hastens receptor desensitization, such that transactivation-dependent ERK1/2 activation is lost within 10–15 min of stimulation. In these cells, reintroducing β-arrestin2 also establishes a sustained phase of EGF receptor-independent ERK1/2 activation that apparently reflects a β-arrestin-dependent signal (14).
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In the case of the wild-type NK1 receptor expressed in HEK-293 cells, the signals supporting NKA-stimulated ERK1/2 activation are clearly distinct from those that support constitutive ERK1/2 activation by the NK1-βArr1 chimera. Although NK1 receptors activate both PKA and PKC, neither kinase is required for rapid NKA-stimulated ERK1/2 activation. Instead, the signal arises primarily from MMP-dependent EGF receptor transactivation. Although NKA stimulation does promote the association of β-arrestin, c-Raf1, and ERK1/2 with the receptor, the initial phase of NK1 receptor-mediated ERK1/2 activation is predominantly β-arrestin-independent. The lack of a significant β-arrestin-dependent component to the early response probably results, at least in part, from overexpression of the wild-type receptor in stoichiometric excess over the endogenous β-arrestin pool.

The stable association of ERK1/2 with GPCR-arrestin complexes appears to influence the function, as well as the time course of ERK1/2 activation. Receptors like the angiotensin AT1a and vasopressin V2 that stably bind β-arrestin tend to promote extranuclear retention of ERK1/2 and attenuate ERK1/2-dependent transcription (18–20). Despite producing an increase in total cellular ERK1/2 phosphorylation, overexpression of β-arrestin2 clearly inhibits AT1a receptor-stimulated ERK1/2-dependent activation of an Elk-1 luciferase reporter, as would be expected of a cytosolic phospho-ERK1/2-binding protein (19). On the other hand, GPCRs that form transient ERK1/2-arrestin complexes, such as the lysophosphatidic acid 1 receptor, appear to be able to drive ERK1/2-dependent transcription through a β-arrestin-dependent mechanism, suggesting that the dissociation of β-arrestin from the receptor may allow activated ERK1/2 to gain access to the cell nucleus (14). Indeed, exchanging the C terminus of the V2 vasopressin receptor for that of the β2-adrenergic receptor, which changes the V2 receptor-arrestin interaction from stable to transient (48), permits the chimeric receptor to stimulate an Elk1-luciferase reporter (20).

In addition to directly phosphorylating nuclear transcription factors, ERK1/2 has numerous plasma membrane, cytoplasmic, and cytoskeletal substrates (49). These include several proteins involved in heptahelical receptor signaling, such as β-arrestin1, G-protein-coupled receptor kinase 2, and the Gα-interacting protein, GAIP. One role of the GPCR-β-arrestin signalosome could be to dictate substrate specificity by targeting ERK1/2 to non-nuclear substrates involved in the regulation of GPCR signaling or intracellular trafficking. In addition, β-arrestin-dependent targeting of ERK1/2 to the leading edge of cells appears to play a role in localized actin assembly in during protease-activated receptor 2-receptor-mediated chemotaxis (23, 24), which may help explain the chemotactic defect observed in leukocytes from β-arrestin2 null mice (50).

Scaffolding of the ERK1/2 kinase cascade by β-arrestin-bound GPCRs appears to be a unique mode of GPCR signaling that confines a distinct time course, spatial distribution, and function on ERK1/2. Our study of constitutive ERK1/2 activation by the NK1-βArr1 chimera suggests that it closely mimics β-arrestin-dependent ERK1/2 activation by native GPCRs, in that it is independent of heterotrimeric G protein activation, results from scaffolding of the Raf-MEK-ERK, and retains active ERK1/2 outside the cell nucleus. As such, it may provide a valuable model for further study aimed at determining the protein composition of the putative GPCR signalosome and identifying plasma membrane, cytosolic, and cytoskeletal ERK1/2 substrates whose phosphorylation may be dependent on β-arrestin-dependent signaling.

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