β-Arrestin 2 Expression Determines the Transcriptional Response to Lysophosphatidic Acid Stimulation in Murine Embryo Fibroblasts*

Diane Gesty-Palmer1, Hesham El Shewy2, Trudy A. Kohout*, and Louis M. Luttrell†

From the 1Department of Medicine, Duke University Medical Center, Durham, North Carolina 27710, 2The Geriatrics Research, Education and Clinical Center, Durham Veterans Affairs Medical Center, Durham, North Carolina 27705, 3Departments of Medicine and Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, South Carolina 29425, 4The Ralph H. Johnson Veterans Affairs Medical Center, Charleston, South Carolina 29401, and 5Departments of Exploratory Discovery and Molecular Biology, Neurocrine Biosciences Inc., San Diego, California 92121

G protein-coupled receptors often employ novel signaling mechanisms, such as transactivation of epidermal growth factor (EGF) receptors or G protein-independent signals transmitted by β-arrestins, to control the activity of extracellular signal-regulated kinases 1 and 2 (ERK1/2). In this study we investigated the role of β-arrestins in lysophosphatidic acid (LPA) receptor-stimulated ERK1/2 activation using fibroblast lines derived from wild type, β-arrestin 1−/−, β-arrestin 2−/−, and β-arrestin 1/2 knock-out mice. LPA stimulation produced robust ERK1/2 phosphorylation in all four backgrounds. In cells lacking β-arrestin 2, >80% of LPA-stimulated ERK1/2 phosphorylation was mediated by transactivated EGF receptors. In contrast, ERK1/2 activation in cells expressing β-arrestin 2 was predominantly EGF receptor-independent. Introducing FLAG epitope-tagged β-arrestin 2 into the β-arrestin 1/2 null background restored EGF receptor-independent ERK1/2 activation, indicating that β-arrestin 2 expression confers ERK1/2 activation via a distinct mechanism. To determine the contributions of β-arrestin 2, transactivated EGF receptors, and ERK1/2 to LPA-stimulated transcriptional responses, we employed gene expression arrays containing cDNA markers for G protein-coupled receptor-mediated signaling. In the β-arrestin 1/2 null background, 1 h of exposure to LPA significantly increased transcription of seven marker genes. Six of these responses were EGF receptor-dependent, and two required ERK1/2 activation. In β-arrestin 2 expressing cells, three of the seven LPA-stimulated transcriptional responses observed in the β-arrestin 1/2 null background were lost. The four residual responses were independent of EGF receptor transactivation, but all were ERK1/2-dependent. These data indicate that β-arrestin 2 functions both to attenuate EGF receptor transactivation-dependent signaling and to promote a distinct subset of ERK1/2-mediated responses to LPA receptor activation.

The G protein-coupled receptors (GPCRs)2 comprise a large superfamily of seven transmembrane-spanning receptors that transmit extracellular signals by activating a variety of signaling pathways. All GPCRs function as ligand-activated guanine nucleotide exchange factors for heterotrimeric guanine nucleotide binding (G) proteins. The binding of ligand to a GPCR stabilizes the receptor in an “active” conformation in which it catalyzes GTP for GDP exchange on the Gα subunit of the GTP-bound heterotrimeric G protein, leading to dissociation of the GTP-bound Gα subunit from the Gβγ heterodimer. Once dissociated, both Gα-GBP and Gβγ subunits regulate the activity of enzymatic effectors, such as adenyl cyclases, phospholipase C isozymes, and ion channels.

Additional GPCR signaling mechanisms have been elucidated that are mechanistically distinct from the classical GPCR signaling paradigm. Most of these novel signals result from the binding of other proteins, such as PDZ domain-containing proteins or kinases, directly or indirectly to the intracellular domains of agonist-occupied GPCRs (1, 2). One such mechanism involves the arrestins, a small family of cytosolic proteins originally identified for their central role in GPCR desensitization (3, 4). Arrestins are recruited to agonist-occupied GPCRs that have been phosphorylated on C-terminal Ser/Thr residues by specialized G protein-coupled receptor kinases. Arrestin binding sterically inhibits receptor-G protein coupling, producing homologous receptor desensitization. In addition, the C-terminal tails of the two non-visual arrestins, β-arrestins 1 and 2, contain binding motifs for clathrin and the β2-adaptin subunit of the AP-2 complex that allow β-arrestin to target the receptor for clathrin-mediated endocytosis. The hypothesis that β-arrestins might also act as GPCR signal transducers originated with the finding that some Src family nonreceptor-tyrosine kinases bind to β-arrestins and are recruited along with it to agonist-occupied GPCRs (5). Subsequent work has identified a number of signaling proteins, including components of the extracellular signal-regulated kinases 1 and 2 (ERK1/2) and c-Jun N-terminal kinase 3 mitogen-activated protein kinase cascades, the E3 ubiquitin ligase, Mdm2, and the cAMP phosphodiesterase, PDE4D, that are recruited in an analogous manner (6–8). The general model is that β-arrestins function as adapter proteins that support the assembly of a multiprotein signaling complex on the receptor and initiate a second wave of G protein-independent signaling that commences as the receptor and G protein disengage.

In many cases the effects of GPCRs on cellular growth and differentiation involve activation of the ERK1/2 mitogen-activated protein kinase cascade. The signaling mechanisms underlying GPCR-mediated ERK1/2 activation are complex and may result from activation of clathrin-regulated kinases 1 and 2; GFP, green fluorescent protein; LPA, lysophosphatidic acid; MEF, murine embryo fibroblast; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; HEK cells, human embryonic kidney cells.

References:
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2. To whom correspondence should be addressed: Division of Endocrinology, Diabetes, and Medical Genetics, Dept. of Medicine, Medical University of South Carolina, 96 Jonathan Lucas St.,816 CSB, P. O. Box 250624, Charleston, SC 29425. Tel.: 843-792-2529; Fax: 843-792-4114; E-mail: luttrell@musc.edu.
3. The abbreviations used are: GPCR, G protein-coupled receptor; G protein, guanine nucleotide-binding protein; EGF, epidermal growth factor; ERK1/2, extracellular sig-
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physiologic levels of expression. Overexpression of β-arrestin 2, which is in effect a cytosolic ERK1/2-binding protein, has clearly been shown to promote cytosolic retention of active ERK1/2 and to attenuate GPCR-stimulated Elk1-luciferase reporter expression and mitogenesis after activation of angiotensin AT1a and vasopressin V2 receptors (12, 13). To better understand the function of β-arrestins in native systems, we capitalized on the existence of spontaneously immortalized clonal murine embryo fibroblast (MEF) lines derived from wild type, β-arrestin 1, β-arrestin 2, and β-arrestin 1/2 knock-out mice as well as β-arrestin 1/2 double knock-out MEFs in which β-arrestin 2 expression has been restored to endogenous levels by stable transfection (14). Using these models we have characterized the contribution of G protein-dependent and β-arrestin-dependent signals to ERK1/2 activation by endogenously expressed LPA receptors and determined the contribution of these signals to the early transcriptional response to LPA. We find that in the absence of β-arrestin 2 expression, cross-talk between LPA and epidermal growth factor (EGF) receptors represents the dominant mechanism of LPA-stimulated ERK1/2 activation and accounts for the majority of the transcriptional responses detected using limited gene arrays. Expression of β-arrestin 2 confers ERK1/2 activation through an EGF receptor-independent mechanism while simultaneously promoting the rapid termination of the EGF receptor-dependent signal, resulting in an altered pattern of transcription. These results underscore the duality of β-arrestin function as both a terminator of G protein-mediated signaling and as a transducer of signals capable of mediating a distinct subset of LPA-stimulated transcriptional responses.

EXPERIMENTAL PROCEDURES

Materials—Tyrothrophin AG1478, PD98059, and EGF were purchased from Calbiochem-EMD Biosciences Inc. (San Diego, CA). LPA and mononclonal M2 anti-FLAG affinity agarose were from Sigma. Trizol reagent, sheared salmon sperm DNA, AlexaFluor 568 goat anti-rabbit IgG, cell culture media, and supplements were from Invitrogen. RNase inhibitor and Moloney murine leukemia virus reverse transcriptase were from Promega Corp. (Madison, WI). RNeasy mini kits were from Qiagen Inc. (Valencia, CA). SuperArray GEArray Q series mouse G protein-coupled receptor signaling pathwayFinder gene arrays were from SuperArray Bioscience Corp. (Frederick, MD). Biotin-16-DUTP and FuGENE 6 were from Roche Diagnostics. Polyclonal phosphorylation state-specific anti-ERK1/2 IgG was from Cell Signaling Technology Inc. (Beverly, MA), and total anti-ERK1/2 IgG was from Upstate Biotechnology, Inc. (Waltham, MA). Horseradish peroxidase-conjugated donkey anti-rabbit and anti-mouse IgG were fromJackson ImmunoResearch Laboratories Inc. (West Grove, PA). Rabbit polyclonal anti-β-arrestin was a gift from Robert J. Lefkowitz (Duke University, Durham, NC). cDNA Constructs—The pcDNA3.1 expression plasmids encoding green fluorescent protein (GFP)-tagged β-arrestin 1 and β-arrestin 2 (15, 16) and influenza virus hemagglutinin epitope-tagged AT1a angiotensin receptor were gifts from Marc G. Caron (Duke University, Durham, NC). The pcDNA3.1 expression plasmid encoding influenza virus hemagglutinin epitope-tagged LPA1 receptor was a gift from Richard T. Premont (Duke University, Durham, NC). The expression plasmid used to stably express FLAG epitope-tagged β-arrestin 2 in MEF lines (pcDNA3.1-FL-β-arrestin 2-Hyg) was generated by substituting a hygromycin resistance cassette in place of the neomycin resistance cassette in pcDNA3.1-FL-β-arrestin 2 and was a gift from William E. Miller (University of Cincinnati, Cincinnati, OH).

Cell Culture and Transfection—Wild type, β-arrestin 1−/−, β-arrestin 2−/−, and β-arrestin 1/2−/− MEF lines were gifts from Robert J. Lefkowitz. These spontaneously immortalized clonal lines were derived from day 10.5 to day 13.5 embryos obtained from crosses between β-arrestin 1 (+/−) or β-arrestin 2 (+/−) mice bred to produce littermate wild type and knock-out embryos, as previously described (14). Double β-arrestin 1/2 knock-out (D2) MEFs were generated from crosses of β-arrestin 1 (+/−) × β-arrestin 2 (+/−) and β-arrestin 1 (−/−) × β-arrestin 2 (−/−) mice. MEFs stably expressing FL-β-arrestin 2 on the β-arrestin 1/2 null background (D2-βarr2) were generated by calcium phosphate transfection of the D2 MEF line with pcDNA3.1-FL-β-arrestin 2-Hyg followed by clonal selection to obtain lines expressing near physiologic levels of β-arrestin 2. All MEF lines were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 100 units/ml penicillin/streptomycin. HEK-293 cells were obtained from the American Type Culture Collection and were maintained in minimal essential medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. Transient transfection of HEK-293 cells was performed using FuGENE 6 according to the manufacturer’s instructions. Before all experiments, cell monolayers were incubated for 16–20 h in serum-free growth medium supplemented with 10 mM HEPES (pH 7.4), 0.1% bovine serum albumin, and penicillin/streptomycin.

ERK1/2 Phosphorylation—To assay ERK1/2 phosphorylation in whole cell lysates, serum-starved cell monolayers in 12-well plates were stimulated at 37 °C as described and lysed directly with 1× Laemmli sample buffer. Sonicated samples were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Phosphorylated ERK1/2 was detected by immunoblotting using rabbit polyclonal anti-phospho-ERK1/2 IgG with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody. Total ERK1/2, measured to confirm equal loading of whole cell lysate samples, was detected using polyclonal anti-ERK1/2 IgG.
Assays measuring the fraction of total cellular ERK1/2 in the phosphoformylated form exploited the fact that phospho-ERK1/2 exhibits faster electrophoretic mobility when subjected to PAGE under native conditions. D2 and D2-βarr2 MEFs in 6-well plates were stimulated as described, lysed in 200 μl of glycerol lysis buffer, and clarified by centrifugation. Aliquots of whole cell lysate containing ~20 μg of protein were mixed with 5× sample buffer lacking SDS and β-mercaptoethanol and resolved by native protein electrophoresis on 10% precast Novex Tris-glycine gels run per the manufacturer’s protocol. Total and phospho-ERK1/2 were detected by immunoblotting with polyclonal anti-ERK1/2 IgG and distinguished based on their differential electrophoretic mobility. Data were expressed as the fraction of total ERK1/2 signal present in the faster migrating phospho-ERK1/2 bands.

Ras Activation—Ras activation was measured using the Ras activation assay kit from Upstate Biotechnology, Inc. according to the manufacturer’s protocol. Briefly, serum-starved D2 and D2-βarr2 MEFs in 10-cm plates were stimulated as described and lysed at 4 °C using the kit-supplied lysis buffer. GTP-bound Ras was isolated by binding to GST-Raf-1 Ras binding domain fusion protein on agarose beads and resolved by SDS-PAGE. Ras in the precipitates was visualized by immunoblotting with murine monoclonal anti-Ras IgG using horseradish peroxidase-conjugated donkey anti-mouse IgG for chemiluminescent detection. Immunoblots of total Ras in each lysate were performed to ensure equal protein content in the starting sample.

Confocal Fluorescence Microscopy—For visualization of β-arrestin recruitment, HEK-293 cells transiently expressing influenza virus hemagglutinin epitope-tagged LPA1 receptors or influenza virus hemagglutinin epitope-tagged AT1a receptors and GFP-tagged β-arrestin 1 or β-arrestin 2 in collagen-coated 35-mm glass-bottom dishes were stimulated as described, washed with Dulbecco’s phosphate-buffered saline, fixed with 4% paraformaldehyde for 30 min at room temperature, and again washed with phosphate-buffered saline before examination. To visualize the subcellular distribution of endogenous phospho-ERK1/2, D2 or D2-βarr2 MEFs were stimulated as described, fixed with paraformaldehyde, permeabilized with 0.2% Triton X-100 in phosphate-buffered saline for 5 min, then incubated in blocking buffer (2% bovine serum albumin in phosphate-buffered saline) for 30 min at room temperature. Phospho-ERK1/2 was stained overnight at 4 °C using a 1:1000 dilution of rabbit polyclonal anti-phospho-ERK1/2 in Tris-glycine gels run per the manufacturer’s protocol. Total and phospho-ERK1/2 were resolved by native protein electrophoresis on 10% precast Novex Tris-glycine gels run per the manufacturer’s protocol. Briefly, serum-starved wild type, β-arrestin 1 knock-out (β-arrestin 1−/−), and β-arrestin 2 knock-out (β-arrestin 2−/−) MEFs, and β-arrestin 1/2 double knock-out (D2) MEFs. B, effect of tyrophosint AG1478 on LPA- and EGF-stimulated ERK1/2 phosphorylation (P) in wild type and β-arrestin knock-out MEFs. Serum-starved wild type, β-arrestin 1−/−, β-arrestin 2−/−, and D2 MEFs were preincubated for 15 min the presence of absence of AG1478 (200 nM) before stimulation for 5 min with LPA (10 μM) or EGF (10 ng/ml). Phosphorylation of endogenous ERK1/2 was determined by immunoblotting of whole cell lysates as described. Results shown are representative of three separate experiments.

RESULTS

Effect of β-Arrestin Content on the Mechanism of LPA Receptor-mediated ERK1/2 Activation in Knock-out Murine Embryo Fibroblasts—To determine the effect of varying the expression of β-arrestin isoforms on the mechanism of LPA receptor-mediated ERK1/2 activation, we initially studied clonal MEF lines derived from wild type, β-arrestin 1, β-arrestin 2, and β-arrestin 1/2 knock-out mice (14). Fig. 1A illustrates the β-arrestin expression profile of each the four lines; wild type, β-arrestin 1−/−, β-arrestin 2−/−, and β-arrestin 1/2−/− (D2 cells). As shown in Fig. 1B, exposure to LPA for 5 min resulted in ERK1/2 activation in all four lines. However, when stimulation was performed in the presence of tyrophosint AG1478, a selective inhibitor of EGF receptor family tyrosine kinases, differences in the pattern of ERK1/2 activation emerged. In the wild type and β-arrestin 1−/− MEFs, both of which express β-arrestin 2, the LPA response was only modestly attenuated by a concentration of AG1478 that completely eliminated the response to EGF. In contrast, the response in the β-arrestin 2−/− and D2 MEFs was almost completely sensitive to AG1478. Because inhibition by AG1478 is a hallmark of GPCR-stimulated ERK1/2 activation caused by EGF receptor transactivation (17–19), these results clearly demonstrate that β-arrestin expression is not required for cross-talk between LPA and EGF receptors. On the other hand, the persistence of LPA-stimulated ERK1/2 activation in AG1478-treated wild type and β-arrestin 1−/− MEFs suggests that the presence of β-arrestin 2, but not β-arrestin 1, confers a mechanism of ERK1/2 activation that is independent of EGF receptor transactivation.

Because each of the β-arrestin knock-out MEF lines was clonally derived, it was possible that the observed LPA signaling phenotypes represented clonal variation rather than a specific consequence of β-arrestin 2 expression. We, therefore, tested whether reintroduction of

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**Figure 1.** Loss of tyrophosint AG1478-sensitive LPA-stimulated ERK1/2 activation in MEFs lacking β-arrestin 2. A, anti-β-arrestin immunoblot (IB) demonstrating expression of β-arrestins 1 and 2 in whole cell lysates from wild type, β-arrestin 1 knock-out (β-arrestin 1−/−), β-arrestin 2 knock-out (β-arrestin 2−/−), and β-arrestin 1/2 double knock-out (D2) MEFs. B, effect of tyrophosint AG1478 on LPA- and EGF-stimulated ERK1/2 phosphorylation (P) in wild type and β-arrestin knock-out MEFs. Serum-starved wild type, β-arrestin 1−/−, β-arrestin 2−/−, and D2 MEFs were preincubated for 15 min the presence of absence of AG1478 (200 nM) before stimulation for 5 min with LPA (10 μM) or EGF (10 ng/ml). Phosphorylation of endogenous ERK1/2 was determined by immunoblotting of whole cell lysates as described. Results shown are representative of three separate experiments.
β-arrestin 2 into the D2 background was sufficient to confer AG1478-insensitive ERK1/2 activation. For these experiments, D2 MEFs were stably transfected with FLAG epitope-tagged β-arrestin 2 (FL-βarr2), and cells expressing FL-βarr2 at approximately endogenous levels (D2-βarr2) were derived using hygromycin resistance selection. A representative β-arrestin immunoblot illustrating the relative level of β-arrestin 2 expression in wild type and D2-βarr2 MEFs is shown in Fig. 2A. To determine whether FL-βarr2 expression qualitatively altered the pattern of endogenous LPA receptor expression, we performed RNA hybridization assays to measure the mRNA level of each of the three LPA receptor isoforms (LPA1–3). As shown in Fig. 2B, LPA1 receptor mRNA represented the major LPA receptor transcripts in the D2 MEFs. The relative abundance of LPA1 receptor mRNA did increase with FL-βarr2 expression, possibly reflecting an increase in LPA1 mRNA transcription related to the restoration of β-arrestin 2-mediated receptor turnover. As shown in Fig. 2C, D2-βarr2 MEFs exhibited a significant increase in LPA-stimulated AG1478-insensitive ERK1/2 activation compared with the parental D2 MEFs. These data indicate that expression of FL-βarr2 at a level comparable with that of endogenous β-arrestin 2 was sufficient to restore EGF receptor transactivation-independent ERK1/2 activation in response to LPA.

We next compared the agonist dose dependence and extent of maximal LPA-stimulated ERK1/2 activation between D2 and D2-βarr2 MEFs. To compare the extent of ERK1/2 activation, we measured ERK1/2 phosphorylation as the fraction of the total ERK1/2 pool that underwent an electrophoretic mobility shift when whole cell lysates were resolved under non-denaturing conditions. As shown in Fig. 3A, 5 min of stimulation with LPA resulted in phosphorylation of 40–45% of the endogenous ERK1/2 pool in both D2 and D2-βarr2 MEFs. This compares with 55–60% ERK1/2 activation by EGF over the same time frame. Thus, despite the qualitative change in the predominant mechanism of ERK1/2 activation between β-arrestin null and β-arrestin 2-expressing MEFs, both pathways elicited responses of similar absolute magnitude. As shown in Fig. 3B, the EC50 for the early phase of LPA-stimulated ERK1/2 activation was also no different between D2 and D2-βarr2 cell lines.

Effect of β-Arrestin 2 Expression on the Contribution of Transactivated EGF Receptors to LPA-stimulated ERK1/2 Activation—Fig. 4A compares the time course of LPA-stimulated ERK1/2 activation in D2 MEFs in the presence and absence of AG1478. In untreated cells, the maximal 10–15-fold increase in ERK1/2 phosphorylation was observed within 5–10 min of stimulation, after which phospho-ERK1/2 levels declined gradually over 30 min to 6–8-fold over basal, where they remained for up to 4 h. In these cells, >80% of the area under the phospho-ERK1/2 curve was eliminated by treatment with AG1478 (shaded area), indicating that LPA-stimulated ERK1/2 activation was primarily dependent on EGF receptor transactivation throughout the entire 4-h time course. As shown in Fig. 4B, a strikingly different pattern emerged when the experiment was repeated using D2-βarr2 MEFs. In the presence of β-arrestin 2, LPA-stimulated phospho-ERK1/2 levels rose to a similar 14–18-fold over basal within 5 min but then declined abruptly until reaching a sustained 6–8-fold over basal after 10–20 min. Moreover, although the early peak of ERK1/2 phosphorylation, like ERK1/2 activation in wild type MEFs (Fig. 1B), was partially AG1478-sensitive, the slower sustained phase of ERK1/2 phosphorylation, between 10 min and 4 h, was not significantly inhibited. As a result, the AG1478-sensitive portion of the curve was largely confined to the first 10 min of stimulation (shaded area). This rapid termination of EGF receptor transactivation in D2-βarr2 MEFs probably reflects β-arrestin-dependent homologous desensitization of LPA receptors. Because an AG1478-sensitive ERK1/2 signal dominated in the β-arrestin null D2 MEFs, it is likely that the gradual decline and sustained duration of this signal reflected persistent G protein activation in the absence of receptor desensitization.
Transactivated EGF receptors catalyze ERK1/2 activation by recruiting Raf to activated Ras at the plasma membrane. In contrast, β-arrestin-dependent ERK1/2 activation appears to be a mechanistically distinct process that can occur under conditions where β-arrestin recruitment is induced in the absence of detectable G protein activation (20–23). Because FL-βarr2 expression led to rapid termination of EGF receptor-dependent ERK1/2 activation, we hypothesized that the duration of LPA-stimulated Ras activation would also be shortened in D2-βarr2 MEFs. To test this, we assayed Ras-GTP loading after stimulation of D2 and D2-βarr2 MEFs with LPA or EGF. As shown in Fig. 4C, exposure of D2 MEFs to either LPA or EGF produced robust Ras-GTP loading that was maintained at 5 and 30 min after stimulation. In D2-βarr2 MEFs LPA-stimulated Ras-GTP loading was detectable at 5 min, but by 30 min Ras-GTP levels had returned to approximately basal levels. EGF-stimulated Ras-GTP loading resembled that observed in D2 MEFs. Because only the early component of LPA-stimulated ERK1/2 activation in D2-βarr2 MEFs involved EGF receptor transactivation, these data suggest that Ras activation in response to LPA is EGFR receptor-dependent, whereas a distinct Ras-independent process transmits the slower β-arrestin 2-dependent signal.

We also determined the effect of FL-βarr2 expression on the subcellular distribution of endogenous phospho-ERK1/2 after stimulation with LPA. As shown in Fig. 4D, exposure of D2 MEFs to LPA for 5 or 30 min increased both cytosolic and nuclear phospho-ERK1/2. Nuclear staining exceeded that in the cytosol at 5 min, consistent with rapid nuclear translocation of ERK1/2 after EGF receptor transactivation. After 30 min of continuous stimulation, phospho-ERK1/2 staining was evenly distributed between the nucleus and cytosol. Stimulation of D2-βarr2 MEFs also increased both nuclear and cytosolic phospho-ERK1/2 staining. Nuclear staining at 5 min was consistently less in D2-βarr2 MEFs compared with D2 MEFs but remained detectable even at 30 min, after the EGF receptor-dependent component of ERK1/2 phosphorylation had waned. These data suggest that both the early EGF receptor-dependent and later β-arrestin 2-dependent pathways are capable of activating phospho-ERK1/2 throughout the cell, but that β-arrestin 2-dependent desensitization of EGF receptor transactivation attenuates the initial rapid increase in nuclear phospho-ERK1/2.

Activation of β-Arrestin 2-bound ERK1/2 by LPA in D2-βarr2 MEFs—If the only role of β-arrestin 2 in LPA signaling was to desensitize the receptor, thereby terminating EGF receptor transactivation, then ERK1/2 activation in the D2-βarr2 MEFs might be expected to decline rapidly after the initial rise and then remain low. What we observed, however, was that FL-βarr2 expression conferred a slow sustained phase of ERK1/2 activation that was not EGF receptor-dependent and not present in D2 MEFs. Several GPCRs have been shown to activate a β-arrestin-bound pool of ERK1/2 (12, 13, 24–27). In addition, the time course of ERK1/2 activation in D2-βarr2 MEFs in the presence of AG1478 resembled that previously described for β-arrestin 2-mediated scaffolding of the ERK1/2 cascade after stimulation of angiotensin AT1a receptors (20).

As shown in Fig. 5A, the activated LPA1 receptor, when overexpressed in HEK-293 cells, recruits β-arrestin 2-GFP but not β-arrestin 1-GFP. The pattern of β-arrestin 2-GFP recruitment, where receptor-arrestin complexes were detected at the plasma membrane but not in endosomes, is similar to that observed for a number of GPCRs, including the β2 and α1 adrenergic receptors. These GPCRs bind β-arrestin 2 on the plasma membrane but dissociate from it as the receptor internalizes (15, 16). This pattern of β-arrestin binding contrasts with the AT1a angiotensin II receptor, which recruits β-arrestin 1-GFP and
FIGURE 4. Effect of AG1478 on the time course of LPA-stimulated ERK1/2 phosphorylation and Ras activation in D2 and D2-βarr2 MEFs. A, serum-starved D2 MEFs were preincubated for 15 min in the absence (open circles) or presence (solid circles) of tyrophostin AG1478 (200 nM) before stimulation for 30 s to 4 h with LPA (10 μM). Phosphorylation (P) of endogenous ERK1/2 was determined by immunoblotting (IB) of whole cell lysates as described. B, results of identical experiments performed using D2-βarr2 MEFs. For each cell line representative phospho-ERK1/2 immunoblots are shown above the graph representing the mean ± S.E. for three independent experiments. The difference between curves obtained in the absence and presence of AG1478 (open triangles, shaded area) represents the fraction of total ERK1/2 activation over time that was attributable to EGF receptor transactivation. C, serum-starved D2 and D2-βarr2 MEFs were stimulated for 0, 5, or 30 min with LPA or EGF, after which GTP-bound Ras was isolated by binding to a GST-Raf-1 Ras binding domain fusion protein on agarose beads and visualized by immunoblotting Ras in the precipitates. Ras-GTP under each condition is shown above the total Ras detected in cell lysates before precipitation. Results shown are representative of three separate experiments. D, serum-starved D2 and D2-βarr2 MEFs were treated with vehicle (NS) or stimulated for 5 or 30 min with LPA, and the distribution endogenous phospho-ERK1/2 was visualized by confocal immunofluorescence microscopy as described. Shown are representative images from one of three separate experiments.
In the β-arrestin null D2 background, cross-talk between the LPA and EGF receptors predominates. In contrast, in D2-βarr2 MEFs, the contribution of transactivated EGF receptors is limited to the early phase of ERK1/2 activation, after which an EGF receptor-independent mechanism generates the bulk of the signal. Thus, β-arrestin 2 expression appears to exert a dual effect on ERK1/2 activation downstream of LPA receptors; that is, early termination of EGF receptor transactivation through desensitization of G protein signaling and initiation of EGF receptor-independent ERK1/2 activation through a β-arrestin 2-dependent mechanism.

To test the hypothesis that β-arrestin 2 modulates the transcriptional response to GPCR activation by influencing the mechanisms of downstream signaling, we determined the contribution of transactivated EGF receptors and ERK1/2 activity to the induction of early response genes by LPA. For these assays we employed gene expression array filters containing gene-specific cDNA fragments representing 60 genes that have been reported to respond to GPCR signaling. Fig. 6 demonstrates the experimental paradigm for one LPA-regulated gene, early growth response 1 (Egr1), in D2 cells. EGR-1 is a zinc finger transcription factor induced as part of the immediate-early gene expression program both during Go to G1 transition in cellular proliferation and in some models of cellular differentiation. As shown for a representative experiment in Fig. 6A and quantitatively in Fig. 6B, 1 h of exposure to either LPA or EGF produced a robust 20–40-fold increase in Egr1 mRNA. When cells were treated with AG1478 before stimulation, both responses were abolished. Exposure to the MEK1/2 inhibitor PD98059 at concentrations sufficient to completely inhibit LPA- and EGF-stimulated ERK1/2 activation (data not shown) strongly inhibited but did not abolish Egr1 transcription. These data suggest that in the β-arrestin null background, LPA-stimulated Egr1 transcription is completely dependent on EGF receptor transactivation but that ERK1/2 activation is only partially responsible for Egr1 induction downstream of the EGF receptor. Such results are not surprising in that EGF receptors generate multiple signals capable of transcriptional regulation, and multiple pathways frequently regulate the same target gene (28, 29).

In all, seven of the 60 GPCR-regulated genes represented on the array showed a significant increase in mRNA abundance after 1 h of exposure of D2 MEFs to LPA: Egr1, Ptgs2, Junb, Myc, Serpine 1, F3, and Scya2. None showed a significant decrease. Fig. 7A depicts the AG1478 sensitivity of each of these responses. Six of the seven responses were either completely or partially inhibited by AG1478, indicating that EGF receptor transactivation contributed substantially to the LPA-mediated response in the β-arrestin null background. One gene that was robustly induced, Scya2, was not inhibited by AG1478, consistent with an EGF receptor-independent LPA response. Fig. 7B shows the results of the same experiment performed in D2-βarr2 MEFs. Several differences were apparent. First, LPA-stimulated induction of three genes, Serpine 1, F3, and Scya2, was lost. This suggests that a subset of the responses in D2 cells depends on prolonged receptor-G protein coupling and that β-arrestin 2 expression, which restores homologous receptor desensitization, attenuates these responses. Second, the four responses that persisted, Egr1, Ptgs2, Myc, and Junb, were no longer significantly inhibited by AG1478. This suggests that the early termination of LPA-EGF receptor cross-talk in the presence of β-arrestin 2 reduces the contribution of transactivated EGF receptors to the transcriptional response. Third, although the magnitude of three of the responses, Egr1, Myc, and Junb, were comparable with that observed in D2 cells, one response, Ptgs2, decreased in magnitude to a level comparable with that observed in the D2 cells in the presence of AG1478. These data suggest that in some cases D2-βarr2 cells can employ alternative, EGF receptor-inde-
**FIGURE 6.** EGF receptor and ERK1/2-dependent increase in Egr1 mRNA abundance in D2 MEFs treated with LPA or EGF. Serum-starved D2 MEFs preincubated for 15 min in the presence or absence of AG1478 (200 nM) or PD98059 (20 μM) were stimulated with LPA (10 μM) or EGF (10 ng/ml) for 1 h before RNA isolation. Biotinylated cDNA probes synthesized from total RNA were hybridized to SuperArray™ mouse G protein-coupled receptor signaling PathwayFinder gene arrays, and mRNA abundance was quantified by autoradiography as described. A, comparison of representative arrays depicting changes in Egr1 mRNA abundance (arrows) under each condition. NS, vehicle. B, a bar graph representing the mean ± S.E. for LPA- and EGF-stimulated Egr1 transcription in three separate experiments.

**FIGURE 7.** Effects of AG1478 on LPA-stimulated transcriptional responses in D2 and D2-βarr2 MEFs. Serum-starved D2 and D2-βarr2 MEFs preincubated for 15 min in the presence or absence of AG1478 (200 nM) were stimulated with LPA (10 μM) for 1 h before RNA isolation and assay of endogenous mRNA abundance as described. A, effect of AG1478 treatment on basal (NS) and LPA-stimulated transcription of Egr1, Ptg2, Junb, Myc, F3, Serpine 1, and Scya2 in D2 MEFs. B, effect of AG1478 treatment on basal and LPA-stimulated transcription of Egr1, Ptg2, Junb, Myc, F3, Serpine 1, and Scya2 in D2-βarr2 MEFs. Each graph represents the mean ± S.E. values from three separate experiments. *, greater than basal (NS), p < 0.05; **, less than LPA stimulated, p < 0.05.

**FIGURE 8.** Effects of PD98059 on LPA-stimulated transcriptional responses in D2 and D2-βarr2 MEFs. Serum-starved D2 and D2-βarr2 MEFs preincubated for 15 min in the presence or absence of PD98059 (20 μM) were stimulated with LPA (10 μM) for 1 h before RNA isolation and assay of endogenous mRNA abundance as described. A, effect of PD98059 treatment on basal (NS) and LPA-stimulated transcription of Egr1, Ptg2, Junb, Myc, F3, Serpine 1, and Scya2 in D2 MEFs. B, effect of PD98059 treatment on basal and LPA-stimulated transcription of Egr1, Ptg2, Junb, Myc, F3, Serpine 1, and Scya2 in D2-βarr2 MEFs. Each graph represents the mean ± S.E. values from three separate experiments. *, greater than vehicle (NS), p < 0.05; **, less than LPA stimulated, p < 0.05.

**DISCUSSION**

As depicted schematically in Fig. 9, our data suggest that two mechanistically distinct sets of signals, one G protein-dependent and one β-arrestin 2-dependent, contribute to the early transcriptional response of cultured MEFs to LPA stimulation. In the β-arrestin 1/2-null background, EGF receptor transactivation is the predominant mechanism ERK1/2 activation (Figs. 1B, 2C, and 4A) and accounts for most of the transcriptional response to stimulation of endogenous LPA receptors (Figs. 6 and 7A). Because activated receptor-tyrosine kinases generate multiplesignals capable of affecting gene expression (28, 29), both ERK1/2-dependent and ERK1/2-independent transcriptional responses occur.
both the GPCR and the cellular context in which the receptor is expressed (9–11). ERK1/2 activation may occur via classic second messenger-dependent pathways including G_{i/o}-adenyl cyclase-, and protein kinase A- and exchange protein directly activated by cAMP (Epac) dependent activation of the small G protein Rap1 (30, 31), protein kinase C-dependent activation of the c-Raf1 isoform (32), and calcium and cell adhesion-dependent activation of the focal adhesion kinase, Pyk2 (33, 34). In addition, GPCRs trigger Ras-dependent ERK1/2 activation by transactivating receptor-tyrosine kinases such as EGF (17, 35) and platelet-derived growth factor receptors (36, 37). Recently, some GPCRs including the protease-activated receptor PAR2, neurokinin NK-1, angiotensin AT1a, vasopressin V2, and \( \beta_2 \) adrenergic receptors, have been shown to activate ERK1/2 using receptor-bound \( \beta \)-arrestins as scaffolds to recruit the component kinases of the ERK1/2 cascade to the agonist-occupied receptor (12, 13, 24–27).

The best understood mechanism of GPCR-mediated EGF receptor transactivation is through the release of EGF receptor ligands from the cell surface via matrix metalloprotease-dependent ectodomain shedding (10). Each of the known ligands for the EGF receptor is synthesized as a transmembrane precursor that is proteolyzed to produce a soluble growth factor (38). In fibroblasts, activation of either \( G_{i/o} \)-coupled or \( G_{q/11} \)-coupled receptors has been shown to stimulate the proteolysis of heparin-binding EGF and produce autocrine/paracrine transactivation of EGF receptors (17–19). Using MEK1/2 single and double knock-out embryos, our data demonstrate that EGF receptor transactivation after LPA stimulation does not require \( \beta \)-arrestin expression. Indeed, in the \( \beta \)-arrestin 1/2 null background, EGF receptor transactivation is the dominant mechanism of LPA-stimulated ERK1/2 activation and mediates many of the early transcriptional responses to LPA. In these cells, the only apparent role of \( \beta \)-arrestins in modulating EGF receptor transactivation is to hasten its termination by promoting receptor desensitization and inhibiting receptor-G protein coupling.

\( \beta \)- Arrestin 2 also plays a stimulatory role by mediating LPA-stimulated ERK1/2 activation through an EGF receptor-independent mechanism. In wild type and \( \beta \)-arrestin 1 null MEFs, which still express endogenous \( \beta \)-arrestin 2, LPA stimulates ERK1/2 via a mechanism that is insensitive to EGF receptor inhibition. Introduction of FLAG-\( \beta \)-arrestin 2 into the \( \beta \)-arrestin 1/2 null background restored EGF receptor inhibitor-insensitive ERK1/2 activation. Furthermore, FLAG-\( \beta \)-arrestin 2 immunoprecipitated from D2-\( \beta \)arr2 MEFs after LPA stimulation coprecipitated with endogenous phospho-ERK1/2, suggesting that it performs a scaffolding function analogous to its previously demonstrated role in mediating ERK1/2 activation by the PAR2, NK-1, AT1a, V2 vasopressin, and \( \beta_2 \) adrenergic receptors (12, 13, 24–27). Whether \( \beta \)-arrestin 2 binding to the LPA receptor initiates a specific stimulus leading to Raf activation is unclear. Our data demonstrate that \( \beta \)-arrestin 2-dependent ERK1/2 activation is independent of EGF receptor transactivation and persists in the absence of increased Ras activity. Our working hypothesis is that \( \beta \)-arrestin 2 promotes ERK1/2 activation by binding the components of the ERK1/2 cascade and recruiting them to agonist-occupied receptors at the plasma membrane, where Raf is activated by an as yet undetermined mechanism. Several lines of evidence support this model. The addition of a C-terminal farnesylated (CAAX) motif to c-Raf1 that promotes its constitutive membrane localization is sufficient to cause activation in the absence of active Ras (39). \( \beta \)-Arrestin 2 binds directly to c-Raf1 and ERK1/2 and recruits them to ligand-activated GPCRs, thereby conferring membrane localization (24, 26). Interestingly, receptor-independent recruitment of \( \beta \)-arrestin 2 to the plasma membrane was recently shown to produce ERK1/2 activation in the absence of any active GPCR (23). Collect-
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...these data suggest that β-arrestin-mediated membrane targeting of Raf coupled with scaffolding of the component kinases of the ERK1/2 cascade may be sufficient to generate sustained ERK1/2 activation even after G protein-dependent signaling has waned as a consequence of receptor desensitization.

Our results demonstrate that β-arrestins 1 and 2 differentially affect LPA receptor signaling, since the knock-out of β-arrestin 2, but not β-arrestin 1, resulted in the loss of EGF receptor-independent ERK1/2 activation. This appears to reflect the β-arrestin binding specificity of Edg receptors (16, 40). The LPA receptor belongs to the group of GPCRs that preferentially binds β-arrestin 2, which includes the β2 and α1B adrenergic, μ opioid, endothelin A, and dopamine D1A receptors. These receptors are also characterized by transient β-arrestin binding such that receptor-β-arrestin 2 complexes form at the plasma membrane but dissociate soon after receptor internalization. On the other hand, some data suggest that β-arrestins 1 and 2 are functionally specialized with respect to supporting ERK1/2 activation by GPCRs that bind both β-arrestin isoforms. In the case of the AT1a receptor, which forms stable receptor-arrestin complexes with both β-arrestin 1 and β-arrestin 2 (16), angiotensin-stimulated ERK1/2 activation is inhibited when the β-arrestin 2 is down-regulated by RNA interference but is enhanced when β-arrestin 1 expression is reduced (22).

Our data illustrate the effect of β-arrestin 2-mediated receptor desensitization on the time course of ERK1/2 activation and the subsequent downstream transcriptional response. In the β-arrestin 1/2 null background, LPA-stimulated EGFR receptor transactivation produced a sustained increase in ERK1/2 activity that reached maximum levels within 5–10 min of stimulation, then declined gradually over 60 min to a steady state level that was maintained for at least 4 h. This translated into an LPA-stimulated transcriptional response at 1 h that was carried primarily by signals originating from transactivated EGFR receptors, a subset of which was mediated through ERK1/2. When β-arrestin 2 expression was restored to endogenous levels, the time course of EGFR receptor-dependent ERK1/2 activation was markedly altered. Although transactivated EGFR receptors still contributed to the rapid early peak in ERK1/2 activity, the effect was rapidly terminated, such that after 10 min ERK1/2 activation was largely via an EGFR receptor-independent mechanism. This early termination of EGFR receptor-dependent ERK1/2 activation probably reflects β-arrestin 2-dependent termination of the G protein-dependent transactivation signal. When viewed at the transcriptional level, β-arrestin 2 expression led to the loss of EGFR receptor-dependent responses, consistent with this rapid termination.

These data are consistent with recent findings on the differences in the time course of G protein-mediated versus β-arrestin 2-mediated ERK1/2 activation by the angiotensin AT1a receptor (20). When the time course of angiotensin 2-stimulated ERK1/2 activation was determined in cells where β-arrestin 2 expression was reduced by RNA interference, AT1a receptors mediated only rapid and transient ERK1/2 activation, which was completely blocked by protein kinase C inhibition. Because the AT1a receptor, unlike LPA receptors, can be desensitized by endogenous β-arrestin 1 (16), these data are consistent with a loss of β-arrestin 2-dependent ERK1/2 activation in the setting of preserved β-arrestin 1-mediated AT1a receptor desensitization. When β-arrestin 1/2-replete cells expressing wild type AT1a receptors were stimulated in the presence of the protein kinase C inhibitor or with [sarcosine 1—ile-4—ile-8]AngII (41), a synthetic AT1a receptor antagonist that promotes β-arrestin binding and internalization without stimulating G protein activation, only slow and sustained ERK1/2 activation was present. An identical time course of ERK1/2 activation was observed in cells expressing a G protein-uncoupled (DRY-AAY) AT1a receptor mutant (21, 42), and in each case the slow sustained phase was eliminated by β-arrestin 2 RNA interference. This time course closely resembles our findings with D2-β-arrestin2 MEFs stimulated in the presence of AG1478 and underscores the duality of β-arrestin 2 function as both a terminator of G protein-dependent signaling and a transducer of β-arrestin 2-dependent ERK1/2 activation.

One surprising result of our study was that the LPA-stimulated transcriptional responses in the D2-β-arrestin2 MEFs were ERK1/2-dependent. Using the AT1a and V2 vasopressin receptors, we have previously demonstrated that ERK1/2 activated via β-arrestin 2 remains associated with the receptor-arrestin complex in early endosomes, is excluded from the cell nucleus, and fails to induce expression of an Elk1-luciferase reporter (12, 13, 26). Indeed, these findings have been recently confirmed using selective RNA interference (20, 21, 43), and similar results have been obtained for the PAR2 receptor (24). Our present data indicate that under at least some circumstances, ERK1/2 activated via a β-arrestin 2-dependent mechanism can elicit an endogenous transcriptional response. The explanation for this apparent contradiction may reflect differences in the stability of the receptor-arrestin interaction. The AT1a, V2, and PAR2 receptors each form receptor-arrestin complexes that remain stably associated as the receptor internalizes (13, 24, 26), and it is clear that under these circumstances β-arrestin 2-bound active ERK1/2 tends to remain associated with the GPCR “signalosome.” In contrast, the LPA receptor forms receptor-arrestin complexes that dissociate upon receptor internalization. Although we are able to detect activated ERK1/2 that coprecipitated with β-arrestin 2 after LPA stimulation, the dissociation of arrestin from the receptor may permit ERK1/2 access to the cell nucleus. Indeed, the 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 (16), confers upon the chimeric receptor the ability to stimulate an Elk1-luciferase reporter (13).

Our data clearly demonstrate that β-arrestin 2 expression alters both the broad pattern of LPA-mediated transcription and the contribution of ERK1/2 to the transcriptional response. Thus, it is likely that the two principal mechanisms of LPA-stimulated ERK1/2 activation in these MEFs, EGFR receptor transactivation and β-arrestin 2-dependent signaling, play different functional roles. Because our results encompass only a limited subset of LPA-responsive genes and a single time point, they do not at present permit us to draw firm conclusions about the nature of these roles. EGFR receptor transactivation, which activates the Ras cascade, has been implicated in the mitogenic response to GPCR stimulation in several cell types (10, 28). Conversely, β-arrestin-dependent ERK1/2 activation does not appear to be involved in mitogenic signaling (13). Although such data suggest that the ultimate function of GPCR-activated ERK1/2 is determined by its mechanism of activation, a thorough understanding of the functions of these different signaling cascades remains the subject of further investigation.

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β-Arrestin 2 Expression Determines the Transcriptional Response to Lysophosphatidic Acid Stimulation in Murine Embryo Fibroblasts
Diane Gesty-Palmer, Hesham El Shewy, Trudy A. Kohout and Louis M. Luttrell

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