Differential Kinetic and Spatial Patterns of β-Arrestin and G Protein-mediated ERK Activation by the Angiotensin II Receptor

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The seven-membrane-spanning angiotensin II type 1A receptor activates the mitogen-activated protein kinases extracellular signal-regulated kinases 1 and 2 (ERK1/2) by distinct pathways dependent on either G protein (likely Gq/G11) or β-arrestin2. Here we sought to distinguish the kinetic and spatial patterns that characterize ERK1/2 activated by these two mechanisms. We utilized β-arrestin RNA interference, the protein kinase C inhibitor Ro-31-8425, a mutant angiotensin II receptor (DRY/AAY), and a mutant angiotensin II peptide (SII-angiotensin), which are incapable of activating G proteins, to isolate the two pathways in HEK-293 cells. G protein-dependent activation was rapid (peak <2 min), quite transient (t1/2 −2 min), and led to nuclear translocation of the activated ERK1/2 as assessed by confocal microscopy. In contrast, β-arrestin-dependent activation was slower (peak 5–10 min), quite persistent with little decrement noted out to 90 min, and entirely confined to the cytoplasm. Moreover, ERK1/2 activated via β-arrestin2 accumulated in a pool of cytoplasmic endosomal vesicles that also contained the internalized receptors and β-arrestin. Such differential regulation of the temporal and spatial patterns of ERK1/2 activation via these two pathways strongly implies the existence of distinct physiological endpoints.

Upon agonist binding, signaling via seven-membrane-spanning (7MS) receptors is classically mediated by receptor coupling to G proteins, leading to dissociation of their α and βγ subunits, which in turn activate a variety of effectors, propagating the signal (1). Termination of this signaling is initiated by phosphorylation of the agonist-occupied receptor by G protein-coupled receptor kinases (GRKs), promoting high affinity binding of cytoplasmic β-arrestins to the receptor (2). Binding of β-arrestins sterically inhibits coupling of the receptor to G protein (desensitization) (2, 3), as well as leading to removal of the receptor from the cell surface (internalization) by interaction with elements of the clathrin-mediated endocytic pathway (4–6).

In addition to these classical functions of β-arrestins as signal terminators for G protein-dependent 7MS receptor signaling, accumulating evidence over the last several years has drawn attention to a novel function of β-arrestins, as signal transducers that scaffold various signaling molecules upon activation of 7MS receptors (7). One such example is that of β-arrestin scaffolding components of mitogen-activated protein kinase (MAPK) cascades, leading to their activation (8–11). In the case of the extracellular signal-regulated kinase (ERK) cascade, it has been demonstrated that upon activation of angiotensin II type 1A (AT1A) (9), neurokinin 1 (10), and protease-activated (11) receptors, β-arrestin scaffolds the components of the ERK cascade, Raf-1, MEK1, and ERK1/2, into the receptor complex, leading to activation of ERK1/2. Furthermore, confocal microscopic studies have revealed that stimulation of the AT1A receptor causes colocalization of activated ERK1/2 with β-arrestin2 in a cytoplasmic vesicular compartment (9, 12). Overexpression of β-arrestin also enhances cytoplasmic or β-arrestin-bound ERK1/2 activation following stimulation of AT1A or vasopressin V2 receptors (12, 13). More recently, studies using the RNA interference technique have demonstrated that β-arrestin2 is involved in AT1A receptor and CXC chemokine receptor 4 (CXCR4)-mediated ERK1/2 activation (14–17). Furthermore, some of these studies have revealed that in the case of ERK1/2 activation by AT1A receptor stimulation, β-arrestin2, but not β-arrestin1, mediates G protein-independent ERK1/2 activation (15, 16).

A wide variety of extracellular signals transduced via numerous cell surface receptors or integrins activate MAPKs including ERK1/2, which in turn play a major role in the integration of multiple biological responses such as cell proliferation, differentiation, and survival (18, 19). Thus, exquisite regulation of MAPK activation is crucial for generating the proper physiological outcomes from a particular stimulus. Two such mechanisms are the duration and subcellular distribution of activated MAPKs, which may be differentially regulated in response to different stimuli (19, 20). For example, it has been shown that CXCR4-mediated ERK2 activation is prolonged in contrast to that by other chemokine receptors (21). Different protease-activated receptors have also been shown to have different kinetic profiles and subcellular localization patterns for activated ERK1/2 (22). Furthermore, it has been suggested that β-arrestin-mediated ERK1/2 activation is likely confined to the cytoplasm (9, 11, 12, 22).

However, up to now, it has not been possible to study the “spatio-temporal” regulation of 7MS receptor-mediated ERK activation by G protein versus β-arrestin-mediated signaling since 7MS receptor stimulation of ERK reflects the simultaneous activation of both pathways. Here we have, for the first time, succeeded in dissecting these two mechanisms by which
the angiotensin II receptor activates ERK1/2 and in determining
the specific spatial and temporal patterns of each. These
results underscore the distinctiveness of the two mechanisms
and point toward physiologically divergent outcomes.

EXPERIMENTAL PROCEDURES

Materials—Radiolabeled 125I-Tyr-angiotensin II (AngII) was ob-
tained from PerkinElmer Life Sciences. (Sar5, Ile8, Ile8) (SII)AngII
(Sar-Arg-Val-Ile-Ile-His-Pro-Ile) was synthesized in the Cleveland
Clinic core synthesis facility. Phorbol 12-myristate 13-acetate (PMA)
and Ro-31-8425 were purchased from Calbiochem. GeneSilencer
and FuGENE 6 transfection reagents were from Gene Therapy
Systems (San Diego, CA) and Roche Applied Science, respectively. All
other reagents were purchased from Sigma. Expression plasmids
encoding the hemagglutinin (HA) epitope-tagged wild type and non-tagged DRy/
AAY mutant AT1A receptors, where the highly conserved DRy (Asp-
Arg-Tyr) motif in the second intracellular loop is changed to AAY
mutant AT1A receptors, have been described before (15). The DNA fragment
encoding rat β-arrestin2 was cloned into the pDredN1 vector to yield
β-arrestin2-RFP.

Synthesis of Small Interfering RNAs (siRNAs)—Chemically synthe-
sized, double-stranded siRNAs, with 19-nucleotide duplex RNA and
2-nucleotide 3’-dTdT overhangs, were purchased from Xeragon
(Germantown, MD) in deprotected and desalted form. The siRNA se-
quence targeting β-arrestin2 is 5’-AAACAGCAAGUGUUGUCG-U-
3’, corresponding to the position 148–168 relative to the start codon
(14). A non-silencing RNA duplex (5’-AAUUCUGCGGACGUGAC-
GU-3’), as the manufacturer indicated, was used as a control.

Cell Culture and RNA Transfection—Human embryonic kidney
(HEK-293 cells were maintained as described (23). Forty to fifty per-
cent confluent, slow growing early passage (<15) cells in 100-mm dishes
were transfected simultaneously with 20 μg of siRNA and 2 μg of
the plasmid encoding the HA-wild-type or DRy/AAY mutant AT1A
receptor using the GeneSilencer transfection reagent as described previously
(14). Forty-eight hours after transfection, cells were divided into poly-
lysine-coated 12-well plates (BD Biosciences) for receptor binding;
6-well plates to prepare cellular extracts, or collagen-coated 35-mm
glass bottom dishes (MatTek, Ashland, MA) for confocal microscopy. All
assays were performed 3 days after transfection. AT1A receptor expres-
sion was determined by radioligand binding assays, as described pre-
viosly (24), and was 200–300 fmol/mg of protein in all experiments.

Preparation of Cellular Extracts and Immunoblotting—HEK-293
cells on 6-well plates were starved for at least 4 h in serum-free medium
prior to stimulation. After stimulation, cells were solubilized by directly adding the 2×
SDS-sample buffer followed by sonication. Aliquotted cells after transfection were solubilized in a lysis buffer, as described previously
(16), to measure the protein concentration. Equal amounts of cellular extracts were separated on 15% SDS–polyacrylamide gels (Invitrogen) and
transferred to nitrocellulose membranes for immunoblotting. Phospho-
ylated ERK1/2, total ERK1/2, and β-arrestins were detected by immu-
noblotting with rabbit polyclonal anti-phospho-p44/42 MAPK (Cell Sig-
naling, 1:2,000), anti-MAP kinase 1/2 (Upstate Technology Inc.,
1:10,000), and anti-β-arrestin (A1CT, 1:3,000) antibodies, respectively.
Chemiluminescence detection was performed using the SuperSignal
West Pico reagent (Pierce), and phosphorylated ERK1/2 immunobLOTS
were quantified by densitometry with a Fluor-S MultiImager (Bio-Rad).

DNA Transfection—HEK-293 cells in 100-mm dishes were tran-
siently transfected with 1.5 μg of the β-arrestin2-RFP-encoding plasmid
and 1 μg of the plasmid encoding the HA-wild-type or DRy/AAY
mutant receptor using the FuGENE 6 reagent according to the manu-
facturer’s instructions. One day after transfection, cells were divided
into collagen-coated 35-mm glass bottom dishes (MatTek, Ashland, MA)
for confocal microscopy.

Confocal Microscopy—HEK-293 cells on collagen-coated 35-mm glass
bottom dishes were starved for at least 2 h in serum-free medium prior to
stimulation. After stimulation, cells were fixed with 6% formalde-
yde diluted in phosphate-buffered saline containing calcium and mag-
nesium. Fixed cells were permeabilized with 0.01% Triton in phos-
hate-buffered saline containing 2% bovine serum albumin for 90 min,
incubated with the rabbit polyclonal anti-phospho-p44/42 MAPK (Cell
Signaling, 1:200) antibody at room temperature overnight, and repeat-
edly washed using phosphate-buffered saline. Incubation of the Bodipy
fluorescein-conjugated secondary antibody (Molecular Probes, 1:100)
deoxygenated at 300 fmol/mg in all experiments.

RESULTS

Using the RNA interference technique, we have previously demonstrated that β-arrestin2 and G protein mediate two sig-
naling pathways for AngII-stimulated ERK1/2 activation, which are independent of each other in HEK-293 cells (15). The
duration of ERK1/2 activation is one of the key determinants
shaping its biological responses (20). To determine the temporal
patterns of ERK1/2 activation mediated via each of these two
pathways, we first examined the effect of RNA interfer-
ence-mediated suppression of β-arrestin2 expression on the
kinetics of ERK1/2 activation following stimulation of tran-
siently expressed AT1A receptors in HEK-293 cells. Fig. 1, A
and B, shows that siRNA targeting of β-arrestin2 effectively
silences expression of β-arrestin2 (80%) with no significant
effect on β-arrestin1 expression. In control siRNA-transfected
cells, ERK1/2 activation reaches maximal levels rapidly (within
2 min of agonist treatment), remains stable for up to 10 min,
and decreases very slowly (Fig. 1, C and D). On the contrary,
depletion of β-arrestin2, which leaves only the G protein-de-
pendent pathway available (15, 16), leads to very rapid and
transient ERK1/2 activation, which decreases rapidly after 2
min of agonist treatment and reaches close to basal levels at 30
min after stimulation (Fig. 1, C and D). By subtracting this G
protein-dependent curve from the control curve, one can obtain
an estimate of the putative β-arrestin2-dependent pathway(s).

In Fig. 1D, the dotted line shows this calculated kinetic curve of
β-arrestin2-mediated ERK1/2 activation. This curve depicts a
slower but much more persistent ERK1/2 activation than that
observed in β-arrestin2-depleted cells. These results suggest
that β-arrestin2-mediated ERK1/2 activation might follow a
very different time course from that due to G protein-depend-
ent ERK activation.

We recently demonstrated that activation of ERK1/2 by the
mutant ligand SII-AngII or by the mutant AT1A receptor that
has mutations in the DRy motif (DRy/AAY), both of which are
unable to elicit receptor coupling to G proteins (15, 25, 26), is
entirely β-arrestin2-dependent (15, 16). This permitted us to verify the calculated kinetic curve for β-arrestin2-mediated
ERK1/2 activation by AngII shown in Fig. 1D by examining the
temporal pattern of ERK1/2 activation by this mutant ligand
and receptor in the HEK-293 cells. As shown in Fig. 2, A and B,
SII-AngII stimulation of the wild type AT1A receptor leads to
relatively delayed but persistent activation of ERK1/2, which
reaches maximal levels by 5 min and which is sustained for much
longer time periods. Wild-type AngII stimulation of the
DRy/AAY mutant receptor causes a similar kinetic pattern of
ERK1/2 activation (Fig. 2, C and D). In both cases, as expected,
there is no detectable ERK1/2 activation at any time point after
agonist treatment, when β-arrestin2 expression is silenced
(Fig. 2E). The kinetic curves in Fig. 2 closely match the calcu-
lated curve (dotted lines) shown in Fig. 1D, thus strongly sup-
porting the idea that β-arrestin2-mediated ERK1/2 activation
stimulated by AngII is relatively slow and prolonged as com-
pared with G protein-dependent activation.

We have previously shown that AngII stimulation of the
β-arrestin2-independent pathway to ERK1/2 activation in
HEK-293 cells is completely blocked by the PKC inhibitor Ro-
31-8425 (15, 16). Thus, another possible way to isolate the
β-arrestin-dependent ERK1/2 activation pathway stimulated by
AngII is to use this inhibitor. Pretreatment with the PKC

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inhibitor results in a dramatic decrease (~75%) in ERK1/2 activation at an early time point (2 min) after AngII stimulation, but little inhibition is observed at longer time points (Fig. 3, A and B). After 30 min of stimulation, there is no significant inhibition of ERK1/2 activation by Ro-31-8425. In general, the pattern (Fig. 3B, closed circles) is similar to the mathematically derived kinetic curve of AngII-stimulated, β-arrestin2-mediated ERK1/2 activation (dotted line), which is calculated as done in Fig. 1D. As expected, the rapid and transient G protein-dependent ERK1/2 activation elicited by AngII in β-arrestin2-depleted cells (Fig. 3C, open triangles) is virtually abolished by pretreatment with the PKC inhibitor (Fig. 3, A and C). In both control and β-arrestin2 siRNA-transfected cells, PMA induces similar levels of ERK1/2 activation, which are abolished by Ro-31-8425 (data not shown). Together with the results shown in Figs. 1 and 2, these data demonstrate that the temporal patterns of β-arrestin2 and G protein-mediated ERK1/2 activation following stimulation of the AT1A receptor are quite distinct from each other; β-arrestin2-mediated activity is retarded and prolonged relative to G protein-dependent activity, which is rapid and transient. Thus, for AT1A receptor-mediated ERK1/2 activation in HEK-293 cells, the majority of early activity (~2 min) is elicited via the G protein-dependent pathway, whereas late activity (>5 min) is predominantly mediated by the β-arrestin2 pathway.

A growing body of evidence has suggested that ERK1/2 activated by β-arrestin-mediated signaling remains exclusively in the cytoplasm (9, 11, 12, 22). Accordingly, we examined the subcellular distribution of activated ERK1/2 (phospho-ERK1/2) by immunofluorescence confocal microscopy at an early (2 min) and late (>30 min) time point after AngII treatment in HEK-293 cells. As demonstrated in Figs. 1–3, these should represent mainly G protein and β-arrestin2-mediated ERK1/2 activation, respectively. As shown in Fig. 4, at 2 min after stimulation, phospho-ERK1/2 is detected in both the nucleus and the cytoplasm, whereas nuclear, but not cytoplasmic, staining of phospho-ERK1/2 is strikingly reduced at the late time point (>30 min). Silencing β-arrestin2 expression by RNA interference leads to no significant effect on the staining pattern at 2 min after AngII treatment as compared with control siRNA-transfected cells but dramatically decreases cytoplasmic staining of phospho-ERK1/2 at the late time point (Fig. 4). Such decreases in the cytoplasmic staining of phospho-ERK1/2 in β-arrestin2-depleted cells is specific for the activation of the AT1A receptor since there is no difference in the pattern and extent of phospho-ERK1/2 staining produced by PMA treatment for 30 min.
with the presence or absence of β-arrestin2 siRNA. In both control and β-arrestin2 siRNA-transfected cells, basal phospho-ERK1/2 is also barely detectable. These results demonstrate that upon stimulation of the AT1A receptor, a pool of ERK1/2 is rapidly activated via the G protein-dependent pathway yet translocates into the nucleus, where it is rapidly dephosphorylated. In contrast, ERK1/2 activated via the β-arrestin2 pathway is retained entirely in the cytoplasm, is not readily dephosphorylated, and is persistent.

Previous confocal microscopic studies using green fluorescent protein (GFP)-β-arrestin have revealed that continuous activation (more than 10 min) of some 7MS receptors results in formation of endocytic vesicular structures, containing receptors and β-arrestin (23). Furthermore, it has been shown that a pool of activated ERK1/2 is colocalized with overexpressed β-arrestin2 in this compartment (9, 12). To further assess the subcellular localization of activated ERK1/2 phosphorylation was visualized (C) and determined (D) as described. Each data point in B and D is expressed as the percentage of the maximal ERK1/2 phosphorylation in response to stimulation for 5 min and represents the mean ± S.E. from six independent experiments. The dotted lines were obtained by normalizing the calculated curve (dotted) in Fig. 1D to express the percentage of the maximal level at 10 min of stimulation. E, HEK-293 cells were transfected simultaneously with β-arrestin2 siRNA and the plasmid encoding either the wild-type HA-AT1A receptor or the DRY/AAY mutant AT1A receptor. After serum starvation and subsequent stimulation, the level of ERK1/2 phosphorylation was visualized as described for A and C. Each lower panel in A, C, and E shows equal amounts of ERK1/2 loaded in different samples.

Fig. 2. Temporal patterns of ERK1/2 activation generated by SII-AngII-stimulated wild-type and AngII-stimulated DRY/AAY mutant AT1A receptors. A and B, HEK-293 cells were transiently transfected with the HA-AT1A receptor-encoding plasmid, and transfected cells were incubated in serum-free medium for at least 4 h followed by stimulation with 30 μM SII-AngII at 37 °C for the indicated periods. Phosphorylation of ERK1/2 was visualized by immunoblotting (A) and subsequently quantified by densitometry (B) as described in the legend for Fig. 1. C and D, HEK-293 cells, transiently expressing the DRY/AAY mutant AT1A receptor, were serum-starved for ~4 h. Cells were stimulated with 100 nM AngII at 37 °C for the indicated periods, and the extent of ERK1/2 phosphorylation was visualized (C) and determined (D) as described. Each data point in B and D is expressed as the percentage of the maximal ERK1/2 phosphorylation in response to stimulation for 5 min and represents the mean ± S.E. from six independent experiments. The dotted lines were obtained by normalizing the calculated curve (dotted) in Fig. 1D to express the percentage of the maximal level at 10 min of stimulation. E, HEK-293 cells were transfected simultaneously with β-arrestin2 siRNA and the plasmid encoding either the wild-type HA-AT1A receptor or the DRY/AAY mutant AT1A receptor. After serum starvation and subsequent stimulation, the level of ERK1/2 phosphorylation was visualized as described for A and C. Each lower panel in A, C, and E shows equal amounts of ERK1/2 loaded in different samples.
restin2 does not interfere with translocation of activated ERK1/2 into the nucleus. At later time points (>30 min) after stimulation, phospho-ERK1/2 is condensed into cytoplasmic endosomal structures that also contain the internalized receptors and restin2-RFP. The overlay image shows complete colocalization (white) of the three proteins to this compartment. In contrast, PMA...

Fig. 3. Effects of the PKC inhibitor Ro-31-8425 on the kinetic pattern of AT1A receptor-mediated ERK1/2 activation. A. HEK-293 cells, transfected with the HA-AT1A receptor-encoding plasmid and the indicated siRNAs simultaneously, were serum-starved for ~4 h and then treated with MeSO (DMSO) vehicle only or 1 μM Ro-31-8425 for 15 min before stimulation. Cells were stimulated with 100 nM AngII at 37 °C for the indicated periods, and then cellular extracts were prepared to visualize phosphorylation of ERK1/2 as described in the legend for Fig. 1. The lower panels show equal amounts of ERK1/2 loaded in each sample. IB: immunoblots; CTL, control. B and C: the amount of ERK1/2 phosphorylation in each lane (A) was quantified by densitometry and expressed as the percentage of the maximal phosphorylation of ERK1/2 obtained at 5 min of stimulation in control siRNA-transfected, MeSO-treated cells. Each data point represents the mean ± S.E. from five independent experiments. The dotted curve in B was generated by subtracting the curve obtained in β-arrestin2 (barr2) siRNA-transfected, MeSO-treated cells (open triangles in C) from the control curve (open circles in B).

Fig. 4. Effects of silencing β-arrestin2 (barr2) expression on the subcellular distribution of phospho-ERK1/2 following different periods of stimulation of the AT1A receptor. HEK-293 cells were transfected with the HA-AT1A receptor-encoding plasmid and the indicated siRNAs simultaneously. After incubation in serum-free medium for at least 2 h, cells were stimulated with 100 nM AngII or 1 μM PMA at 37 °C for the indicated periods, and then subsequently fixed and permeabilized. Cellular distribution of phospho-ERK1/2 was visualized by immunolabeling with a polyclonal anti-phospho-ERK1/2 antibody and then a Bodipy fluorescein-conjugated secondary antibody followed by confocal microscopy, as described under "Experimental Procedures." Fluorescent confocal images shown were collected using a single line excitation (488 nm) and emission (515–540 nm) filter set and represent similar results obtained from four independent experiments. CTL, control.
For the Alexa633-labeled HA-AT1A receptor, HEK-293 cells transiently expressing the HA-AT1A receptor along with β-arrestin2-RFP were starved in serum-free medium at least for 2 h and stimulated 100 nM AngII or 1 μM PMA at 37 °C for the indicated periods. Cells were then immunolabeled for phospho-ERK1/2 as described in the legend for Fig. 4. Subsequently, the HA-AT1A receptor was stained with the 12CA5 monoclonal anti-HA antibody and an Alexa633-conjugated secondary antibody as described under “Experimental Procedures.” Fluorescent confocal images were obtained using multitrack sequential excitation (488, 568, 633 nm) and emission filter sets; emission was at 515–540 nm for detecting Bodipy fluorescein-labeled phospho-ERK1/2 (green), at 585–615 nm for β-arrestin2-RFP (red), and at 650 nm for the Alexa633-labeled HA-AT1A receptor (blue). The data shown represent similar results obtained from four independent experiments.

To further validate the spatio-temporal pattern of ERK1/2 activation mediated via the β-arrestin2 pathway, we monitored the subcellular distribution of phospho-ERK1/2 stimulated by the DRY/AAY mutant AT1A receptor in β-arrestin2-RFP-expressing cells (Fig. 6). In Fig. 6, we documented that this mutant receptor cannot transduce G protein-dependent ERK1/2 activation, in agreement with previous results (15). Unlike the wild-type receptor (Figs. 4 and 5), the DRY/AAY receptor fails to generate nuclear staining of phospho-ERK1/2 at 2 min after AngII treatment (Fig. 6). However, the overall staining pattern of phospho-ERK1/2 at later time points (>30 min) is similar to that obtained with the wild-type receptor (Fig. 5). Although less robust than activation by the wild-type receptor, the overlay image shows that activation of the DRY/AAY receptor leads to colocalization of phospho-ERK1/2 with β-arrestin2-RFP in endosomes. These data further support the distinct spatio-temporal pattern of β-arrestin2-mediated ERK1/2 activation as compared with ERK1/2 activated via G proteins as observed in Figs. 4 and 5.

Next, we examined the effect of the PKC inhibitor Ro-31-8425, which abolishes AngII stimulation of G protein-dependent ERK1/2 activation in HEK-293 cells (Fig. 3) (15), on the subcellular localization of phospho-ERK1/2 after stimulation of the AT1A receptor in β-arrestin2-RFP-expressing cells (Fig. 7). After pretreatment with PKC inhibitor, not only is nuclear staining of phospho-ERK1/2 eliminated, but also, cytoplasmic staining seems to be reduced at 2 min after stimulation. Interestingly, colocalization of phospho-ERK1/2 with β-arrestin2-RFP at the cell membrane at 2 min, which is not visible in the absence of the PKC inhibitor, presumably due to extensive cytoplasmic staining (Fig. 5), is also detected. At later time points (>30 min) after stimulation, however, pretreatment with the PKC inhibitor has no effect on the colocalization of phospho-ERK1/2 with β-arrestin2-RFP in the cytoplasmic endosomal compartment. Overall, the staining pattern at 30 min is the same as that observed in cells with no inhibitor treatment (Fig. 5). Application of Ro-31-8425 ablates PMA-stimulated staining of phospho-ERK1/2. These results demonstrate that following stimulation of the AT1A receptor in HEK-293 cells, transient nuclear staining of phospho-ERK is mediated by G protein-dependent signaling, whereas sustained cytoplasmic and endosomal localization of phospho-ERK1/2 is entirely β-arrestin2-dependent.

**DISCUSSION**

MAPK signaling is important for the control and coordination of multiple cellular responses, such as proliferation, differentiation, and viability (18–20). Even within a single cell, activation of ERK can result in opposite cellular responses, depending on the stimulation. How do cells ensure that MAPK activation leads to the correct responses to a variety of signals? Clearly, the duration and subcellular distribution of MAPK activity will largely shape the response to a particular stimulus. Although the temporal and spatial characteristics of ERK1/2 activation following stimulation by growth factors have been well characterized (19, 20), these characteristics of 7MS receptor-mediated ERK1/2 activation have been poorly studied. The task is complicated by the multiple signaling pathways emanating from a receptor, which lead to ERK activation. Moreover, the combinational proportions of such multiple pathways can vary in different cell types (27), as well as within a given cell type with different forms of stimulation (13, 22).

Our data reveal that upon stimulation of the AT1A receptor, ERK1/2 are immediately, but transiently, activated via the G protein-dependent pathway, whereas β-arrestin2-mediated ERK1/2 activation is relatively slow but very persistent. ERK activity measured very shortly after stimulation (e.g. <2 min)
is largely G protein-dependent, whereas that measured late (e.g., >30 min) is β-arrestin2-dependent. The traditional functions of β-arrestin are to turn off G protein signaling through desensitizing the coupling of the activated 7MS receptor to G protein (2, 3) and through sequestration of the receptor away from the cell surface by triggering clathrin-dependent receptor internalization (4–6). Thus, it seems likely that at least part of the mechanism that rapidly terminates G protein-dependent ERK1/2 activation is mediated by binding of β-arrestin to the activated receptor. However, recently mounting evidence (9, 12, 13, 15, 16) as well as the results in the present study, demonstrate a new role for β-arrestin2, scaffolding components of the ERK cascade, leading to activation of ERK1/2. It is also possible that the prolonged activity of β-arrestin-scaffolded ERK1/2 is due to some effects of the β-arrestin to shield the bound phospho-ERK from MAPK phosphatases. Thus, our results indicate that β-arrestin2 simultaneously acts as a signal terminator and transducer. Binding of β-arrestin2 to the activated receptor functions to end the “first wave” of G protein-dependent signaling but at the same time to initiate a “second wave” of β-arrestin2-mediated signaling.

The present data show that a pool of ERK1/2 activated via the G protein-dependent pathway translocates into the nucleus, whereas ERK1/2 activated via β-arrestin2 stays in the cytoplasm, suggesting that substrates of ERK1/2 activated via β-arrestin2-mediated signaling are exclusively cytoplasmic. Although nuclear substrates of ERK1/2, particularly transcription factors, have been the most often studied, cytoplasmic ERK substrates have been the focus of much less scrutiny. Nonetheless, several examples suggest that cytoplasmic ERK activity is important in the regulation of cell morphology, migration, and viability. ERK1/2 have been shown to phosphorylate cytoskeletal proteins and microtubule-associated proteins, which are involved in cell migration and morphological alterations (28–31). It has also been recently shown that some cytoplasmic proteins important in cell viability and apoptosis are regulated through their phosphorylation by ERK1/2 (32–34). A number of proteins involved in various signaling pathways are phosphorylated by ERK1/2, including phospholipase A$_2$ (35), some components of the ERK cascade itself, such as Raf-1 (36) and MEK1 (37), phosphodiesterase 4D (38), GRK2 (39), β-arrestin1 (40), and others. Our data show that β-arrestin2 restricts the activity of ERK1/2 to the receptor complex that moves from the cell surface to the endosomal compartment, which presumably targets the activated ERK1/2 to such potential cytoplasmic substrates. Nevertheless, the specific substrates for β-arrestin-dependent ERK activity have yet to be determined. However, β-arrestin-mediated activation of ERK1/2 and another MAPK, p38, has recently been shown to be involved in protease-activated receptor-2 and CXCR4-induced chemotaxis, respectively (17, 22). Determination of substrates for ERK1/2 activated by β-arrestin2-dependent pro-
cesses is thus an important next step to understand the physiological consequences of β-arrestin2-mediated ERK signaling.

It has been increasingly accepted that the mechanism of MAPK activation is a major determinant of MAPK function. As discussed above, our results strongly suggest that the cellular responses mediated by two distinct pools of ERK1/2 activated via separate β-arrestin2 and G protein-dependent signaling must be distinct. In support of this idea, previous studies have shown that β-arrestin overexpression, which increases cytoplasmic or β-arrestin2-bound pools of activated ERK1/2, decreases translocation of activated ERK1/2 into the nucleus, leading to inhibition of transcriptional activation and DNA synthesis in response to stimulation of AT1a and V2 receptors (12, 13). In addition, it has been suggested that the ability of a 7MS receptor to maintain the β-arrestin-scaffolded signaling complex in the endosomal compartment can determine the effectiveness and signaling consequences of agonist-mediated activation of MAPK. Two different 7MS receptors that have different affinities for β-arrestin binding have been shown to lead to distinct profiles of ERK1/2 activation and physiological consequences (13). In this regard, our results not only demonstrate the markedly different spatio-temporal patterns of ERK1/2 activation via β-arrestin2 and G protein-mediated pathways but also emphasize that a single readout of cellular ERK1/2 activity as is commonly done is likely to be misleading.

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