Arrestin Serves as a Molecular Switch, Linking Endogenous α2-Adrenergic Receptor to SRC-dependent, but Not SRC-independent, ERK Activation*

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Our previous studies have demonstrated that neither receptor endocytosis nor arrestin is required for ERK activation by the α2-adrenergic receptor (Wang, Q., Zhao, J., Brady, A. E., Feng, J., Allen, P. B., Lefkowitz, R. J., Greengard, P., and Limbird, L. E. (2004) Science 304, 1940–1944). The present studies address whether arrestin plays a role in determining the route of α2AR-evoked ERK signaling activation, taking advantage of endogenous expression of the α2AAR subtype in mouse embryonic fibroblasts (MEFs) and the availability of MEFs without arrestin expression (derived from Arr2,3−/− mice). Our data demonstrate that the endogenous α2AAR evokes ERK phosphorylation through both a Src-dependent and a Src-independent pathway, both of which are G protein dependent and converge on the Ras-Raf-MEK pathway. Arrestin is essential to recruit Src to this process, as α2AAR-mediated ERK signaling in Arr2,3−/− MEFs does not involve Src. Stimulation of α2AAR enhances arrestin-Src interaction and promotes activation of Src. α2 agonists have similar potencies in stimulating Src-dependent and Src-independent ERK phosphorylation in wild-type and Arr2,3−/− cells, respectively. However, Src-independent α2AAR-mediated ERK stimulation has both a longer duration of activation and a more rapid translocation of pERK into the nucleus when compared with Src-dependent activation. These data not only affirm the role of arrestin as an escort for signaling molecules such as Src family kinases but also demonstrate the impact of arrestin-dependent modulation on both the temporal and spatial properties of ERK activation.

Mitogen-activated protein (MAP) kinases are important effectors for G protein-coupled receptors (GPCRs)2 to regulate cell growth, survival, and movement (1). GPCRs activate the MAPK cascades through divergent pathways involving tyrosine kinases, small GTPases, and other effectors (1). Recent studies have suggested that activation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) by GPCRs can occur through G protein-dependent and G protein-independent pathways and that arrestin is the essential mediator of the latter pathway (1).

Arrestin is a family of proteins with multifunctions in GPCR regulation (2, 3). As originally revealed in the visual system (4–7), arrestin associates with the agonist-evoked conformations of GPCRs, especially when the receptor is phosphorylated by G protein receptor kinases, and mediates homologous desensitization of GPCR pathways (4, 5, 8–11). In non-sensory systems, ubiquitously expressed arrestin 2 (β-arrin 1) and 3 (β-arrin 2) also serve as adaptors for linking GPCRs to endocytosis machinery by directly binding to both the heavy chain of clathrin (12) and to β-adaptin (13). Depending on their association with arrestin post-endocytosis, GPCRs are classified into two families (3, 14, 15). Class A receptors dissociate from arrestin after internalization and are rapidly recycled back to the cell surface. By contrast, class B receptors stay associated with arrestin after internalization and are eventually subjected to lysosomal degradation.

In addition to its functions in mediating desensitization and internalization, arrestin has been implicated as scaffolding protein linking receptors to downstream signaling pathways including the ERK pathway (16–19) in a G protein-independent manner (20). Arrestin-mediated ERK activation has been mostly demonstrated by class B receptors, and this process occurs on endocytic vesicles (17–19). Compared with G protein-dependent activation of ERK signal, which is transient and translocated into nuclei, ERK signal activated through an arrestin-dependent, G protein-independent pathway is sustained and restricted to the cytosol (20, 21). Endocytosis is not always required for ERK activation (22–

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‡ The abbreviations used are: GPCR, G protein-coupled receptor; AR, adrenergic receptor; ERK, extracellular signal-regulated kinase; pERK, phosphorylated ERK; MEF, mouse embryonic fibroblast; PI3K, phosphoinositide 3 kinase; EGF, epidermal growth factor; WT, wild type; GFP, green fluorescent protein; HA, hemagglutinin; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase.
Arrestin and Src Involvement in α₂AR Activation of ERK

26), and arrestin also seems to be dispensable for ERK activation by some GPCRs, including the gonadotropin-releasing hormone receptor (27), the D2 and D3 dopamine receptors (24), and the α₂AR (26). The regulatory role of arrestin in ERK activation by these receptors, if any, has not been fully clarified to date.

Our previous studies have demonstrated that the α₂-adrenergic receptor (AR), a class A receptor, evokes phosphorylation of ERK1/2 through G protein-dependent pathways and this process does not require arrestin-mediated receptor endocytosis (22, 26). Nonetheless, arrestin does regulate the time course of α₂AR-mediated ERK activation, and the presence of arrestin accelerates the rate of ERK activation by the α₂AR (26).

To further explore the role of arrestin in the α₂AR-mediated G protein-dependent activation of ERK, we analyzed ERK phosphorylation stimulated by endogenous α₂AR in mouse embryonic fibroblasts (MEFs) with or without (Arr2,3−/−) arrestin expression. Here we report that the α₂AR mediates ERK activation through Src-dependent and Src-independent pathways. Even though arrestin is dispensable for α₂AR-mediated ERK activation 

**EXPERIMENTAL PROCEDURES**

**Materials**—Dulbecco’s modified Eagle’s medium (Invitrogen) was prepared by the Cell Culture Core, a facility sponsored by the Diabetes Research and Training Center at Vanderbilt University Medical Center (supported by National Institutes of Health Grant DK20593) or was purchased from Invitrogen. UK 14,304 was from Research Biochemical International. Pertussis toxin was ordered from List Biological Laboratories Inc. AG1478, LY294002, genistein, PP2 (AG1879), Ftsase inhibitor I, totoxin was ordered from List Biological Laboratories Inc. AG1478, LY294002, genistein, PP2 (AG1879), Ftsase inhibitor I, totoxin was ordered from List Biological Laboratories Inc. AG1478, LY294002, genistein, PP2 (AG1879), Ftsase inhibitor I, 

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**Measurement of ERK Activation**—ERK activation (active/total) was measured as described previously (22). Active (pERK) and total ERKs were detected by Western analysis using corresponding antibodies (Cell Signaling). For pertussis toxin treatment, 200 ng/ml was added to culture for 20 h before and during the time of stimulation of cells. For other inhibitor treatment, drugs in the amounts indicated in figure legends were added to culture for 1 h before and during the time of stimulation of cells.

**Immunofluorescence of pERK in MEFs**—pERK in MEFs was detected by pERK antibody (Cell Signaling) using the immunostaining protocol provided by the manufacturer. Briefly, MEFs cultured on coverslips were fixed with 3% paraformaldehyde for 30 min at 4 °C followed by three washes in TBST (Tris-buffered saline (50 mM Tris-HCl, pH 7.4, 150 mM NaCl) and 0.1% Triton X-100). After one wash in TBS buffer, cells were further fixed/permeabilized with 100% MeOH for 5 min at −20 °C. Following three washes with TBST, cells were blocked with 5% horse serum in TBST for 1 h at room temperature and then incubated with pERK antibody (diluted 1:400 in TBST containing 5% bovine serum albumin) overnight at 4 °C. AlexaFluor-488-conjugated anti-mouse antibody (Molecular Probes) was used as the secondary antibody at 1:1000 (diluted in TBST containing 3% bovine serum albumin) for 1 h at room temperature. Fluorescent images were taken using a LSM510 confocal microscope (in the Vanderbilt University shared imaging facility).

**Co-immunoprecipitation of c-Src and Arrestin3**—Direct interaction between c-Src and arrestin was assessed using co-immunoprecipitation strategy. CosM6 cells cotransfected with cDNA encoding α₂AR (29), HA-Src (30), and GFP-arrestin3 (31) protein (0.6, 1, and 6 µg/10-cm plate, respectively) were serum starved overnight and treated with or without 100 µM epinephrine for 5 min at 37 °C in the presence of 1 µM propranolol (to block trace endogenous βAR) and 1 µM prazosin...
Arrestin and Src Involvement in α2AR Activation of ERK

FIGURE 1. Endogenously expressed α2ARs evoke ERK activation through the G<sub>G_{i,o}</sub> family of G proteins in MEFs. A, MEFs express endogenous α<sub>2</sub>AAR. Total RNA was extracted from MEFs, and reverse transcription PCR was performed using primers specific for amplification of the α<sub>2</sub>A, α<sub>2</sub>B, or α<sub>2</sub>C AR subtype as described under “Experimental Procedures.” PCR products of plasmids (psld) containing each subtype were loaded as positive controls, and reverse transcription PCR reactions without reverse transcriptase were run as negative controls. B, stimulation of endogenous α<sub>2</sub>AAR activates ERK signaling. WT and Arr<sub>2,3</sub>Δ<sup>−/−</sup> MEFs were treated with α<sub>1</sub>AR agonist (10<sup>−4</sup> M epinephrine + 10<sup>−5</sup> M propranolol to block βARs + 10<sup>−8</sup> M prazosin to block α<sub>1</sub>ARs) in the presence or absence of yohimbine (an α<sub>2</sub>AR antagonist) or pertussis toxin (PTx) for 5 min. For pertussis toxin treatment, cells were pretreated with 200 ng/ml pertussis toxin for 20 h before and during stimulation. Total cell lysates were separated on SDS-PAGE and blotted for pERK and total ERK as outlined under “Experimental Procedures.”

RESULTS
Endogenously Expressed α<sub>2</sub>ARs in MEFs Activate ERK through the G<sub>G_{i,o}</sub> Family of G Proteins—To explore the function of arrestin on endogenous α<sub>2</sub>AR-mediated signaling, we evaluated MEFs, which express the α<sub>2</sub>AR subtype (but not the α<sub>2</sub>B or α<sub>2</sub>C AR subtypes) endogenously, as revealed by reverse transcription PCR (Fig. 1A). Functional α<sub>2</sub>AR expression in these cells was documented by [3H]Rauwolscine binding (74.3 ± 10.7 fmol/mg protein detected at the K<sub>D</sub> for [3H]Rauwolscine; data not shown). Stimulation of endogenous α<sub>2</sub>AAR in MEFs with α<sub>2</sub>AR agonists allows an assessment of α<sub>2</sub>AR activation of ERK in native cells (Fig. 1B). For the studies in Fig. 1B, we used the endogenous α<sub>2</sub> AR agonist epinephrine in the presence of propranolol to block βAR and prazosin to block α<sub>1</sub>AR (see “Experimental Procedures”) to stimulate the α<sub>2</sub>AAR. ERK activation by the α<sub>2</sub>AR in MEFs can also be evoked by clonidine, UK 14,304 (data not shown), and dexmedetomidine (Fig. 1D) and can be blocked by the α<sub>2</sub> antagonist yohimbine (Fig. 1B).

As discussed earlier, there is evidence for GPCR activation of ERK by G protein-dependent and -independent pathways (19–21, 32). ERK activation by α<sub>2</sub>ARs in MEFs is eliminated by pretreatment of MEFs by pertussis toxin (Fig. 1, B and C, PTx), demonstrating that endogenous α<sub>2</sub>AAR activation of ERK in MEFs is mediated by G proteins of the G<sub>G_{i,o}</sub> subtype, consistent with the properties of α<sub>2</sub>AR activation of ERK in heterologous systems (33–35). Furthermore, the G protein dependence of ERK activation by endogenous α<sub>2</sub>AR in MEFs occurs both in WT cells, which express arrestins, and in cells derived from mice null for arrestin 2 and 3 (Arr<sub>2,3</sub>Δ<sup>−/−</sup>).

C-terminal, endogenous α<sub>2</sub>AR-evoked ERK activation is pertussis toxin sensitive. ERK activity (active/total) was quantitated from five independent experiments, and percent increase of ERK activity over basal (±S.E.) was plotted using GraphPad Prism<sup>®</sup>. *, p < 0.01. D, ERK phosphorylation in response to increasing doses of α<sub>2</sub>AR agonist in WT versus Arr<sub>2,3</sub>Δ<sup>−/−</sup> MEFs. Top, representative blots for pERK following 5 min of treatment of α<sub>2</sub>AR agonist dexmedetomidine (Dex) at indicated doses. Bottom, dose response curves of ERK activation by Dex in WT versus Arr<sub>2,3</sub>Δ<sup>−/−</sup> MEFs. ERK activation (active/total) was quantitated from five independent experiments and plotted using GraphPad Prism. The EC<sub>50</sub> values for Dex to induce ERK phosphorylation in WT and Arr<sub>2,3</sub>Δ<sup>−/−</sup> MEFs are 8.4 × 10<sup>−8</sup> M and 4.0 × 10<sup>−8</sup> M, respectively.

The data in Fig. 1, B and C, emphasize that arrestin–dependent receptor endocytosis is not a requirement for α<sub>2</sub>AR-mediated ERK signaling, although arrestin–dependent endocytosis, followed by recycling, leads to an arrestin–dependent acceleration of α<sub>2</sub>AR activation of ERK (26).

We asked whether
arrestins would alter the potency of α2-AR agonists in activating ERK as a result of the rapid regeneration of native α2A-AR in arrestin-expressing cells. As shown in Fig. 1D, the EC50 of the α2-AR agonist/partial agonist dexmedetomidine in activating ERK in Arr2,3−/− MEFs (4.0 × 10−8 M) is comparable with that in WT cells (0.84 × 10−8 M). In addition, the maximal activation of ERK by dexmedetomidine in Arr2,3−/− MEFS (4.0 × 10−8 M) is comparable with that in WT cells (0.84 × 10−8 M). In terms of the magnitude of ERK activation, the maximal stimulation of ERK by dexmedetomidine in Arr2,3−/− MEFS is similar to that in WT cells (Fig. 1D). A similar potency for ERK activation in WT versus Arr2,3−/− MEFs was also observed in response to the α2-AR partial agonist UK 14,304 (data not shown). We chose partial agonists for these studies as they are most sensitive to revealing differences in coupling efficiency between receptors and effectors (36, 37). These data indicate that the presence of arrestins, although accelerating the rate of ERK activation in MEFs (Fig. 2, below, and Ref. 26), does not significantly change the potency or the efficacy of α2-AR agonists in activating ERK.

Lack of Arrestins Alters the Temporal Pattern of α2A-AR-elicited ERK Activation—Although the data in Fig. 1, B–D, indicate that arrestins are not required for α2A-AR activation in MEFs, we nonetheless wanted to explore whether arrestin had any regulatory impact on endogenous α2A-AR-evoked, G protein-dependent ERK signaling. We noted, as shown in Fig. 2, that arrestin alters both the time of onset and the duration of α2A-AR-evoked activation of ERK in MEFs. In WT cells, endogenous α2A-AR-evoked ERK activation peaks at 2 min and then declines, due to desensitization of receptor-mediated signaling. Virtually no stimulation is detected after 20 min of treatment with α2A-AR agonists in WT MEFs that express arrestin (Fig. 2). In cells without arrestin expression, the time to peak stimulation appears to be delayed (Fig. 2B). In addition, the desensitization of α2A-AR-evoked ERK signal is less rapid and occurs to a lesser extent; thus, ~40% of ERK activity remains detectable after 20 min of treatment with the α2 agonist (Fig. 2). This extended ERK activation in Arr2,3−/− cells lasts for another 3 h in the presence of agonist before becoming fully desensitized.
**Arrestin and Src Involvement in α2AR Activation of ERK**

**A**

| Agent:          | p-ERK | ERK | α2-agonist | PI3K inhibitor | Ras inhibitor | Raf inhibitor | MEK inhibitor |
|-----------------|-------|-----|------------|----------------|---------------|---------------|---------------|
| WT              |       |     |            |                |               |               |               |
| Arr2,3−/−       |       |     |            |                |               |               |               |

**B**

![Graph showing differential responses of α2AR-evoked ERK signals in WT versus Arr2,3−/− MEFs to tyrosine kinase inhibitors.](image)

**C**

![Phosphorylation of ERK and Akt in WT and Arr2,3−/− MEFs following indicated inhibitor treatments.](image)

**D**

![Graph showing summary of quantitative data.](image)

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**FIGURE 4.** Endogenous α2AR-evoked ERK activation is Ras-Raf dependent and does not require PI3K. A and B, WT and Arr2,3−/− MEFs were pre-treated with or without indicated inhibitors for 1 h and then treated with the α2 agonist (10⁻⁴ M epinephrine + 10⁻⁶ M propranolol + 10⁻⁶ M prazosin) for 5 min in the presence or absence of the indicated inhibitors. Cell lysates were blotted for pERK and total ERK, A, representative blots of α2AR-evoked ERK at 5 min in WT and Arr2,3−/− MEFs in response to indicated inhibitor treatments. B, summary of quantitative data. ERK signals (active/total) were quantitated from five independent experiments, and percent increase of ERK activity over basal (±S.E.) was plotted using GraphPad Prism®. *p < 0.01.

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**FIGURE 5.** Differential responses of α2AR-evoked ERK signals in WT versus Arr2,3−/− MEFs to tyrosine kinase inhibitors. WT and Arr2,3−/− MEFs were pre-treated with or without indicated inhibitors for 1 h and then treated with α2 agonist (10⁻⁴ M epinephrine + 10⁻⁶ M propranolol + 10⁻⁶ M prazosin) for 5 min in the presence or absence of indicated inhibitors. Cell lysates were blotted for pERK and total ERK, A, representative blots of α2AR-evoked ERK at 5 min in WT and Arr2,3−/− MEFs following indicated inhibitor treatments. B, summary of quantitative data. ERK signals (active/total) were quantitated from five independent experiments, and percent increase of ERK activity over basal (±S.E.) was plotted using GraphPad Prism®. *p < 0.01.

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The impaired desensitization of α2AAR signaling seen in Arr2,3−/− cells is consistent with the well appreciated roles of arrestins in the receptor desensitization process (38).

**Enrichment of Phosphorylated ERK in the Nucleus following Agonist Treatment Is Independent of Arrestin Expression**—We examined the morphological distribution of pERK following α2A agonist activation of MEFs. Detection of pERK in WT MEFs first occurs in cytosol (see the 1 min time point in Fig. 3), but pERK becomes enriched in nuclei after agonist stimulation for 10 min (Fig. 3). In Arr2,3−/− MEFs, the α2AAR-evoked pERK signal is highly enriched in nuclei at all time points examined, including the earliest time point measured, i.e. 1 min (Fig. 3). Whether pERK is activated in the nucleus, without required cytosolic to nuclear transport, is not known, but cytosolic pERK was barely detectable at the earliest time points in our studies of Arr2,3−/− MEFs.

**Endogenous α2AAR-evoked ERK Activation Is Ras-Raf Dependent Both in the Presence and Absence of Arrestins**—It has been reported that the α2AR activates ERK signaling through a Gβγ- and a Ras-Raf-dependent pathway (39). Here we examined whether this is also true in cells without arrestin expression. As shown in Fig. 4, inhibitors of Ras (Ftase inhibitor I) and Raf (Raf inhibitor II) blocked ~80% of ERK activation elicited by endogenous α2AAR in both WT and Arr2,3−/− cells, indicating that the α2AAR still activate ERK through the G protein-Ras-Raf pathway even without arrestin expression. It has been reported that Gβγ activation of Ras is sensitive to phosphoinositide 3 kinase (PI3K) inhibitors (40, 41), suggesting that PI3K can serve as an intermediate linking GPCRs to ERK signaling. Therefore, we examined the effect of PI3K inhibitor treatment in ERK activation mediated by the endogenous α2AAR in MEFs. The PI3K inhibitor LY294002 failed to alter α2AAR-evoked ERK activation in either WT or Arr2,3−/− MEFs (Fig. 4, A and B), although control studies confirmed that LY294002 was active, as it blocked serum-induced phosphorylation of Akt (a known downstream target of PI3K) in parallel experiments (Fig. 4, C and D). These data indicate that PI3K is not involved in this process.

**Lack of Arrestins Eliminates Sensitivity of α2AAR Activation of pERK to Src Family Kinase Inhibitors**—Because arrestins have been reported to serve as a scaffold for the β3AR, MEK, ERK, and Src (16), we explored whether activation of Src kinase is a critical link between α2AAR activation and ERK phosphorylation and whether arrestin is necessary for this role of Src, if
it occurs. The overall involvement of a tyrosine kinase (or kinases) in α₂AR activation of ERK in WT cells is evident in Fig. 5, where we observed elimination of agonist-stimulated ERK phosphorylation in the presence of the general tyrosine kinase inhibitor genistein. This involvement of tyrosine kinases is not due to transactivation of the epidermal growth factor (EGF) receptor tyrosine kinase, because α₂AR activation was insensitive to inhibition by the EGF receptor kinase inhibitor, AG1478 (Fig. 5), whereas this agent completely eliminated effects of EGF on ERK phosphorylation in parallel control experiments (data not shown). For WT MEFs, the Src family tyrosine kinase inhibitor PP2 eliminated the α₂AR-evoked activation of ERK (Fig. 5), suggesting that sensitivity to genistein may be accounted for by the role of Src family kinase in linking α₂AR activation to ERK phosphorylation. The involvement of Src kinase in α₂AR-mediated ERK activation is confirmed by the fact that expression of a kinase-inactive dominant negative Src (SrcK297R) (42) remarkably reduced ERK phosphorylation following α₂AR stimulation (Fig. 6). However, a different pathway appears to link the α₂AR to ERK phosphorylation in cells lacking arrestin expression, because stimulation of p-ERK accumulation in Arr2,3−/− MEFs is independent of either genistein or Src kinase inhibitors (Fig. 5). Thus, in the absence of arrestin expression, the α₂AR appears to engage an additional signaling pathway to phosphorylate ERK, a Src kinase-independent pathway.

We further examined α₂AR-mediated ERK activation in cells lacking expression of the Src family kinases using Src-null SYF cells derived from Src, Yes, and Fyn triple knock-out mouse embryos. Consistent with the above findings, the α₂AR activated ERK in SYF cells, affirming its ability to do so in a Src-null, thus Src-independent, environment (Fig. 7A). Interestingly, similar to findings in Arr2,3−/− MEFs, the α₂AR-evoked pERK signal in SYF cells is highly enriched in nuclei at the earliest time point of measurement (Fig. 7B). In contrast, when c-Src is reconstituted in SYF cells (i.e. SYF+c-Src cells), α₂AR-evoked pERK signal is mainly distributed in cytosol at this early 2-min time point (Fig. 7B).

Agonist Stimulation of the α₂AR Enhances Arrestin3 Interaction with c-Src as Well as Src Activation—Our data suggest that arrestin may serve as a scaffold linking Src kinase to α₂AR-evoked ERK signaling pathways. Direct interaction between c-Src and arrestin has been reported previously (16, 43). Here we examined whether Src and arrestin interact under our experimental conditions and whether activation of α₂AR has any impact on this interaction. As shown in Fig. 8, agonist occupancy of the α₂AR remarkably enriched the amount of arrestin3 in the HA-Src immunoisolated complex by 3.1-fold, indicating that activation of α₂AR enhances formation of the arrestin-Src complex. Moreover, the amount of active Src (detected by antibody against p-Tyr-416) in cells was increased...
Arrestin and Src Involvement in α₂AR Activation of ERK

Following agonist stimulation, even though the amount of total Src (detected by the anti-HA antibody) is not changed (Fig. 8). These data strongly suggest that stimulation of α₂AR promotes arrestin-Src interaction and Src activation.

**DISCUSSION**

Arrestin regulates GPCR-mediated signaling in a variety of ways. Arrestin binding to activated and phosphorylated receptors uncouples G proteins from these receptors, thus desensitizing G protein-dependent signaling (2). Arrestin also is required for GPCR internalization (12, 13), which leads either to receptor degradation or to recycling (3, 14, 15). Arrestin-dependent endocytosis and subsequent recycling enhances the initial rate of GPCR-mediated signaling, at least in some settings (26). Furthermore, arrestin can serve as a scaffold to directly link receptors to downstream signaling effectors in a G protein-independent manner (20). Our current studies demonstrated that in the process of G protein-dependent ERK activation by the α₂AR, arrestin not only is critical for desensitization of this process (Fig. 2) but also is required for involvement of the Src family of tyrosine kinases in this event (Figs. 5 and 9).

As shown schematically in Fig. 9, ERK stimulation by the α₂AR requires the Gi/Go subfamily of G proteins. With arrestin present, G proteins activate Src tyrosine kinase, which subsequently activates the Ras-Raf pathway and results in ERK phosphorylation. In this process, arrestin serves as a scaffold recruiting Src to the plasma membrane for activation by G proteins (Figs. 8 and 9), by analogy to its role in ERK activation by β₂AR (16). In contrast to β₂AR-mediated ERK activation, which appears to require arrestin-dependent endocytosis via clathrin-coated pits (44), α₂AR-evoked ERK activation does not require endocytosis (22, 26). Furthermore, even without arrestin expression (in Arr2.3−/− MEFs), α₂AR agonists are still able to evoke ERK phosphorylation and do so with a potency similar to that observed in WT cells (Fig. 1D). Arrestin-independent ERK activation occurs through a G protein-dependent but Src-independent pathway that requires Ras activation (Figs. 4, 5, and 9).

Therefore, our data reveal that arrestin serves as a molecular switch determining the signal transduction route of ERK activation by the α₂AR.

Our data also demonstrate an important role of arrestin in modulating the temporal and spatial profiles of α₂AR-mediated ERK activation. In cells without the arrestin-mediated desensitization process, the duration of the α₂AR-evoked pERK signal is considerably extended (Fig. 2), lasting up to 5 h (data not shown). In Arr2.3−/− cells, α₂AR-evoked phosphorylation of ERK is detected principally in the nucleus, without detectable prior phosphorylation in the cytosol (Figs. 3 and 9), indicating a role of arrestin in retaining ERK localization in the cytosol. The ERK signaling pathway is critical for multiple cellular processes, including proliferation, differentiation, survival, and migration, and the duration and subcellular localization of pERK are the major factors determining what cellular response active ERK signaling would trigger (45, 46). Therefore, the changes in duration and localization of α₂AR-evoked pERK due to absence of arrestin observed in this study may result in significant alteration of α₂AR-mediated cellular responses. For example, the sustained nuclear-localized pERK in the absence of arrestin may lead to transcription of certain responsive genes and sub-
Arrestin and Src Involvement in \( \alpha_{2A}R \) Activation of ERK

Sequentially activate cellular response pathways mediated by these genes.

Our data also eliminate PI3K and transactivation of EGF receptor tyrosine kinase as contributors to \( \alpha_{2A}R \)-mediated ERK activation in native cells. Our present findings revealed that \( \alpha_{2A}R \)-evoked ERK phosphorylation in MEFs is not altered by PI3K inhibitor LY290004 (Fig. 4), indicating that PI3K is not involved in the \( \alpha_{2A}R \)-stimulated G protein-Ras-Raf signaling pathway. This is in contrast to previous findings that PI3K is employed by some GPCRs such as dopamine D2 and D3 receptors to activate ERK (24). Similarly, although some GPCRs activate ERK by transactivation of receptor tyrosine kinases, such as the EGF receptor (32), our findings revealed that \( \alpha_{2A}R \) activation of ERK signaling is not significantly altered by the EGF receptor inhibitor AG1478 in either WT or Arr2,3−/− MEFs (Fig. 5).

Taken together, our findings provide the first evidence of the role of arrestin as a determinant of Src kinase-dependent versus Src kinase-independent activation of p42/44 ERK by endogenous \( \alpha_{2A}R \) despite the absence of an obligatory role of arrestin in linking \( \alpha_{2A}R \) to ERK activation. These findings add arrestin to the list of “molecular switches” in GPCR signaling. Furthermore, the data demonstrate a possible role for arrestin and Src kinase-dependent pathway of ERK activation in dictating the rate and extent of nuclear localization of pERK.

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