Molecular Medicine

Arrestin Orchestrates Crosstalk Between G Protein–Coupled Receptors to Modulate the Spatiotemporal Activation of ERK MAPK

David Cervantes, Catherine Crosby, Yang Xiang

Rationale: G protein–coupled receptors (GPCRs) respond to diversified extracellular stimuli to modulate cellular function. Despite extensive studies investigating the regulation of single GPCR signaling cascades, the effects of concomitant GPCR activation on downstream signaling and cellular function remain unclear.

Objective: We aimed to characterize the cellular mechanism by which GPCR crosstalk regulates mitogen-activated protein kinase (MAPK) activation.

Methods and Results: Adrenergic receptors on cardiac fibroblasts were manipulated to examine the role of arrestin in the spatiotemporal regulation of extracellular signal-regulated kinase (ERK)1/2 MAPK signaling. We show a general mechanism in which arrestin activation by one GPCR is capable of regulating signaling originating from another GPCR. Activation of Gq coupled–receptor signaling leads to prolonged ERK1/2 MAPK phosphorylation, nuclear accumulation, and cellular proliferation. Interestingly, coactivation of these receptors with the β-adrenergic receptors induced transient ERK signaling localized within the cytosol, which attenuated cell proliferation. Further studies revealed that recruitment of arrestin3 to the β2-adrenergic receptor orchestrates the sequestration of Gq-coupled receptor–induced ERK to the cytosol through direct binding of ERK to arrestin.

Conclusions: This is the first evidence showing that arrestin3 acts as a coordinator to integrate signals from multiple GPCRs. Our studies not only provide a novel mechanism explaining the integration of mitogenic signaling elicited by different GPCRs, but also underscore the critical role of signaling crosstalk among GPCRs in vivo. (Circ Res. 2010;106:79-88.)

Key Words: adrenergic receptor ■ cardiac fibroblast ■ ERK ■ arrestin

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G protein–coupled receptors (GPCRs) respond to diversified extracellular stimuli to modulate cellular function. Traditionally, activated receptors couple to G proteins, which transduce downstream signals via second messengers and membrane channels.1 Active GPCRs are phosphorylated by specific GPCR kinases (GRKs) leading to receptor desensitization. Arrestin proteins then bind to the phosphorylated receptor initializing clathrin-mediated internalization.2 Despite extensive studies investigating the regulation of single GPCR signaling cascades, the effect of concomitant GPCR activation by endogenous stimuli on downstream signaling remains poorly understood. There is a great deal of evidence supporting functional crosstalk between different GPCRs both in vitro and in vivo.3-5 The majority of these studies focus on short-term stress responses involved in modulation of common effectors such as G proteins, phospholipases (PLs), and adenylyl cyclases.4 However, chronic activation of multiple GPCR signaling pathways during maladaptive tissue and organ remodeling suggests the potential of downstream crosstalk away from the plasma membrane.

One potential nexus for GPCR signaling crosstalk are the multifunctional scaffold proteins known as arrestins. Arrestins not only scaffold proteins for the activation of different MAPK families under single receptor activation, but also mediate transactivation of epidermal growth factor receptor signaling pathways2,6 and activation of many other non-GPCR signaling cascades.7 In the case of GPCR-induced extracellular signal-regulated kinase (ERK) mitogen-activated protein kinase (MAPK) activation, both G proteins and arrestins are capable of mediating ERK activation via independent mechanisms, with each pathway leading to unique spatiotemporal consequences.8 Whereas G protein–dependent ERK translocates to the nucleus for gene transcription, arrestin-dependent ERK remains within the cytoplasm. Because arrestins preferentially bind to some, but not all, GPCRs in a ligand-dependent manner, we envision that arrestins may play a role in GPCR crosstalk by coordinating MAPK activation in distinct subcellular compartments. Such a regulatory mechanism is essential for modulating MAPK signaling in divergent cellular functions such as cell proliferation and growth,9 mobility,10 and apoptosis.11
We chose cardiac fibroblasts as a model to study GPCR signaling crosstalk. Both α1-adrenergic receptor (α1ARs) and βARs are expressed in cardiac tissue and are activated by catecholamines to modulate maladaptive cardiac remodeling, including cardiac fibroblast proliferation, by activation of distinct pathways. These pathways transduce their proliferative signal via members of the MAPK family, including ERK1/2. Stimulation of the α1AR leads to Gq coupling and subsequent PLC and protein kinase (PK)C activation.12 PKC via Gq signaling leads to ERK activation in a Gi-dependent manner has been shown to directly activate the Raf-MEK1-ERK axis.14a Cardiac fibroblasts or MEF cells were then subjected to Western blotting with antibodies accordingly. Preplating of neonatal cardiac fibroblasts and embryonic fibroblasts (MEFs) was the first step in the protocol. Animal care and use was in accordance with institutional guidelines.

### Methods

**Non-standard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
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<tr>
<td>AR</td>
<td>adrenergic receptor</td>
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<td>Arr</td>
<td>arrestin</td>
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<tr>
<td>Epi</td>
<td>epinephrine</td>
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<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
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<td>GPCR</td>
<td>G protein–coupled receptor</td>
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<td>GRK</td>
<td>G protein–coupled receptor kinase</td>
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<tr>
<td>HA</td>
<td>hemagglutinin</td>
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<tr>
<td>Iso</td>
<td>isoproterenol</td>
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<tr>
<td>KO</td>
<td>knockout</td>
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<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
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<tr>
<td>MEF</td>
<td>mouse embryonic fibroblast</td>
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<tr>
<td>MEK</td>
<td>mitogen-activated protein extracellular kinase</td>
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<td>Phe</td>
<td>phenylephrine</td>
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<tr>
<td>PK</td>
<td>protein kinase</td>
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<tr>
<td>PL</td>
<td>phospholipase</td>
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<tr>
<td>PMA</td>
<td>phorbol myristate acetate</td>
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<tr>
<td>Tim</td>
<td>timolol</td>
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<tr>
<td>WT</td>
<td>wild type</td>
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Animal care and use was in accordance with institutional guidelines. Neonatal cardiac fibroblasts were isolated from new born mice using a collagenase dispersion procedure with a preplating step, as described previously.14a Cardiac fibroblasts or MEF cells were then transfected, if indicated, with either arrestin, GRK, Src, or AR constructs using Lipofectamine 2000 according to the instructions of the manufacturer (Invitrogen). For Western blotting, cells were pretreated with the indicated drugs and then stimulated with adrenergic agonists or antagonists for the indicated times. Cell lysates were then subjected to Western blotting with antibodies accordingly. The cell proliferation ELISA was carried out using bromodeoxyuridine (BrdU) Labeling and Detection Kit III (Roche) according to the instructions of the manufacturer. Student’s t test was performed using Prism software (GraphPad).

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

### Results

**Agonist-Dependent Spatiotemporal Activation of ERK on Stimulation of ARs in Cardiac Fibroblasts**

Both α1ARs and βARs are implicated in promoting ERK activation on stimulation with catecholamines.15 Using cardiac fibroblasts, we investigated the mechanism leading to the modulation of ERK signaling on potential crosstalk between these GPCRs. We examined agonist-dependent ERK phosphorylation induced by epinephrine (Epi). Because α1ARs are the primary ARs responsible for ERK activation in cardiac tissues, we used the α1AR-specific agonist phenylephrine (Phe). Both Epi and Phe activated ERK by increasing the phosphorylation of ERK (phospho-ERK). The maximal phospho-ERK levels peaked at 5 minutes after stimulation, with the peak levels induced by Phe significantly lower than that by Epi. Moreover, the Epi-induced phospho-ERK underwent a rapid decrease to baseline, whereas, the Phe-induced phospho-ERK signal was prolonged and returned to baseline levels slowly (Figure 1A).

Previous studies have indicated that subcellular ERK distribution may shape the temporal profile of ERK activation.16 We found that Epi-induced phospho-ERK accumulated in the cytosol and returned to baseline levels rapidly (Figure 1B). Interestingly, Phe-induced phospho-ERK translocated to the nucleus and remained elevated throughout stimulation (Figure 1B). The subcellular distribution of phospho-ERK was confirmed in fractionation studies. The Epi-induced phospho-ERK was enriched in the cytosolic fraction; however, the Phe-induced phospho-ERK was enhanced in both the cytosolic and nuclear fractions (Figure 1C). Thus, concomitant activation of the β and α1ARs by Epi induces a distinct spatiotemporal ERK signal than that elicited by activation of the α1AR alone by Phe.

**ERK Activation Is Induced by a Classic α1AR/Gq Pathway in Cardiac Fibroblasts**

To investigate the mechanism underlying the modulation of ERK signaling on AR crosstalk, we first examined which AR was responsible for ERK phosphorylation. Stimulation with Epi and Phe induced potent ERK activation (Figure 2A). Stimulation with the βAR agonist isoproterenol (Iso) resulted in minimal ERK activation, whereas, stimulation of the α1ARs with clonidine did not activate ERK (Figure 2A). In addition, pretreatment with the α1AR antagonist prazosin, but not the βAR antagonist timolol (Tim) or the α1AR antagonist yohimbine, significantly blocked Epi-induced ERK activation (Figure 2B and data not shown). As a control, prazosin and...
these results confirmed that ERK activation resulted primarily from α1AR stimulation in cardiac fibroblasts, which was supported by the expression of both α1AR and α2βAR genes (Online Figure III). Accordingly, stimulation of Gq with

Figure 2. ERK activation on adrenergic stimulation occurs via α1AR signaling. A, WT cardiac fibroblasts were stimulated with 10 μmol/L either Epi, Iso, Phe, or clonidine (Clo) for 5 minutes or PMA for 10 minutes to obtain maximal stimulation. B, WT fibroblasts were pretreated with 10 μmol/L either prazosin (Prazo) or Tim for 5 minutes. Cells were then stimulated with 10 μmol/L either Epi or Phe for 5 minutes or PMA for 10 minutes. C and D, WT fibroblasts were pretreated for 30 minutes with the PLC inhibitor U73122 (1 μmol/L), PKC inhibitor (PKC inhibitory peptide [PKCi] 20 μmol/L), or a MEK inhibitor (1 μmol/L) and then stimulated with either Epi or Phe for 5 minutes. Phospho-ERK (ERKp) was normalized against total ERK, *P<0.05; **P<0.01 by unpaired Student’s t test (n=3).

Pasteurella multocida toxin and stimulation of PKC with phorbol myristate acetate (PMA) was sufficient to induce potent ERK activation (Figure 2 and data not shown). In contrast, direct inhibition of Gi with pertussis toxin had no effect on agonist-induced ERK activation (Online Figure IV). Pretreatment with the PKA inhibitor H89, which blocks Gs signaling, also had no significant effect on agonist-induced ERK activation (Online Figure IV). Together, these data confirm that the α1ARs are the primary adrenergic subtypes responsible for ERK phosphorylation via Gq activation in cardiac fibroblasts.

Classic α1AR/Gq coupling activates PLC to produce diacyl glycerol, leading to PKC activation. Pretreatment with the PLC inhibitor U73122 significantly blocked both Epi- and Phe-induced ERK phosphorylation (Figure 2C and 2D). Similarly, pretreatment with a myristoylated PKC inhibitory peptide significantly prevented ERK activation on either Epi or Phe stimulation (Figure 2C and 2D). Moreover, inhibition of mitogen-activated protein extracellular kinase (MEK) with
U1026 also prevented both Epi and Phe-induced ERK activation (Figure 2C and 2D). These data confirm that α₁AR-induced ERK signaling is dependent on activation of PLC and PKC leading to the Raf-MEK pathway.18

**BAR Activation Prevents Nuclear Translocation of α₁AR-Induced phospho-ERK and Cardiac Fibroblast Proliferation**

One possible explanation for the differences in the spatiotemporal ERK activation profile between Epi and Phe is that βAR activation by Epi may lead to cytosolic retention of α₁AR-induced phospho-ERK. To test this hypothesis, we blocked the βARs with Tim before Epi stimulation. Inhibition of the βARs with Tim redistributed the Epi-induced phospho-ERK to both the cytosol and the nucleus, resulting in a similar spatial profile to that induced by Phe (Figure 3A and 3B). As a result, βAR blockade significantly prolonged the phospho-ERK signal (Figure 3C). Moreover, simultaneous stimulation of βARs with Iso prevented nuclear translocation of Phe-induced phospho-ERK and promoted phospho-ERK signal attenuation (Online Figure V). Together, our data suggest that activation of βARs can prevent nuclear translocation of phospho-ERK induced by the α₁AR/Gq signaling pathway.

To understand the physiological consequence of cytosolic ERK sequestration on α₁AR and βAR crosstalk in cardiac fibroblasts, we examined cell proliferation using BrdU incorporation on different ligand stimulation. Activation of the α₁ARs with Phe, but not the βARs with Iso, induced a significant increase in BrdU incorporation, which was blocked by the MEK inhibitor U1026. Further, activation of both the α₁AR and βARs with Epi did not significantly enhance BrdU incorporation. However, inhibition of βARs with Tim enabled Epi to stimulate BrdU incorporation (Figure 3D), presumably because of the redistribution of phospho-ERK to the nucleus for gene transcription (Figure 3A). This increase in BrdU incorporation was again blocked by U1026.

**β₂AR, but Not β₁AR, Activation Prevents Nuclear Translocation of α₁AR-Induced ERK Activation**

We then sought to identify the βAR subtype responsible for cytosolic sequestration of α₁AR-induced ERK. Cardiac fibroblasts lacking the β₂AR, but not those lacking the β₁AR, displayed nuclear phospho-ERK accumulation on Epi stimulation (Figure 4A and 4B), which also prolonged the
phospho-ERK signal (Figure 4C). Stimulation of double knockout (KO) cells, which lack both βAR subtypes, with Epi induced phospho-ERK in both the cytosol and nucleus (Figure 4D and 4E). We then reintroduced either β1AR or β2AR into double KO cells with similar expression levels (data not shown). Expression of β2AR, but not β1AR, recovered the cytosolic retention of phospho-ERK induced by Epi (Figure 4D and 4E). Thus, the effect is β2AR-specific, and is not attributable to the higher endogenous expression levels of the β1AR than the β2AR in cardiac fibroblasts. Together, these data suggest that activation of the β2AR modulates both the spatial and temporal profile of α1AR-induced ERK activation in cardiac fibroblasts.

β2AR-Dependent Recruitment of Arrestin Is Necessary to Sequester α1AR-Induced ERK Signaling

We further examined the molecular mechanism explaining this signaling crosstalk. Previous studies reported that α1DARs form a heterodimer with β2ARs in HEK293 cells, thus altering α1D signaling, but this α1AR subtype is not expressed in cardiac fibroblasts (Online Figure III). Moreover, neither α1AAR nor α1BAR dimerized with β2AR (Online Figure VI). Alternatively, on phosphorylation of the β2ARs via GRKs, arrestins are recruited leading to receptor internalization, and the internalized β2AR/arrestin complexes propagate numerous signaling pathways, including...
ERK pathways. Because of their role in arrestin recruitment, we investigated the role of GRKs in Epi-induced ERK activation. MEF cells were used for selective knockdown individual GRKs. In wild-type (WT) MEF cells, Epi stimulated cytosolic phospho-ERK, similar to cardiac fibroblasts. Selective knockdown of GRK2, but not other GRKs, in MEF cells significantly promoted nuclear accumulation of phospho-ERK induced by Epi (Figure 5A and Online Figure VII). Expression of βARKct, a GRK2 inhibitor that prevents β-arrestin recruitment and thus βAR internalization, significantly promoted nuclear accumulation of phospho-ERK (Figure 5B) and prolonged ERK signaling (Figure 5C). These data indicate that the GRK2-mediated phosphorylation of the β2AR modulates ERK activation, presumably through β-arrestin-mediated scaffolding of ERK.

In addition, Src is necessary for arrestin-dependent βAR internalization on agonist binding. Inhibition of Src with either overexpression of dominant negative Src (DN-Src) or treatment with Src inhibitor PP2 prolonged ERK activation induced by Epi, but had no effect on Phe-induced ERK (Online Figure VIII and data not shown). Fractionation studies revealed that DN-Src expression enhanced nuclear phospho-ERK translocation on Epi stimulation (Online Figure VIII). These data suggest that Src-dependent and arrestin-mediated β2AR internalization is necessary for cytosolic sequestration of the α1AR-induced ERK signal under Epi stimulation.

To identify the arrestin(s) responsible for this crosstalk, MEF cells lacking either Arr2 (Arr2-KO) or Arr3 (Arr3-KO) were used. In comparison to WT MEF cells, Epi promoted phospho-ERK translocation to the nucleus in Arr3-KO, but not Arr2-KO MEF cells (Figure 6A and 6B). Thus, Arr3 is primarily responsible for the crosstalk between the β2 and α1ARs. We then examined the association between ERK and the β2AR/Arr3 complex on agonist stimulation. Epi induced a significant increase in the association between ERK and the β2AR/Arr3 complex, which was attenuated by Tim (Figure 6C and Online Figure IX). In contrast, neither Phe nor Iso enhanced the association between ERK and the β2AR/Arr3 complex; but costimulation with Iso and Phe promoted formation of the complex (Figure 6C). Furthermore, a dominant-negative Arr3 (GFP-V54DArr3) also formed a complex with the β2AR, but was not sufficient to promote the binding of ERK to the β2AR/Arr3 complex (Figure 6C).
then used GFP-V54DArr3 to further perturb the crosstalk between the β2 and α1-ARs. Expression of GFP-V54DArr3, but not GFP-Arr3, promoted nuclear translocation of the phospho-ERK induced by Epi, which did not colocalize with GFP-V54DArr3 in the cytosol (Figure 6D and 6E). In contrast, expression of neither GFP-Arr3 nor GFP-V54DArr3 affected the Phe-induced nuclear accumulation of phospho-ERK (Figure 6D and 6E). In cells expressing GFP-V54DArr3, the cytosolic and the nuclear accumulation of Epi-induced phospho-ERK was further confirmed by fractionation studies (Figure 6F). Not surprisingly, nuclear translocation also prolonged the Epi-induced phospho-ERK signal (Figure 6G). Together, these data show that internalization of the β2-AR via Arr3 induces the crosstalk between the α1-AR and β2-ARs, leading to ERK sequestration within the cytosol.

**Arrestin Modulation of Cellular ERK Signaling via GPCR Crosstalk Is a General Mechanism**

To test whether this arrestin-mediated GPCR crosstalk is a general MAPK regulatory mechanism on concomitant acti-
vation of multiple GPCRs, we used MEF cells lacking both Arr2 and Arr3 (Arr2/3-KO) to investigate phospho-ERK distribution induced by different Gq-coupled receptors in the absence and presence of H9252 activation. Stimulating both WT MEFs and cardiac fibroblasts with either Phe or PMA induced nuclear accumulation of phospho-ERK. Interestingly, stimulation of WT MEF and cardiac fibroblast cells with 2 other Gq-coupled receptor agonists, (Val5) angiotensin II or thrombin, also induced nuclear phospho-ERK accumulation (Figure 7A and 7B), likely through Gq-dependent pathways.26 However, on Iso costimulation, Phe-, (Val5) angiotensin II–, thrombin-, and PMA-induced phospho-ERK was sequestered within the cytoplasm in both WT MEF and cardiac fibroblast cells (Figure 7A and 7B). As expected, in MEF cells lacking β-arrestins, or in cardiac fibroblasts expressing the GFP-V54DArr3 mutant, ERK signaling induced by the different stimuli was able to translocate into the nucleus. However, both arrestin deficiency and GFP-V54DArr3 expression blocked the effect of β2AR activation under Iso stimulation (Figure 7A and 7B). Together, these data suggest that arrestin activation by the β2AR can sequester Gq-coupled receptor–induced phospho-ERK within the cytosol in both cardiac fibroblasts and MEF cells.

Discussion

In this study, we have identified a novel mechanism regulating Gq-coupled receptor–induced ERK MAPK signaling via crosstalk with β2AR-recruited Arr3 in cardiac fibroblasts and MEFs (Figure 8). This is the first evidence suggesting that arrestin activation by one receptor is capable of regulating signaling originating from another GPCR. G protein-independent regulation of GPCR signaling via arrestins is an emerging mechanism explaining the regulation of a growing list of GPCR-mediated signaling including α2AR,16 angiotensin receptors,22 β1AR,14 β2AR,8 opioid receptors,27 and the vasopressin receptors.28 Recruitment of arrestins to a phosphorylated GPCR regulates not only receptor internalization but also intracellular signaling such as transactivation of receptor tyrosine kinases.2,6 Arrestin, in addition, scaffolds ERKs, leading to activation, cytosolic retention,22,29 and decreased transcription in the nucleus.30 In reconstituted systems this provides a linear mechanism, however, this model fails to reflect the convoluted signaling networks in vivo. Endogenous ligands, including neurotransmitters and hormonal peptides, bind to multiple receptors present in a cell, activating numerous signaling cascades. Indeed, accumulative evidence supports signal crosstalk among GPCRs, such as between the β1AR and the angiotensin 1 receptor,5 the α2AR and the opioid receptors,11 as well as the c5a receptor and the UDP receptor.32 Here, our results suggest that arrestin functions as a master regulator, coordinating subcellular ERK activation under multiple extracellular stimuli to inhibit nuclear translocation and facilitate signal attenuation. Considering the ability of arrestin to scaffold different cytosolic signaling components besides ERK, such as Src, Jun N-terminal kinase, and p38,26 and the ability of arrestin to selectively bind to some, but not all, GPCRs in an agonist-dependent fashion,33 our data suggest a general mechanism of

![Figure 7](http://circres.ahajournals.org/figs/7.png)
arrestin-mediated crosstalk among GPCRs with broad implications in physiological responses under neurohormonal regulation in vivo.

In cardiac fibroblasts and MEFs, our data indicate that the α1Rs make the primary contribution to ERK activation, supporting the dominant roles of these receptor subtypes in cardiac remodeling.34 Consistent with previous studies,35 stimulation of the α1Rs with Phe induces the classic Gq-dependent activation of PLC and PKC, leading to ERK activation through Raf-MEK1 kinase cascade. Under this signaling cascade, activated ERK translocates to the nucleus.35 Interestingly, this scenario is completely reshaped when β2Rs are coactivated with the α1R on Epi stimulation. Coactivation of the α1 and β2Rs leads to sequestration of phospho-ERK within the cytosol. Under Epi stimulation, it was possible that two pools of ERK existed; a transient pool activated by the β2AR and a prolonged pool activated by the α1R. The first pool becomes dominant simply because β2Rs are more prominent in cardiac tissues than α1Rs. This explanation is unlikely for several reasons. First, α1R antagonist prazosin blocked Epi-induced ERK phosphorylation. Second, stimulation with isoproterenol (a βAR agonist) alone induces minimal ERK activation. Third, blockade of the β2AR prolonged ERK activation and only slightly decreased maximal ERK levels (Figure 3C). Taken together, these data support that ERK activation by Epi and Phe originates from α1R activation. However, under Epi stimulation, β2AR activation changes the spatiotemporal profile of ERK signaling by policing ERK to the β2AR/Arr3 complex. Cytosolic retention may allow targeting to nonnuclear ERK substrates involved in different cellular stress processes36 and receptor desensitization.37 This unique observation highlights the potential diversified physiological consequences under a given extracellular environment when multiple receptors are activated simultaneously. As expected, this Arr3-mediated crosstalk is dependent on the receptor phosphorylation by GRK. Inhibiting GRK2, but not other GRKs, is sufficient to prevent the β2AR-dependent modulation of α1R-induced ERK signaling. In addition to sequestering ERK within proximity of cytosolic ERK targets, our data indicate that interaction with Arr3 may facilitate the attenuation of ERK signaling in the cytosol.38 However, inhibition of phosphatase 2A or dual-specificity phosphatases failed to prevent ERK dephosphorylation (data not shown); thus, the phosphatases involved remain to be identified.

The distinct spatiotemporal profile of ERK activation induced by Gq-coupled receptors in the presence or absence of β2AR (and potentially other GPCRs) activation has broad implications in the maladaptive remodeling of different tissues in vivo. Indeed, in cardiac myocytes, the β2AR, but not the β1AR, appears to crosstalk with Gq-coupled receptors in modulating ERK activation (unpublished data). Gq-coupled receptor signaling and ERK activation play essential roles in long-term pathophysiological cardiac remodeling.9,34 Traditionally, activated ERK MAPK translocates to the nucleus to activate transcription factors including Elk-139 and GATA-4,40 leading to fibroblast proliferation and myocyte hypertrophy. Here, this nuclear translocation of ERK is abrogated by arrestin binding to activated β2ARs in cardiac fibroblasts; interestingly it can be restored with the clinically-relevant β blocker Tim. Our data thus provide insights into understanding the tissue remodeling observed in patients under chronic treatment with β-blockers in various clinical and physiological conditions.

In summary, our findings provide the first evidence for the role of β2AR-recruited arrestin in regulating signaling from another GPCR. These findings provide a novel mechanism to significantly advance our understanding of the growing profiles of GPCR regulatory pathways. These data further underscore the critical role of signaling crosstalk in the complex regulation of receptor signaling via subcellular localization of signaling components, which will have significant implications in numerous clinical and physiological conditions.

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Disclosures
None.

References


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Online Material and Methods

Cell culture, transfection, and adenoviral infections Animals protocols are approved by the IACUC of the University of Illinois according to NIH regulation. Cardiac fibroblasts were isolated from the hearts of either wild-type, $\beta_1$KO or $\beta_2$KO neonatal FVB mice by a collagenase dispersion procedure utilizing a preplating step to remove cardiac myocytes as detailed in previous publications $^1, 2$. Cells were cultured on 10 cm tissue culture treated dishes until 90% confluent. Cells were then plated on 35 mm tissue culture treated dishes at a confluence of 60-70%. Primary cardiac fibroblasts were maintained for 24 h in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and experiments were performed, unless otherwise noted, on cells serum-deprived for 24 h. MEFs fibroblasts (wild type and $\beta$arrretin KO that lacks both $\beta$arrretin 1 and $\beta$arrretin 2 genes) were maintained 24 h in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum $^3$. FLAG-tagged mouse arrestin 3 constructs were transfected into primary neonatal cardiac fibroblasts utilizing Lipofectamine2000 (Invitrogen, Carlsbad, CA). Alternatively, neonatal cardiac fibroblasts were infected with recombinant adenoviruses as indicated after being cultured for 24 h. Recombinant plasmids expressing $\beta$ARKct, GFP- arrestin 3, and GFP- arrestin 3 V54D have been generated as previously described $^2, 4$. GFP- arrestin 3 and GFP- arrestin 3 V54D plasmids were gifts from Dr. Gang Pei (Chinese Academy of Sciences, Shanghai). Dominant negative Src plasmids were gifts from Dr. Qin Wang (University of Alabama at Birmingham). Mouse embryonic fibroblasts (MEF) cell lines were from Dr. Robert Lefkowitz (Duke University). GRK3ct is a gift from Dr. Blaxall Burns (University of Rochester). Co-immunoprecipitation experiments were conducted after 48 h expression.

Drug Treatment Fibroblasts were treated with the following agonists or antagonists for the indicated times: epinephrine (Epi, 10 $\mu$M; Sigma, St. Louis, MO) or norepinephrine (NE, 10 $\mu$M; Sigma, St. Louis, MO) as general AR agonists, phenylephrine (Phe, 10 $\mu$M; Sigma, St. Louis, MO) as an $\alpha$1AR agonist, isoproterenol (Iso, 10 $\mu$M; Sigma, St. Louis, MO) as a $\beta$AR agonist, clonidine (Clo, 10 $\mu$M; Sigma, St. Louis, MO) as an $\alpha$2AR agonist, timolol (Tim, 10 $\mu$M; Sigma, St. Louis, MO) as a $\beta$AR antagonist, prazosin (Prz, 10 $\mu$M; Sigma, St. Louis, MO) as an $\alpha$1AR antagonist, yohimbine (Yoh, 10 $\mu$M; Sigma, St. Louis, MO) as a $\alpha$2AR antagonist,
(Val5)angiotensin II (1μM; Sigma, St. Louis, MO) as an angiotensin agonist, thrombin (1nM; Sigma, St. Louis, MO) as a thrombin receptor agonist, phorbol 12-myristate 13-acetate (PMA, 10μM; Sigma, St. Louis, MO) as a PKC agonist. Fibroblasts were treated with the following inhibitors for the indicated times: persussis toxin (PTX, 500 ng/mL; Sigma, St. Louis, MO) as a Gi specific inhibitor, U73122 (1 μM; Calbiochem, San Diego, CA) as a non-specific phospholipase C (PLC) inhibitor, N-[2-(p-Bromocinnamylamino) ethyl]-5-isoquinolinesulfonamide dihydrochloride; (H89, 10 μM; Sigma, St. Louis, MO) as a protein kinase A (PKA) inhibitor, or the membrane-permeable myristoylated PKC inhibitor fragment 19-27 (PKCi, 100 μM; MP Biomedicals, Irvine, CA).

**Immunoblotting** Antibodies specific to both phosphorylated and total ERK1/2 were purchased from Cell Signaling (Danvers, MA). Fibroblasts were starved for 24 h prior to the addition of 10 μM of the indicated agonist or antagonist for the indicated times. Alternatively, fibroblasts were pretreated with inhibitors as indicated in the figure legends. After stimulation, fibroblasts were chilled, washed, and harvested in lysis buffer (5 mM Tris-HCl pH 7.4, 1 mM EDTA, 1% NP-40, 2.5 mM Na₃P₂O₇, 2.5 mM NaF, 0.5 mM PMSF, 40 μg ml⁻¹ leupeptin and aprotinin). Lysates were clarified and resolved on 10% SDS-PAGE gels for western blot. In order to obtain nuclear extracts, stimulations were stopped on ice with PBS containing Ca²⁺ and Mg²⁺. Cells were scraped in hypotonic buffer (5mM NaCl, 20mM HEPES, 2.5mM PMSF, 2mM EDTA, 40μg ml⁻¹ leupeptin and aprotinin, 10mM NaF, 1mM NaVO₄, 0.4% NP-40) and disrupted by mechanical lysis. Nuclear extracts were pelleted by centrifugation at 12,000g for 1 min and resuspended in hypotonic buffer. Nuclear and cytoplasmic extracts were resolved via SDS PAGE. Signal intensity was quantitated by densitometry of Western blots. Phosphorylated and total ERK were detected via incubation with the corresponding antibodies (1:1000, Cell Signaling, MA) at 1:1000 for 2hr RT. Primary antibodies were visualized with IRDye 680CW goat-anti mouse or with IRDye 800CW goat-anti rabbit secondary antibodies using an Odyssey scanner (1:10,000; LI-COR Biosciences, Lincoln, NE). Phospho-ERK signals were corrected for total ERK levels and plotted as increase over basal levels.

**Immunofluorescence microscopy** Fluorescent images were taken with a Zeiss Axioplan 2 microscope (Carl Zeiss Inc., Thornwood, NY) with an attached CCD camera and Metamorph
software (Molecular Devices, Sunnyvale, CA). Cells were plated on laminin coated coverslips and then serum starved for 24 h. Fibroblasts were then stimulated with 10 μM of the indicated agonists or antagonists for the indicated times. Cells were then fixed with 4% paraformaldehyde for 1 hr at 4°C and then permeabilized with PBST (0.1% Triton X-100 in PBS) for 15 min at RT, rinsed and blocked, and then incubated with 1:400 HA antibody or 1:400 ERKp antibody diluted in PBST 2 hr at RT. AlexaFluor-594-conjugated goat anti-mouse IgG1 antibody or AlexaFluor-488-conjugated goat anti-rabbit IgG1 were utilized as the secondary antibody at 1:1000 diluted in PBST with 10% FBS for 1 hr at RT.

**Co-immunoprecipitation** Wild-type MEFs were transfected with FLAG-tagged mouse βAR together with either GFP-arrestin3 or together with FLAG-tagged α1A-AR, α1B-AR, α1D-AR, or β2AR constructs as described above. MEFs were stimulated with 10 μM of the indicated agonist or antagonist for 10 min before being lysed with lysis buffer. Lysates were cleared by centrifugation and subjected to immunoprecipitation with M2 anti-FLAG affinity resin (25 μL, Sigma, St. Louis, MO). The immunoprecipitates were resolved via SDS-PAGE and blotted with antibodies against ERK (1:500, Cell Signaling, MA), FLAG (1:1,000; Sigma, St. Louis, MO), HA (1:1000, Covance, CA), GFP (1:1000, Rockland Immunocchemicals, PA), or γ tubulin (1:5,000; Sigma, St. Louis, MO). Primary antibodies were visualized with IRDye 680CW goat-anti mouse or with IRDye 800CW goat-anti rabbit secondary antibodies using an Odyssey scanner (LI-COR Biosciences, Lincoln, NE).

**Reverse Transcriptase PCR** RNA was extracted from neonatal cardiac fibroblasts according to protocol supplied with the Aurum total RNA minikit (Biorad, Hercules, CA). cDNA was created and amplified via PCR in a one step method using the protocol supplied with the Access RT-PCR system (Promega, Madison, WI). Briefly, 100ng RNA was amplified using 2mM MgSO4 utilizing the following thermocycler profile: reverse transcription at 45°C for 45 min, deactivation of reverse transcriptase at 94°C for 2 min, 45 cycles of denaturation at 94°C for 1 min, annealing at 57°C for 1 min and extension at 68°C for 1 min. Primers against α1A-AR (forward: 5’-GTAGCCAAGAGAAAGCCG-3’; reverse: 5’-CATCCCCACCACAATGCCCAG-3’) α1B-AR (forward: 5’-GCTCCTTCTACATCCCCACTGG-3’; reverse: 5’-AGGGGAGCCAACATAAGATGA-3’) and α1D-AR (forward: 5’-
CGTATGCTCCTTCTACCTCCC-3’; reverse: 5’-GCACAGGACGAAGACCCAC-3’) were designed based on previous publications⁵,⁶. PCR products were visualized on a 2% DNA gel.

**Cardiac Fibroblast Cell Proliferation ELISA** The cell proliferation ELISA was carried out utilizing BrdU Labeling and Detection Kit III (Roche) according to the manufacturer’s instructions. Briefly, cardiac fibroblasts were cultured in a 96-well plate as described above and left overnight to attach. 10 μM BrdU was added to cells prior to drug stimulation for 8 hours at 37°C. Cells were then fixed and nucleases were allowed to partially digest cellular DNA. Cells were then incubated with an anti-BrdU antibody (200mU/ml) for 30 min at 37°C. Colorimetric analysis was performed with a Spectramax M2 fluorometer reader. The data were normalized against the control.

**GRK Knockdown** GRK2, GRK3 and GRK5 shRNA plasmids were obtained from Sigma. WT MEF cells were co-transfected with a GRK shRNA plasmid and a YFP plasmid for visualization. After overnight transfection, 10 μg/ml of puromycin was added to dishes to select for positive transfection. After 48 hr puromycin selection, cells were serum starved for another 24 h. Cells were then either processed for imaging or harvested as described above for Western blot. Cell lysates were resolved via SDS-PAGE and blotted with antibodies against GRK2, GRK3 or GRK5 (SCBT,CA). Primary antibodies were visualized with IRDye 680CW goat-anti mouse or with IRDye 800CW goat-anti rabbit secondary antibodies using an Odyssey scanner (LI-COR Biosciences, Lincoln, NE).

**Statistical analysis** Data analysis was performed using GraphPad Prism version 4.0 (GraphPad Software, San Diego CA).


Online Supplementary Figures

Supplementary Figure I. Adrenergic receptor antagonists do not alter basal phospho-ERK levels. A, WT cardiac fibroblasts were treated for 5 min with 10 μM of either prazosin (Prz) or timolol (Tim) or yohimbine (Yoh). B, WT cardiac fibroblasts were treated for 5 min with 10 μM of either Tim, Propanolol (Pro), Alprenolol (Alp), Carvedilol (Carv) or PMA for 10 min. Cells were harvested and Phospho-ERK (ERKp) and total ERK (ERK_T) were detected with western blotting and signals were normalized as ERKp/ERK_T. N=3.

Supplementary Figure II. Iso-induced ERK signaling is both cell- and development specific. Neonatal cardiac fibroblasts, HEK 293 cells, Neonatal cardiac myocytes, Adult cardiac myocytes and MEF cells were treated for 5 min with 10 μM of Isoproterenol (Iso) or 10 min with 10 μM PMA for 10 min. Cells were harvested and Phospho-ERK (ERKp) and total ERK (ERK_T) were detected with western blotting.

Supplementary Figure III. Neonatal Cardiac fibroblasts predominantly express α_1A and α_1B but not α_1D α1AR subtypes. RNA was extracted from neonatal cardiac fibroblasts according to protocol supplied with the Aurum total RNA minikit (Biorad, Hercules, CA). cDNA was created and amplified via PCR in a one step method using the protocol supplied with the Access RT-PCR system (Promega, Madison, WI). Both α_1A-AR and α_1B-AR, but not α_1D-AR, were detected in neonatal cardiac fibroblasts.

Supplementary Figure IV. ERK activation does not occur via classical Gs-PKA signaling. A, WT cardiac fibroblasts were pretreated, if indicated, with 200 ng of pertussis (PTX) O/N before stimulation with either 10 μM epinephrine or phenylephrine (Phe) for 5 min. B, WT cardiac fibroblasts were pretreated, if indicated, with 10 μM H89 for 30 min before stimulation with either 10 μM epinephrine or phenylephrine (Phe) for 5 min. Cells were harvested and Phospho-ERK (ERKp) and total ERK (ERK_T) were detected with western blotting and signals were normalized as ERKp/ERK_T. N=3.

Supplementary Figure V. Co-activation of βARs prevents nuclear accumulation of ERK signal induced by α_1ARs. A, WT cardiac fibroblasts were stimulated with Phe (10 μM) in the presence or absence of Iso (10 μM). Phospho-ERK (ERKp) was detected by
immunofluorescence imaging. B, WT fibroblasts were stimulated with Phe (10 μM) in the presence or absence of Iso (10 μM) for 5 mins. Phospho-ERK (ERKp) and total ERK (ERK_T) were detected with Western blotting and signals were normalized as ERKp/ERK_T N=3; ***, p<0.001.

Supplementary Figure VI. α1ARs do not form heterodimers with β2AR. FLAG-tagged α1-A-AR, α1-B-AR or α1-D-AR constructs were transfected into MEF cells along with HA-β2AR. After 48 hr expression, cells were lysed for immunoprecipitation with an anti-flag antibody to examine the association between β2AR and α1AR subtypes and ERK. HA and FLAG were detected with Western blotting.

Supplementary Figure VII. Effective GRK knockdown in MEF cells. WT MEF cells were transfected with shRNA constructs target at GRK2, GRK3 or GRK5. After expression and selection, cells were lysed and harvested and lysates were subjected to SDS-PAGE. Individual GRK isoforms were visualized with Western Blotting.

Supplementary Figure VIII. Src is involved in βAR cross-talk with the α1AR in cardiac fibroblasts. A and B, WT fibroblasts expressing a dominant negative Src (HA-DN-Src) were stimulated with 10 μM Epi or Phe for the indicated times. Phospho-ERK (ERKp) and total ERK (ERK_T) were detected via Western blotting and signal was quantitated as ERKp/ERK_T. C, WT fibroblasts expressing a dominant negative Src (HA-DN-Src) were stimulated with Epi for the indicated times. Cells were lysed and separated into nuclear and cytosolic fractions. Phospho-ERK (ERKp) and total ERK (ERK_T) in each fraction were detected via Western blotting and signal was quantitated as ERKp/ERK_T N= 4. *, p<0.05; ***, p<0.001.

Supplementary Figure IX. β2AR, Arrestin 3 and phosphor-ERK colocalize intracellularly upon Epi stimulation. DKO MEF cells were cotransfected with FLAG-β2AR and GFP-Arr3 constructs. Cells were then stimulated for 10 min with Epi (10 μM), then fixed and stained with both FLAG or anti-ERKp antibodies for fluorescence imaging.
Supplementary Figure I

A

B

ERK\(^{p}\)   ERK\(^{t}\)

ERK\(^{p}\)   ERK\(^{t}\)

Timolol +  +  +  +
Prazosin +  +  +  +
Yohimbine +  +  +  +
PMA +  +  +  +

ERK\(^{p}\)/ERK\(^{t}\)

0  1  2  3  4

Timolol +  +  +  +
Propanolol +  +  +  +
Alprenolol +  +  +  +
Carvedilol +  +  +  +
PMA +  +  +  +

***
Supplementary Figure III

- Void   Fibroblast   Plasmid
  α1A

- Void   Fibroblast   Plasmid
  α1B

- Void   Fibroblast   Plasmid
  α1D
Supplementary Figure V

A

Control
Phe
ISO

B

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ERK\(\beta\) / ERK\(\beta\)

***

0' 5' 20' 0' 5' 20'

Time
Supplementary Figure VI

**IP:** FLAG-α1AR

**IB:** α1A ρ1B ρ1D

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**Int.**
Supplementary Figure VII