β-Arrestin1 is an adapter/scaffold for many G protein-coupled receptors during mitogen-activated protein kinase signaling. Phosphorylation of β-arrestin1 at position Ser-412 is a regulator of β-arrestin1 function, and in the present study, we showed that insulin led to a time- and dose-dependent increase in β-arrestin1 Ser-412 phosphorylation, which blocked isoproterenol- and lysophosphatidic acid-induced Ser-412 dephosphorylation and impaired ERK signaling by these G protein-coupled receptor ligands. Insulin treatment also led to accumulation of Ser-412-phosphorylated β-arrestin1 at the insulin-like growth factor 1 receptor and prevented insulin-like growth factor 1/Src association. Insulin-induced Ser-412 phosphorylation was partially dependent on ERK as treatment with the MEK inhibitor PD98059 inhibited the insulin effect (62% reduction, p = 0.03). Inhibition of phosphatidylinositol 3-kinase by wortmannin did not have a significant effect (9% reduction, p = 0.41). We also found that the protein phosphatase 2A (PP2A) was in a molecular complex with β-arrestin1 and that the PP2A inhibitor okadaic acid increased Ser-412 phosphorylation. Concomitant addition of insulin and okadaic acid did not produce an additive effect on Ser-412 phosphorylation, suggesting a common mechanism. Small t antigen specifically inhibited PP2A, and in HIRcB cells expressing small t antigen, β-arrestin1 Ser-412 phosphorylation was increased, and insulin had no further effect. Insulin treatment caused increased β-arrestin1 Ser-412 phosphorylation, which blocked mitogen-activated protein kinase signaling and internalization by β-arrestin1-dependent receptors with no effect on β-adrenergic receptor Gαi-mediated cAMP production. These findings provide a new mechanism for insulin-induced desensitization of ERK activation by Gαi-coupled receptors.

Many hormone signaling systems are mediated by G protein-coupled receptors (GPCRs), and several have been found to utilize β-arrestin as a key regulatory protein (1–8). In addition, the insulin-like growth factor-1 (IGF-1) receptor, a receptor tyrosine kinase, has recently been found to activate MAP kinase signaling in a Gαq- and β-arrestin1-dependent manner (9). Thus, changes in β-arrestin function can have widespread implications for hormone signaling. We have recently found that insulin treatment is associated with down-regulation of β-arrestin1 protein (10) as well as phosphorylation of β-arrestin1 Ser-412 (11), representing possible new mechanisms of insulin-induced impairment of β-arrestin1-dependent GPCR signaling.

Activation of adenylate cyclase and generation of cAMP following ligand binding to some GPCRs coupled to Gαi is attenuated once β-arrestin1 binds to the activated GPCR and sterically uncouples the receptor from further interaction with Gαi (5). Other GPCRs, however, utilize β-arrestin as a scaffold for assembly of a multiprotein signaling complex that leads to activation of ERK1/2 (12–14). In addition, β-arrestin interacts with clathrin, targeting activated receptors to clathrin-coated pits for endocytosis (6, 15, 16). Therefore, β-arrestin performs a dual role during intracellular signaling by GPCRs: desensitizing signals leading to adenylate cyclase activation while coordinating and enhancing signals leading to ERK1/2 activation.

As one would predict from the known functions of β-arrestin discussed above, a reduction of β-arrestin protein content using antisense oligonucleotide (17), knock-out gene technology (18), small interfering RNA (19) or by insulin treatment (11) is associated with both impaired desensitization of Gαi signaling (Gαi signal “supersensitization”) and impaired ERK activation.

β-Arrestin1 function during G protein-mediated ERK signaling is also regulated by phosphorylation of the carboxyl terminal Ser-412 residue. Dephosphorylation of Ser-412 is required for the isoproterenol-stimulated association between β-arrestin1 and Src, a non-receptor tyrosine kinase involved in β1- and β2-adrenergic receptor (AR) mitogenic signaling (12). In addition, Ser-412 dephosphorylation is also required for β-arrestin1/clathrin association, which is essential for β2AR internalization (16). Mutants of β-arrestin1 that have a serine to aspartic acid substitution (S412D) and thus mimic the phosphorylated state at the 412 residue have reduced affinity for Src and for clathrin and function as dominant negative inhibitors of G protein-mediated MAP kinase signaling (16). The mechanism behind the regulation of β-arrestin1 Ser-412 phosphorylation/dephosphorylation has not been described. We have recently shown that insulin treatment leads to β-arrestin1 degradation (10) as well as increased β-arrestin1 Ser-412

**This work was supported by National Institutes of Health Grant K08 DK65127-01 (to C. J. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1744 solely to indicate this fact.

**To whom correspondence should be addressed: Dept. of Medicine, Division of Endocrinology and Metabolism, University of California, San Diego, La Jolla, California 92037, the Department of Reproductive Medicine, University of California, San Diego, La Jolla, California 92037, the Department of Medicine, Division of Endocrinology and Metabolism and the Department of Reproductive Medicine, University of California, San Diego, La Jolla, California 92037, the Department of Medicine, Division of Endocrinology and Metabolism, University of California, San Diego, La Jolla, California 92037, and The Whittier Diabetes Institute, La Jolla, California 92037.

†‡§¶**

Christopher J. Hupfeld‡, Jamie L. Resnik§, Satoshi Ugi‡, and Jerrold M. Olefsky‡¶**

From the ‡Department of Medicine, Division of Endocrinology and Metabolism and the §§Department of Reproductive Medicine, University of California, San Diego, La Jolla, California 92037, the ¶Veterans Affairs Hospital, Research Service, San Diego, California 92161, and ¶¶The Whittier Diabetes Institute, La Jolla, California 92037.

Received for publication, April 2, 2004, and in revised form, October 12, 2004

Published, JBC Papers in Press, November 1, 2004, DOI 10.1074/jbc.M403674200

This paper is available online at http://www.jbc.org

This paper is available online at http://www.jbc.org

β-Arrestin1 Ser-412 Phosphorylation Is a Mechanism for Desensitization of ERK Activation by Gαi-coupled Receptors*
phosphorylation (11). In these studies, insulin-induced changes in β-arrestin1 were associated with increased βAR/Gαi signal- ing (due to loss of Gαi signal desensitization) and with im- paired Gαi-mediated MAP kinase activation (due to defective β-arrestin/Src and β-arrestin/clathrin interaction). However, the relative importance of insulin-induced protein degradation versus insulin-induced Ser-412 phosphorylation in these pro- cesses remains unclear.

In addition, the mechanism of regulation of Ser-412 phos- phorylation is unknown. Previous studies have described a role for ERK during Ser-412 phosphorylation (16, 20), while others have found the protein phosphatase 2A (PP2A) and PP2B in complexes that contain β-arrestin (21). In this regard, we have recently found that insulin-induced reduction of PP2A activity is involved in the desensitization of ERK signaling (22), but the role of PP2A in regulating β-arrestin1 Ser-412 phosphorylation has not been described.

In the current study, we present a new mechanism of insulin- induced desensitization of GPCR signaling. Thus, insulin- inducades β-arrestin1 Ser-412 phosphorylation, and this disrupts many of the known β-arrestin1 functions in GPCR signaling. In addition, we demonstrate a novel role for PP2A in this process.

**EXPERIMENTAL PROCEDURES**

**Materials**—IGF-1, lysophosphatidic acid (LPA), PD98059, wortman- nin, and okadaic acid were purchased from Calbiochem. CGP-12177 was purchased from Pierce. Anti-β-arrestin1 was from Transduction Laboratories. Anti-phospho-β-arrestin1 (Ser-412) and anti-phospho-Akt (Ser-473) were from Cell Signaling Technology. Anti-phospho-ERK1/2, anti-PP2, and anti-Src were from Santa Cruz Biotechnology. Insulin, isoproterenol, BRL 37344, and all other chemicals were from Sigma.

**Cell Culture**—Confluent 3T3-L1 preadipocytes were differentiated and maintained at 37 °C with 10% CO2 in Dulbecco’s modified Eagle’s medium supplemented with 4.5g/liter glucose, 10% fetal calf serum, 0.1% Glutamax, and penicillin/streptomycin. On day 1, medium was supplemented with 0.1% Glutamax, and penicillin/streptomycin. On day 4, the differentiation mixture was replaced with regular medium. On day 7, cells were re- seeded, and experiments were performed on days 10–14 postdifferentiation. Cells were placed in serum-free medium (Dulbecco’s modified Eagle’s medium with 1.0 g/liter glucose, 0.5% bovine serum albumin, 0.1% Glutamax, and penicillin/streptomycin) for 16 h prior to ligand stimulation unless otherwise indicated.

**Immunoprecipitation and Western Blotting**—Cells were lysed, and protein concentrations were determined by the Bradford assay as described previously. For Western blotting, 25 μg of cellular protein were dissolved in Laemmli buffer supplemented with dithiothreitol, and boiled for 5 min. For immunoprecipitations, 500 μg of total protein were diluted to 1 μg/μl in PBS to which 3 μg of the appropriate antibody were added overnight at 4 °C. Immune complexes were captured by adding 40 μl of a 50% protein A/G bead mixture for an additional 1 h. Beads were then washed three times with PBS, dissolved in Laemmli buffer plus dithiothreitol, and boiled for 5 min. Proteins were separated by 7.5–10% SDS-PAGE and then transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline/Tween 20 and probed with the appropriate primary and secondary antibodies. Horseradish peroxidase-conjugated secondary antibody binding was detected by chemiluminescence.

**Generation of Small t Antigen-expressing Cell Lines**—We have previously described the generation of stable cell lines expressing small t antigen in our laboratory (22). Rat-1 fibroblasts overexpressing human insulin receptors (HIRβC) cells were maintained as described previ- ously (23). A plasmid encoding small t antigen, pCMV5-small t (24), was a gift from Marc C. Mummy (University of Texas Southwestern Medical Center, Dallas, TX). cDNA from pCMV5-small t was digested with HindIII and BamHI and subcloned into pCDNA3/Hygro (Invitrogen), which contains the hygromycin B phosphotransferase gene. The result- ant plasmid, pCDNA3.1/Hygro-small t, was transfected into HIRβC cells by using FuGENE according to the manufacturer’s instructions. Stable cell lines were selected in 400 μg of hygromycin B/ml. Clonal cell lines were isolated by limiting dilution and then screened by immunoblotting with anti-small t antibody. Parental cells transfected with pCDNA3.1/ Hygro alone (Hyg cells) were used as a control.

**Insulin Treatment Leads to a Time- and Dose-dependent Increase of β-Arrestin1 Ser-412 Phosphorylation**—We have previously shown that insulin treatment (100 ng/ml) for 8–12 h leads to proteosomally mediated degradation of β-arrestin1, reducing cellular β-arrestin1 function (10). Phosphorylation of Ser-412 can also impair β-arrestin1 activity, providing an alter- native mechanism to functionally down-regulate this protein. As shown in Fig. 1, insulin treatment of 3T3-L1 adipocytes led to a time- and dose-dependent increase in Ser-412 phosphorylation (Fig. 1, C and D) increase in Ser-412 phosphorylation of β-arrestin1. As indicated in Fig. 1B, the 6-h insulin pretreatment did not cause substantial β-arrestin1 protein degradation (reduced ~10–15%). In contrast, treatment with isoproterenol and LPA, whose cognate GPCRs require β-arrestin1 Ser-412 dephosphorylation for ERK signaling (10), led to Ser-412 dephosphorylation (Fig. 1E). EGF receptor signaling is independent of β-arrestin, and EGF stimulation did not have a significant effect on Ser-412 phosphorylation (Fig. 1F).

**Insulin Treatment Inhibits GPCR Ligand-mediated Ser-412 Dephosphorylation**—Because increased β-arrestin1 Ser-412 phosphorylation could lead to desensitization of Gαi-coupled GPCR activation of MAP kinase, we determined whether insulin treatment prevented Ser-412 dephosphorylation induced by treatment with isoproterenol or LPA. Insulin pretreatment for 6 h prevented isoproterenol- and LPA-induced dephosphory- alation of Ser-412 (Fig. 2, A and B) and also inhibited isoproter- enol- and LPA-mediated ERK phosphorylation (Fig. 2C). Insu- lin treatment itself can impair ERK signaling due to Raf-1 hyperphosphorylation and uncoupling of Grb2 and SOS (26–28), an effect that is fully resolved within 4–6 h. Consistent with this, the insulin pretreatment protocol did not cause general- ized desensitization of ERK signaling as EGF-mediated ERK activation proceeded normally (Fig. 2D).

Isoproterenol is a nonspecific activator of all βAR subtypes (β1, β2, and β3). The β1- and β2ARs utilize β-arrestin during ERK activation, while the β3AR activates ERK by a β-arrestin-independent but Src-dependent mechanism (29). As insulin treatment causes a partial rather than complete inhibition of isoproterenol-mediated ERK activation, we hypothesized that β3AR-mediated ERK signaling, which is β-arrestin-independent, is unaffected by insulin treatment. To test this, we measured ERK phosphorylation following treatment with the β3AR-specific ligand BRL 37344 in the presence or absence of insulin.
ERK signaling is pertussis toxin-sensitive (dependent on Gαt1 recruitment to the IGF-1R and that IGF-1R-mediated ERK signaling is Src-dependent (30), and Phosphorylated β-arrestin1, Prevents IGF-1R Association with Src, and Blocks IGF-1-mediated ERK Activation—β-arrestin1 is a scaffold for several signaling proteins involved in G protein-mediated ERK activation. Best characterized are the interactions between β-arrestin1 and Src, a non-receptor tyrosine kinase, and clathrin, a protein involved in receptor internalization. Interaction between β-arrestin1 and these proteins requires dephosphorylation at the β-arrestin1 Ser-412 site. We have previously shown that IGF-1 treatment leads to β-arrestin1 recruitment to the IGF-1R and that IGF-1-mediated ERK signaling is pertussis toxin-sensitive (dependent on Gαi) (9) and β-arrestin1-dependent (10). Others have shown that IGF-1R-mediated ERK signaling is Src-dependent (30), and internalization of the IGF-1R is β-arrestin- and clathrin-dependent (15).

Our preliminary studies showed that, unlike isoproterenol and LPA, IGF-1 treatment did not lead to detectable Ser-412 dephosphorylation in total cell lysates (not shown). We next determined whether the β-arrestin1 recruited to the IGF-1R following IGF-1 treatment is Ser-412-phosphorylated. We measured the amount of Ser-412-phosphorylated β-arrestin1 in IGF-1R immunoprecipitates and did not detect significant amounts either before or after IGF-1 treatment (Fig. 3A). In contrast, insulin pretreatment greatly increased the amount of Ser-412-phosphorylated β-arrestin1 associated with the IGF-1R following IGF-1 treatment. Strikingly the same insulin treatment protocol blocked IGF-1R/Src association (Fig. 3B) and reduced IGF-1-mediated ERK phosphorylation (Fig. 3C). Thus, IGF-1 normally recruits dephosphorylated β-arrestin1 to the receptor, which is fully competent to associate with Src and relay signals to ERK. In contrast, after insulin pretreatment, IGF-1 recruits Ser-412-phosphorylated β-arrestin1 to the receptor, which cannot couple to Src, and displays impaired downstream signaling.

Insulin-induced Ser-412 Phosphorylation Is Partially Dependent on ERK Signaling—The Ser-412 site is an ERK consensus sequence, and therefore we questioned whether insulin-induced Ser-412 phosphorylation proceeds by an ERK-dependent mechanism in our system. To assess this, we used the MEK inhibitor PD98059 and found that it substantially reduced insulin-induced β-arrestin1 Ser-412 phosphorylation, while the phosphatidylinositol 3-kinase inhibitor wortmannin had no effect (Fig. 4A). We quantified eight independent experiments using PD98059 and found it led to a 62% reduction in insulin-stimulated Ser-412 phosphorylation (Fig. 4B, p = 0.03). Quantification of a similar number of experiments using wortmannin failed to detect a significant effect (9% reduction, p = 0.41, data not shown). Blots showing the effectiveness of PD98059 (preventing MEK-dependent ERK phosphorylation) and wortmannin (preventing phosphatidylinositol 3-kinase-dependent Akt phosphorylation) are shown in Fig. 4, C and D.
Insulin-induced β-Arrestin1 Ser-412 Phosphorylation

Insulin-induced Ser-412 Phosphorylation Is Lost in Small t Antigen-expressing Cells—To further test the hypothesis that insulin-induced inactivation of PP2A leads to increased Ser-412 phosphorylation, we used an HIRcB cell line stably expressing small t antigen (ST) derived from SV40 (22). The PP2A heterotrimer includes a scaffolding subunit (A), a catalytic subunit (C), and one of several possible regulatory subunits (B), each of which localize PP2A to specific subcellular compartments and direct interaction with specific protein targets (34). In cells infected with SV40, B subunits are displaced from their natural binding to A subunits by ST, resulting in inhibition of PP2A phosphatase activity (35). PP2A is the only protein known to interact with ST, and when expressed in cells, ST inhibits PP2A, but not PP1, activity (22). As shown in Fig. 6, A and B, basal Ser-412 phosphorylation was increased in ST-expressing cells, whereas insulin-induced Ser-412 phosphorylation was prevented.

Insulin-induced β-Arrestin1 Ser-12 Phosphorylation Impairs β-Adrenergic Receptor Internalization—Down-regulation of β-arrestin1 protein levels leads to inhibition of G protein-mediated ERK activation. However, β-arrestin1 Ser-412 phosphorylation itself could be an independent mechanism for inhibiting G protein-mediated ERK activation. Therefore, as an alternative approach, we took advantage of the fact that insulin causes β-arrestin1 degradation in a dose-responsive manner, and low doses of insulin do not cause proteasomal degradation of β-arrestin1, although they are still capable of increasing Ser-412 phosphorylation. As shown in Fig. 7, A and B, treatment with insulin (1 ng/ml) for 6 h had no effect on β-arrestin1 protein levels but led to increased Ser-412 phosphorylation. As shown in Fig. 7C, we found that insulin (1 ng/ml) pretreatment had no effect on isoproterenol-mediated cAMP generation, a Goα-coupled, β-arrestin1-desensitized signaling event that is unaffected by Ser-412 phosphorylation (16). Since β-arrestin1 Ser-412 phosphorylation impairs the association of β-arrestin1 to Src and also disrupts clathrin-mediated internalization of the β2AR, we reasoned that insulin treatment would impair endocytosis of βARs. We measured isoproterenol-induced βAR internalization following insulin treatment using the CGP-12177 assay (25). CGP-12177 is a βAR agonist that is hydrophilic and not internalized once bound to the receptor, providing a quantitative measure of surface βAR before and after ligand stimulation. Pretreatment with insulin (1 ng/ml) to cause β-arrestin1 Ser-412 phosphorylation with no change in β-arrestin1 protein levels inhibited isoproterenol-mediated βAR internalization (75%, Fig. 7D).

Insulin-induced Ser-412 Phosphorylation Leads to Desensitization of β-Arrestin-dependent, G Protein-mediated MAP Kinase Signaling—We next hypothesized that Ser-412-phosphorylated β-arrestin1 would be inefficient at directing ERK phosphorylation. Again using the lower dose of insulin, we found that insulin (1 ng/ml) pretreatment blocked ERK phosphorylation by three independent Goα-coupled ligands (Fig. 8, A and B). In contrast, insulin treatment had no effect on EGF-mediated ERK phosphorylation, which is not β-arrestin1/Goα-coupled but does utilize many of the same MAP kinase signaling intermediates, such as Grb2/SOS, Ras, Raf-1, and MEK. Finally, as a positive control, we measured LPA-mediated ERK phosphorylation in the Hyg and ST cell lines. In ST cells where Ser-412 phosphorylation was increased due to PP2A inactivity, LPA-mediated ERK phosphorylation was impaired (reduced 48% at 2.5 min, p = 0.09; reduced 29% at 5 min, p = 0.31).

PP2A Associates with β-Arrestin1, and Treatment with the PP2A Inhibitor Okadaic Acid Leads to Increased Ser-412 Phosphorylation—The multiprotein complex that forms at the activated β2AR includes not only β-arrestin and other ERK signaling cascade intermediates but also the phosphatases PP2A and PP2B (21). In addition, a recent study has shown that isoproterenol treatment acutely activates PP2A (31). Since two groups, including ours, have previously shown that insulin treatment leads to decreased PP2A phosphatase activity (22, 32), we speculated that inhibition of PP2A could contribute to increased β-arrestin1 Ser-412 phosphorylation.

First, we found that β-arrestin1 and PP2A were associated in the basal state and that isoproterenol treatment led to an increase in this association in 3T3-L1 adipocytes (Fig. 5A), suggesting that PP2A is involved in isoproterenol-mediated Ser-412 dephosphorylation. If PP2A can decrease Ser-412 phosphorylation, inhibition of PP2A activity should lead to an increase in Ser-412 phosphorylation. Low (1 μM) concentrations of okadaic acid have been shown to specifically inhibit PP2A (33), and when cells were incubated with 1 μM okadaic acid, Ser-412 phosphorylation was clearly increased (Fig. 5B). When insulin and okadaic acid were added together, insulin did not significantly increase Ser-412 phosphorylation above the level induced by okadaic acid alone (Fig. 5, C and E), suggesting that insulin and okadaic acid act through a common mechanism or that okadaic acid treatment alone leads to stoichiometric phosphorylation at Ser-412, which cannot be enhanced further. In addition, treatment of 3T3-L1 adipocytes with okadaic acid prevented isoproterenol-mediated dephosphorylation of Ser-412 (Fig. 5, D and E), suggesting that isoproterenol treatment leads to increased Ser-412 dephosphorylation.

Insulin-induced Ser-412 Phosphorylation Is Activated Phorylation—PP2A Inhibitor Okadaic Acid Leads to Increased Ser-412 Phosphorylation. Inhibition of PP2A activity should lead to an increase in Ser-412 dephosphorylation. If PP2A can decrease Ser-412 phosphorylation, inhibition of PP2A activity should lead to an increase in Ser-412 phosphorylation. Low (1 μM) concentrations of okadaic acid have been shown to specifically inhibit PP2A (33), and when cells were incubated with 1 μM okadaic acid, Ser-412 phosphorylation was clearly increased (Fig. 5B). When insulin and okadaic acid were added together, insulin did not significantly increase Ser-412 phosphorylation above the level induced by okadaic acid alone (Fig. 5, C and E), suggesting that insulin and okadaic acid act through a common mechanism or that okadaic acid treatment alone leads to stoichiometric phosphorylation at Ser-412, which cannot be enhanced further. In addition, treatment of 3T3-L1 adipocytes with okadaic acid prevented isoproterenol-mediated dephosphorylation of Ser-412 (Fig. 5, D and E), suggesting that isoproterenol treatment requires activation of PP2A to induce Ser-412 dephosphorylation.
In the present study, we found that insulin led to \( \beta \)-arrestin1 Ser-412 phosphorylation and impaired \( \beta \)-arrestin1 function. We demonstrated the roles of a kinase (ERK) and a phosphatase (PP2A) in mediating this effect of insulin on the Ser-412 site. We showed that, by increasing \( \beta \)-arrestin1 Ser-412 phosphorylation, insulin treatment led to desensitization of \( \beta \)-arrestin1-dependent, G protein-mediated ERK signaling. This molecular mechanism was demonstrated using the IGF-1R, which is G\( \alpha \)-coupled and associates with \( \beta \)-arrestin1 and Src following IGF-1 stimulation. When IGF-1 stimulation was preceded by insulin pretreatment, the \( \beta \)-arrestin1 associated with the IGF-1R was clearly phosphorylated at Ser-412. Perhaps more importantly, insulin pretreatment led to impaired...

**DISCUSSION**

In the present study, we found that insulin led to \( \beta \)-arrestin1 Ser-412 phosphorylation and impaired \( \beta \)-arrestin1 function. We demonstrated the roles of a kinase (ERK) and a phosphatase (PP2A) in mediating this effect of insulin on the Ser-412 site. We showed that, by increasing \( \beta \)-arrestin1 Ser-412 phosphorylation, insulin treatment led to desensitization of \( \beta \)-arrestin1-dependent, G protein-mediated ERK signaling.

This molecular mechanism was demonstrated using the IGF-1R, which is G\( \alpha \)-coupled and associates with \( \beta \)-arrestin1 and Src following IGF-1 stimulation. When IGF-1 stimulation was preceded by insulin pretreatment, the \( \beta \)-arrestin1 associated with the IGF-1R was clearly phosphorylated at Ser-412. Perhaps more importantly, insulin pretreatment led to impaired...
IGF-1-mediated recruitment of Src to the IGF-1R and inhibition of ERK activation by IGF-1. This insulin-induced increase in β-arrestin1 Ser-412 phosphorylation represents a novel mechanism for insulin-induced desensitization of G protein-mediated ERK activation.

The importance of β-arrestin1 Ser-412 dephosphorylation during initiation of ERK signaling from the activated β-adrenergic receptor has been described previously (16). We definitively demonstrated ligand-mediated Ser-412 dephosphorylation in 3T3-L1 adipocytes using two different β-arrestin-dependent GPCR ligands, isoproterenol and LPA. In contrast
to treatment with GPCR ligands, insulin treatment led to an increase in phosphorylation at Ser-412 and in fact prevented Ser-412 dephosphorylation induced by isoproterenol and LPA. This was associated with desensitization of ERK signaling by both isoproterenol and LPA. Interestingly while stimulation of the IGF-1R, which is Go-coupled and β-arrestin1-dependent for MAP kinase signaling, did not lead to detectable reductions in total cellular Ser-412-phosphorylated β-arrestin1, receptor-bound β-arrestin1 was clearly dephosphorylated, suggesting that the IGF-1R interacts preferentially with Ser-412-dephosphorylated β-arrestin1. Insulin treatment greatly increased the amount of Ser-412-phosphorylated β-arrestin1 at the IGF-1R and blocked the association of the IGF-1R and Src. Therefore, ligand-mediated dephosphorylation of Ser-412 plays a critical role in G protein-mediated ERK signaling, and this was prevented by insulin treatment.

β-Arrestin1 has an ERK consensus sequence around Ser-412, and a previous study (20) using in vitro phosphorylation and two-dimensional tryptic phosphopeptide mapping identified ERK as the kinase responsible for phosphorylating Ser-412. We found that, using the MEK inhibitor PD98059, insulin-induced Ser-412 phosphorylation in 3T3-L1 adipocytes was reduced by 62%. We conclude that ERK, or another MEK-dependent kinase, was responsible for phosphorylating the Ser-412 site in response to insulin treatment. Interestingly other ERK-activating ligands (isoproterenol, LPA, IGF-1, and EGF) failed to increase Ser-412 phosphorylation, suggesting that non-ERK mechanism also plays a partial role in determining Ser-412 phosphorylation status.

Our findings suggest that changes in phosphatase activity, specifically PP2A, can have a profound effect on Ser-412 phosphorylation and may partially explain the effects of insulin. PP2A is activated upon isoproterenol stimulation (31), and we found that PP2A co-immunoprecipitated with β-arrestin1 in 3T3-L1 adipocytes both at base line and in response to isoproterenol treatment. This is consistent with a previous study in which both PP2A and PP2B were recovered from β-arrestin-containing β2AR immunoprecipitates (21), suggesting a role for PP2A in isoproterenol-mediated Ser-412 dephosphorylation. Consistent with this, we found that inhibition of PP2A activity by okadaic acid treatment enhanced Ser-412 phosphorylation and prevented isoproterenol-mediated dephosphorylation of this residue.

We have previously shown that insulin treatment inhibits PP2A activity (22, 32). In the current study, insulin treatment did not lead to further Ser-412 phosphorylation in cells where PP2A was already inactivated by another method. When cells were treated with insulin and okadaic acid together, the effect on Ser-412 phosphorylation was not additive. In addition, we found that insulin treatment had no effect on Ser-412 phosphorylation in HIRcB cells that express the ST, an SV40-derived protein that specifically inhibits PP2A. Interestingly basal phosphorylation of Ser-412 was increased both by okadaic acid treatment and in ST-expressing cells in the absence of ligand stimulation. This suggests that the basal activity of a serine kinase targeting Ser-412 is held in check by PP2A activity and that a balance between the activity of this kinase and PP2A normally regulates Ser-412 phosphorylation levels. It is also possible to conclude from these experiments that okadaic acid treatment and ST expression lead to stoichiometric phosphorylation at Ser-412, and therefore insulin is unable to further increase Ser-412 phosphorylation irrespective of the mechanism involved. However, the results presented here provide a plausible mechanism to explain why ERK activation alone does not fully account for the increase in Ser-412 phosphorylation with insulin treatment.
1. DeFea, K. A., Zalevsky, J., Thoma, M. S., Dery, O., Mullins, R. D., and Bunnett, N. W. (2000) J. Cell Biol. 148, 1267–1282
2. Luttrell, L. M., Roudabush, F. L., Choy, E. W., Miller, W. E., Field, M. E., Pierce, K. L., and Lefkowitz, R. J. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 2449–2454
3. Lin, F. T., Daaka, Y., and Lefkowitz, R. J. (1998) J. Biol. Chem. 273, 31640–31643
4. Lin, F. T., Kreuger, K. M., Kendall, H. E., Daaka, Y., Fredericks, Z. L., Pitcher, J. A., and Lefkowitz, R. J. (1997) J. Biol. Chem. 272, 31051–31057
5. Mundell, S. J., Loudon, R. P., and Benovic, J. L. (1999) Biochemistry 38, 8723–8732
6. Kohout, T. A., Lin, F. T., Perry, S. J., Conner, D. A., and Lefkowitz, R. J. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 1601–1606
7. Ahn, S., Nelson, C. D., Garrison, T. R., Miller, W. E., and Lefkowitz, R. J. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 1740–1744
8. Lin, F. T., Miller, W. M., Luttrell, L. M., and Lefkowitz, R. J. (1999) J. Biol. Chem. 274, 15871–15974
9. Shih, M., Lin, F. T., Scott, J. D., Wang, H., and Malbon, C. C. (1999) J. Biol. Chem. 274, 1588–1595
10. Ugi, S., Imamura, T., Ricketts, W., and Olefsky, J. M. (2002) Mol. Cell. Biol. 22, 2375–2387
11. Ricketts, W. A., Rose, D. W., Shoelson, S., and Olefsky, J. M. (1996) J. Biol. Chem. 271, 26165–26169
12. Sontag, E., Sontag, J. M., and Garcia, A. (1997) EMBO J. 16, 5662–5671
13. Staehelin, M., and Simons, P. (1982) EMBO J. 1, 187–190
14. Klarlund, J. K., Cherniack, A. D., and Czech, M. P. (1995) J. Biol. Chem. 270, 23421–23428
15. Fucini, R. V., Okada, S., and Pessin, J. E. (1999) J. Biol. Chem. 274, 18651–18658
16. Langlois, W. J., Sasaoka, T., Saito, A. R., and Olefsky, J. M. (1995) J. Biol. Chem. 270, 25320–25323
17. Cao, W., Luttrell, L. M., Medvedev, A. V., Pierce, K. L., Daniel, K. W., Dixon, T. M., Lefkowitz, R. J., and Collins, S. (2000) J. Biol. Chem. 275, 38131–38134
18. Boney, C. M., Sekimoto, H., Gruppuso, P. A., and Frackelton, A. R., Jr. (2001) Cell Growth Differ. 12, 379–386
19. Pullar, C. E., Chen, J., and Isseroff, R. R. (2003) J. Biol. Chem. 278, 22555–22562
20. Bejum, N., and Ragnolia, L. (1996) J. Biol. Chem. 271, 31166–31177
21. Favre, B., Turowski, P., and Hemmings, B. A. (1997) J. Biol. Chem. 272, 13856–13863
22. Kambayashi, C., Estes, R., Lickteig, R. L., Yang, S. I., Craft, C., and Mumbay, M. C. (1994) J. Biol. Chem. 269, 20139–20148
23. Mateer, S. C., Fedorov, S. A., and Mumbay, M. C. (1998) J. Biol. Chem. 273, 35339–35346
24. Cammisotto, P. G., and Bukowiecki, L. J. (2002) Am. J. Physiol. 283, C244–C250
25. Greenberg, A. S., Shen, W.-J., Muliro, K., Patel, S., Souza, S. C., Roth, R. A., and Kraemer, F. B. (2001) J. Biol. Chem. 276, 45456–45461
26. Lindquist, J. M., and Rehman, S. (1998) J. Biol. Chem. 273, 30147–30156