β2-Adrenergic Receptor Activates Extracellular Signal-regulated Kinases (ERKs) via the Small G Protein Rap1 and the Serine/Threonine Kinase B-Raf*

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G protein-coupled receptors can induce cellular proliferation by stimulating the mitogen-activated protein (MAP) kinase cascade. Heterotrimeric G proteins are composed of both α and βγ subunits that can signal independently to diverse intracellular signaling pathways including those that activate MAP kinases. In this study, we examined the ability of isoproterenol, an agonist of the β₂-adrenergic receptor (β₂AR), to stimulate extracellular signal-regulated kinases (ERKs). Using HEK293 cells, which express endogenous β₂AR, we show that isoproterenol stimulates ERKs via β₂AR. This action of isoproterenol requires cAMP-dependent protein kinase and is insensitive to pertussis toxin, suggesting that Ga₃ activation of cAMP-dependent protein kinase is required. Interestingly, β₂AR activates both the small G proteins Rap1 and Ras, but only Rap1 is capable of coupling to Raf isoforms. β₂AR inhibits the Ras-dependent activation of both Raf isoforms Raf-1 and B-Raf, whereas Rap1 activation by isoproterenol recruits and activates B-Raf. β₂AR activation of ERKs is not blocked by expression of RasN17, an interfering mutant of Ras, but is blocked by expression of either RapN17 or Rap1GAP1, both of which interfere with Rap1 signaling. We propose that isoproterenol can activate ERKs via Rap1 and B-Raf in these cells.

Cell proliferation is regulated by extracellular signals including growth factors and hormones. Growth factors activate receptor tyrosine kinases to stimulate a number of intracellular signaling cascades. One cascade, the MAP kinase (or ERK) cascade triggers cellular proliferation through multiple mechanisms including inducing stimulation of progression through the G₁/S transition of the cell cycle and by activating rate-limiting proteins involved in both DNA and protein synthesis (1, 2). ERKs are activated in cancerous cells through the action of proto-oncogenes like ras that lie upstream of the MAP kinase cascade. Hormones can also activate the MAP kinase cascade to stimulate proliferation in many cell types (3). Some hormones, like insulin, act like growth factors to activate receptor tyrosine kinases to stimulate intracellular cascades leading to ERK (4, 5). However, most hormones act via serpine (or seven-transmembrane receptors), and couple to heterotrimeric GTP-binding proteins (G proteins) to elicit their effects (6, 7).

Heterotrimeric G proteins are composed of two functional units, an α subunit and a βγ subunit. Both α and βγ are released from hormone receptors upon ligand binding and can directly bind to and activate specific effectors. For α, one of these effectors is adenylyl cyclase. Historically α subunits that stimulate adenylyl cyclase are called α₂ for stimulatory, whereas those that inhibit adenylyl cyclase are termed αi, for inhibitory. Over the past 5 years, cross-talk between G protein-coupled signaling pathways have been identified for many G protein-coupled receptors (3, 8). The activation of MAP kinase cascades has been established for G proteins of diverse classes, including Gα₁, Gα₂, and Gα₃ (9–11). For some of these, direct or indirect involvement of cytoplasmic tyrosine kinases has been shown (12–16). For others, association with regulatory molecules like RasGAP (17) or Rap1GAP1 (18, 19) provides the cross-talk necessary to modulate signals to the small G proteins Ras or Rap1, respectively, to regulate the MAP kinase cascade.

Perhaps the best studied mechanism of cross-talk between G proteins and the MAP kinase cascade involves the βγ subunit of heterotrimeric G proteins. Activation of both Gα₁ and Gγ-coupled receptors releases βγ to activate the tyrosine kinase c-Src, which can activate Ras via the phosphorylation of the adaptor molecule Shc, which then recruits a complex consisting of Grb2 and SOS, the Ras-specific guanine nucleotide exchange factor (GEF), to the membrane where it can activate Ras (20). In some cases, a role for phosphoinositol 3-kinase γ in Src activation has been shown (21). In other cases, Src is activated by a calcium-sensitive kinase PYK2 (12). Despite variations on the mechanisms used, all examples of βγ signaling to ERKs require Ras activation.

Recently, the α subunits of heterotrimeric G proteins have also been shown to signal to the MAP kinase cascade. The α subunits of Gα₁ and Gα₂ (which share extensive sequence homology and PTX sensitivity) both bind to Rap1GAP1, a GTPase-activating protein specific for a distinct small G protein Rap1 (19). Rap1 is a cell type-specific antagonist of Ras-dependent signaling, and its inhibition by Rap1GAP1 can allow Ras to signal effectively to ERKs. The α subunit of Gα₃ has also been implicated in MAP kinase activation. For example, constitutively activated mutants of Gα₃ are oncogenic (22–25). These mutants encode an oncogene called gsp that can activate ERKs when expressed in transfected cells. Activated Gα₃ triggers the synthesis of the second messenger cAMP through direct association with specific adenylyl cyclases (26, 27). The major...
target of cAMP is the cAMP-dependent protein kinase PKA (28, 29). PKA has cell-type-specific actions on MAP kinase signaling. In many cell types, PKA antagonizes Ras-dependent activation of Raf-1, an ubiquitously expressed MAP kinase kinase (30–33) to inhibit cellular proliferation and Ras-dependent transformation (34). In other cell types, PKA can activate MAP kinase through a distinct pathway involving Rap1 and a cell type-specific isoform of Raf called B-Raf (9, 35, 36). Recently, a second enzyme target for cAMP, cAMP-GEF (or Epac), was identified as a Rap1-specific GEF (37, 38). Therefore, in B-Raf-dependent pathways, both PKA and Epac can potentially activate ERKs through Ras-independent pathways, one via PKA and another through direct activation of Rap1-GEFs.

The ability of hormones that couple to Goα, to activate Rap1 and ERKs has been examined in transfected cell lines overexpressing specific serpentine receptors. In Chinese hamster ovary cells overexpressing the adenosine A2A receptor, adenosine has been shown to activate ERKs via Rap1 (39). In HEK293 cells, a well studied model of G protein coupling, overexpression of β2-adrenergic receptor (β2AR) was shown to couple to ERKs via a Ras-dependent pathway (40, 41). The best studied receptor system coupled to Goα is the βAR and its activation by the agonist isoproterenol. In this study, we examine the mechanism by which isoproterenol activates ERKs in HEK293 cells expressing endogenous levels of β2AR.

**EXPERIMENTAL PROCEDURES**

**Materials—**Antibodies to Rap1, B-Raf, Rap1, recombinant MEK-1 protein, and aggarose-conjugated antibodies to ERK1, ERK2 (c-16), and myc-ERK were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA). Anti-Ras antibody was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Phosphorylation-specific ERK antibodies (pERK) that recognize phosphorylated ERK1 (pERK1) and ERK2 (pERK2), at residues threonine 183 and tyrosine 185 were purchased from New England Biolabs (Beverly, MA). Isoproterenol, thrombin, carbachol, Flag (M2) antibody, and lysophosphatidic acid were purchased from Sigma. Forskolin, clonidine, PTX, alprenolol, atenolol, epidermal growth factor (EGF), AG1478, and N-[2-(p-bromocinnamylamino)ethyl]-5-isouquinolinesulfonamide (H89) were purchased from Cal Biochem (Riverside, CA). Nickel-nitrilotriacetic acid-agarose and nickel-nitrilotriacetic acid-agarose-Sepharose (BRL) were purchased from New England Biolabs. The control vector, pcDNA3 (Invitrogen Corp.), was included in each set of transfections to assure that each plate received the instructions. The control vector, pcDNA3 (Invitrogen Corp.), was included in each set of transfections to assure that each plate received the instructions. 

**Immune Complex Assays**—For ERK assays, all cell treatments were performed by transfecting HEK293 cells using LipofectAMINE reagent. Dermal growth factor (EGF), AG1478, and thrombin-activated Rap1 and B-Raf Mediate

**Affinity Assay for Rap1 Activation in HEK293 Cells—**GST fusion protein of the Rap1-binding domain of Raf1p was expressed in Escherichia coli following induction by isopropyl-ß-D-galactopyranoside (GST-RaigD was a gift from Dr. Bos (Utrecht University, Utrecht, The Netherlands) to P. S. S.). B-Raf was confirmed by Western blotting. Isoproterenol Activates ERK via Endogenous β2ARs—Isoproterenol activates ERKs in B-Raf and Rap1 proteins were identified as a Rap1-specific GEF (37, 38). Therefore, in B-Raf-dependent pathways, both PKA and Epac can potentially activate ERKs through Ras-independent pathways, one via PKA and another through direct activation of Rap1-GEFs.

**RESULTS**

Isoproterenol Activates ERK via Endogenous β2ARs—Isoproterenol treatment of HEK293 cells with the β2-adrenergic agonist, isoproterenol, induces phosphorylation of MAP kinase ERKs in a cell-type-dependent manner (Fig. 1A). Thirteen minute stimulations with increasing concentrations of isoproterenol revealed maximal ERK kinase activity at concentrations over 10 μM. Similar to previously published data, 10 μM isoproterenol induced endogenous ERK kinase activity maximally between 3 and 5 min (Fig. 1B) (43). Isoproterenol-induced ERK kinase activation was completely blocked by pretreatment with...
the selective β1,2-adrenergic antagonist alprenolol (Fig. 1C). Pretreatment with the selective β1-adrenergic antagonist, atenolol, did not inhibit isoproterenol-mediated activation of MAP kinase. These results suggest that isoproterenol activates ERKs via endogenously expressed β2ARs with maximal activation between 3 and 5 min.

β2ARs mediate their intracellular signals via Gαo, which, upon isoproterenol binding is released to activate adenylate cyclase. This results in the rapid elevation of intracellular cAMP levels and activation of the cAMP-dependent protein kinase PKA. To determine whether PKA plays a role in mediating endogenous ERK activation we utilized the selective PKA inhibitor H89 (44). Pretreatment of serum-starved HEK293 cells with H89 completely eliminated the ability of isoproterenol to activate ERK kinase (Fig. 2). As a positive control, we treated cells with forskolin, an activator of adenylate cyclase. Forskolin activated ERKs, and H89 abolished forskolin activation of ERKs (Fig. 2). Taken together, the above data demonstrate that isoproterenol activates endogenous signaling pathways that utilize both the β2AR and the cAMP-dependent kinase PKA.

**FIG. 2. Endogenous β2-adrenergic receptors in HEK293 cells activate ERKs via PKA.** Serum-starved HEK293 cells were treated with isoproterenol for 3 min or forskolin for 5 min in the absence or presence of the PKA inhibitor H89 (10 μM), as indicated. Cells were then lysed, and equal protein amounts per treatment condition were used for Western blot with pERK or kinase assay using MBP as a substrate. A representative experiment showing both pERK (upper panel) and kinase activity (middle panel) is shown. The lower panel demonstrates equal protein levels as evidenced by Western blot probe for ERK2.

**FIG. 3. β2AR-mediated activation of ERKs via endogenous receptors is insensitive to PTX.** A, HEK293 cells were serum-starved and received either no pretreatment or pretreatment with 100 ng/ml PTX for 16 h. Cells were then stimulated with 10 μM isoproterenol for the indicated times. As negative and positive controls, respectively, HEK293 cells were also treated with 100 ng/ml EGF for 5 min and 10 μM carbachol for 5 min in the presence or absence of PTX. HEK293 cells were lysed, and equal amounts of protein were analyzed by Western blotting with pERK antibody (upper panel). B, HEK293 cells were prepared similarly to those in panel A with PTX pre-treatment for 16 h. Cells were then treated with 10 μM isoproterenol for 3 min, 100 ng/ml EGF for 5 min, and 50 μM clonidine for 5 min. Cells were lysed, and endogenous ERK1/2 were immunoprecipitated from equivalent amounts of protein using agarose-coupled ERK antibodies (as in Fig. 1B). A representative immune complex kinase assay with the location and phosphorylation of the MBP substrate is shown (upper panel). The lower panel represents a Western blot identifying the levels of ERK2 to control for protein loading.

**ERK Activation by Isoproterenol Is Insensitive to PTX Treatment**—Recent reports using HEK293 cells transiently transfected with cDNA encoding the β2AR have shown that isoproterenol-induced activation of ERK was blocked by PTX (41, 45). These data imply that ERK activation utilizes a Gαi (or Gαo) pathway to stimulate ERK activity. To investigate whether β2AR can activate endogenous signaling pathways in the presence of PTX, we pretreated HEK293 cells overnight with PTX and assessed the ability of isoproterenol to activate endogenous ERKs. In an extended time course measuring ERK activation by isoproterenol, no differences between PTX-treated and untreated cells were seen (Fig. 3A). ERK activation following treatment of HEK293 cells with both the muscarinic agonist carbachol (Fig. 3A) and lysophosphatidic acid (data not shown) was blocked by PTX, consistent with their ability to couple to Gαi. To further confirm that the activation of ERKs by isoproterenol was insensitive to PTX, immune complex kinases assays were performed on endogenous ERK1/2. As can be seen in Fig. 3B, isoproterenol’s activation of ERKs was not blocked by PTX. However, activation of ERKs by the α-adrenergic receptor agonist, clonidine, was blocked by PTX. As a negative control,
Therefore, we sought to determine whether endogenous Rap1 has a role in signaling via G proteins (9, 49). We determined if endogenous Rap1 is sensitive to H89. Cells were pretreated with 10 μM isoproterenol for 3 min and 10 μM forskolin for 5 min, following a pretreatment with H89 (10 μM); equal amounts of cell lysate were used to assay for GTP-loaded Rap1. Thrombin was used as a positive control for Rap1 activation and a negative control for H89. C. isoproterenol activation of Rap1-sensitive to PKI and CBR. HEK293 cells were co-transfected with Flag-Rap1 and the indicated cDNAs, serum-starved, and stimulated with 10 μM isoproterenol for 3 min. Cells transfected with Crk-L/C3G were not stimulated. Equal amounts of cell lysate were incubated with GST-RalGDS and a Flag (M2) antibody to identify Flag-Rap1 protein. D. HEK293 cells express C3G. Western blotting of equal amounts of protein were used to represent cell lysates from various cell types: COS 7 (lane 1), PC12 (lane 2), and HEK293 (lane 3).

We show that EGF-mediated activation of ERKs was not blocked by Ptx (Fig. 3A). These results would indicate that β2AR is able to activate endogenous ERKs via a Gαi/Gαo-independent pathway.

**ERK Activation by β2AR Requires Rap1**—Recent studies have identified a role for Rap1 in signaling via G proteins (9, 18, 19). We sought to determine whether endogenous β2AR stimulation by isoproterenol could activate Rap1. To determine whether Rap1 was activated in response to isoproterenol treatment, we performed a time course of Rap1 activation. Endogenous Rap1 was activated at the earliest time point examined with maximal activation observed from 3 to 5 min, and a return to base line by 20 min (Fig. 4A). As previously demonstrated, thrombin was also able to induce endogenous Rap1 activity in these cells (39). To investigate the requirement for PKA in activating Rap1, cells were pretreated with H89. Pretreatment of HEK293 cells with 10 μM H89 blocked the ability of either forskolin or isoproterenol to activate Rap1 at 3 min, but had no effect on thrombin’s action (Fig. 4B). Taken together, these results would suggest that β2AR activates Rap1 in a PKA-dependent manner. Recent studies have suggested that the guanine-nucleotide exchange factor, C3G, may play a role in activating Rap1 (46). C3G is constitutively associated with a member of the Crk adaptor family and is stabilized by its association with Crk-L (47). As can be seen in Fig. 4C, cotransfection of Flag-Rap1 along with Crk-L and C3G resulted in Rap1 activation in HEK293 cells as in other cell types (47). To determine whether C3G is playing a role in activating Rap1 in response to isoproterenol we used a truncated mutant of C3G containing the CRK-binding region, CBR, which interferes with CRK function (46, 47). Transfection of CBR along with Flag-Rap1 blocked the ability of isoproterenol to activate Rap1 (Fig. 4C). To further confirm the role for PKA in activating Rap1 in response to isoproterenol we co-transfected the PKA-specific inhibitory protein, PKI, which abolished the ability of isoproterenol to activate Rap1 (Fig. 4C). These results would suggest that Rap activation in response to β2AR stimulation is PKA-dependent and also utilizes the guanine-nucleotide exchange factor C3G. Indeed, HEK293 cells express endogenous levels of C3G (Fig. 4D) raising the possibility that the β2AR may utilize C3G to activate Rap1.

Recent data have suggested that the small G protein Ras may play a role in mediating ERK activation by β2AR (41, 48). To examine the ability of β2AR to activate Ras, we examined a time course of Ras activation. Similar to Rap1 activation, Ras appeared to be activated very early following isoproterenol stimulation and was inactive by 5–10 min (Fig. 5A). HEK293 cells were treated with EGF as a positive control for Ras activation. To determine whether Ras activation was PKA-dependent, HEK293 cells were pretreated with H89 and stimulated with isoproterenol. H89 pretreatment had no effect on Ras activation (Fig. 5B), suggesting that Ras is activated by isoproterenol in a PKA-independent fashion. Consistent with this result, forskolin did not activate Ras. Moreover, EGF stimulation of Ras was not blocked by H89, suggesting that H89’s effect was specific for PKA. These data would indicate that Ras activation by β2AR did not require cAMP or PKA and suggests that Gαi stimulation of adenylate cyclase was not directly involved in Ras activation.

Based on the finding that both Rap1 and Ras were rapidly activated in response to isoproterenol treatment, we next examined the role of these small G proteins in mediating ERK activation. HEK293 cells were transiently transfected with cDNAs encoding an interfering mutant of Rap1, RapN17, the Rap1 antagonist Rap1GAP1, and the interfering mutant of Ras, RasN17. These mutants have previously been characterized by our laboratory and others and function as selective blockers of Rap1 or Ras signaling (9, 49, 50). Cells transfected...
with myc-ERK and stimulated with 10 μM isoproterenol for 3 min displayed robust ERK kinase activity (Fig. 6A). Isoproterenol-induced ERK activation was significantly reduced when cells were co-transfected with either RapN17 or Rap1GAP1. RasN17 did not appear to have a significant effect (Fig. 6A). The differences in kinase activity were not attributed to varying levels of myc-ERK expression (Fig. 6A, lower panel). Quantification of three independent experiments revealed that ERK kinase activity, induced by isoproterenol for 3 min, was significantly reduced by either RapN17 or Rap1GAP1 (Fig. 6B). These data indicate that endogenous Rap1, but not endogenous Ras, is required for β2AR to activate MAP kinase at this time point.

**Isoproterenol Induces Rap1/B-Raf Association and B-Raf Kinase Activity**—To further investigate the function of active Rap1 in mediating MAP kinase activation in HEK293 cells, we examined the downstream target of Rap1, B-Raf. Prior studies from our laboratory have demonstrated in PC12 cells, which express high levels of B-Raf, that cAMP is able to activate ERKs through a PKA/Rap1/B-Raf pathway (9). HEK293 cells also express high levels of endogenous B-Raf protein (Fig. 7A). HEK293 cells were left untransfected or transfected with His-Rap or a constitutively active mutant of His-Rap, His-RapV12 (9, 52), serum-starved, and treated with isoproterenol for 3 min in the absence or presence of H89. Isoproterenol stimulation induced Rap1/B-Raf association and B-Raf kinase activity (Fig. 7B). Both the association and kinase activity was blocked by the PKA inhibitor H89. Results from three independent experiments are shown in Fig. 7C.

Isoproterenol stimulation of HEK293 cells induced the activation of Ras (Fig. 5A). To determine whether active Ras could couple to relevant downstream effectors, we investigated its ability to associate with the Raf isoforms B-Raf and Raf-1. Previous studies have suggested that recruitment of Raf to Ras is necessary for its activation (53–56). HEK293 cells were transfected with His-tagged Ras cDNA (His-Ras) and treated with either isoproterenol or EGF, or pretreated with isoproterenol and then treated with EGF. Results presented in Fig. 7D suggest that isoproterenol stimulation did not induce the association of endogenous Raf-1 with Ras. More importantly, pre-treatment with isoproterenol inhibited the ability of EGF to induce the association of endogenous Raf-1 with Ras (Fig. 7D). Parallel experiments examining the association of B-Raf with Ras indicated that isoproterenol alone inhibited basal as well as EGF-induced association of B-Raf with Ras (Fig. 7E). These results suggest that, although Ras is activated by β2AR, it is unable to couple to either Raf-1 or B-Raf kinases.

**ERK Activation by β2AR Occurs Independently of EGF Receptor Phosphorylation**—A recent study has suggested a role for the EGF receptor in mediating β2AR-induced ERK activation (57). To address the requirement for the EGF receptor in β2AR signaling, we treated cells with the EGF receptor kinase inhibitor AG1478, which specifically inhibits kinase activity of the receptor. Pretreatment of cells with AG1478 did not block isoproterenol-induced activation of endogenous ERKs (Fig. 8A). The above results would suggest that Rap1-dependent activation of ERKs by β2AR does not require EGF receptor transactivation.

Recent studies have also suggested that the activation of Ras by β2AR may also utilize the EGF receptor, via non-classical coupling to Gαs (57). To further elucidate the mechanism by which Ras is activated by β2AR, we determined whether endogenous Ras activation by isoproterenol was dependent on EGF receptor activation. Pretreatment of HEK293 cells with AG1478 did not block Ras activity by isoproterenol at 3 min (Fig. 8B). To investigate the possibility that Gαs may signal to Ras, we pretreated HEK293 cells with PTx and stimulated cells with either isoproterenol or carbachol for 3 and 5 min, respectively. Representative data presented in Fig. 8C demonstrate that Ras activation by isoproterenol, but not by carbachol, was insensitive to PTx. As a positive control, we show that Ras activation by carbachol was sensitive to PTx (Fig. 8C). The above data as well as that presented in Fig. 5A indicate that Ras is activated by the endogenously expressed β2AR independently of either the EGF receptor or Gαs.

**DISCUSSION**

The second messenger cAMP is the best studied intracellular signal. Its major action, the activation of PKA (28, 29) allows hormonal signals to couple to intracellular phosphorylation events. Hormonal elevation of cAMP levels is triggered by the specific heterotrimeric G protein subunit Gαs. The range of extracellular ligands that couple to Gαs is extensive and includes moderately sized peptides, including vasoactive intestinal peptide-like, members of the glucagon/secretin superfamily, adrenocorticotropic hormone, parathyroid stimulating hormone, and a large family of hypothalamic releasing factors, as well as the family of large glycoproteins thyroid stimulating hormone, follicle-stimulating hormone, and luteinizing hormone. Small molecules can also activate Gαs to stimulate adenylate cyclases, including dopamine (via the D1 receptor), adenosine (via the A2A receptor), prostaglandin E, and the family of adrenergic molecules, including epinephrine and norepinephrine (58–60). The cognate receptors for all these ligands are heptahelical transmembrane proteins (also called serpentine receptors) that associate with Gαs.
In the unliganded, resting state, these receptors bind inactive GDP-bound Goa subunits that are associated with specific βγ subunits. Upon ligand binding, exchange of GTP for GDP converts α into its active GTP-bound state, causing it to be released from the receptor, where it is free to bind to, and activate, membrane-associated adenylate cyclases. At the same time Goa dissociates from the receptor, βγ is released from Goa and can activate effectors independently of Goa. βγ signaling from Goa-coupled receptors has not been reported. However, βγ release from Go and Goa is well known to activate a number of intracellular kinases, including phosphoinositol 3-kinase (20, 21), phospholipase C (61), Src (16), and ERK (15, 62).

The ability of Goa-coupled receptors to modulate the MAP kinase (or ERK) cascade provides a mechanism for cAMP-coupled signaling pathways to regulate cell growth (3). The best studied actions of cAMP on ERK signaling are inhibitory and lead to a decrease in cellular proliferation (30–32). This is achieved, in part, by a PKA-dependent phosphorylation of the MAP kinase kinase kinasesase Rap-1 on serine 43, which un couples Rap-1 from its upstream activator Ras (30). In cells that express the Raf isoform B-Raf (which does not contain a PKA site corresponding to serine 43), cAMP can activate ERKs (9, 35, 63). Although this has been shown in multiple cell types, additional factors may influence cAMPs ability to activate B-Raf. Indeed, cAMP has also been reported to inhibit the activation of B-Raf through a PKA phosphorylation near the kinase domain itself. However, this effect is only seen in truncated proteins lacking the N terminus of B-Raf (64). In cells that express a truncated splice variant of B-Raf that also lacks the N terminus, cAMPs inhibitory effects may predominate (65). However, cAMP robustly activates the full-length B-Raf protein, which is achieved via the activation of the small G protein Rap1 (9, 66, 67). Interestingly, Rap1 is also an antagonist of Ras-dependent signaling (52, 68, 69) and blocks Ras-dependent activation of Raf-1 (62, 70–72). Unlike Ras, Rap1 is activated by increased cAMP levels via PKA. Recently, Rap1 activators have been identified that can be directly activated by cAMP, suggesting that cAMP can activate Rap1 via both PKA-dependent and PKA-independent mechanisms (37, 38). The ability of β2AR to inhibit ERK signals has been demonstrated in adipocytes (32) and smooth muscle cells (31). Recently, β2AR has been shown to activate ERKs in HEK293 cells (40, 41, 73). In this study, we show that β2AR can activate ERKs in HEK293 cells by activating a Rap1/B-Raf pathway, while simultaneously blocking Ras-dependent signals.

HEK293 cells are commonly used to examine signaling pathways downstream of transfected receptors (39–41, 74). We show that these cells express endogenous β2AR and upon isoproterenol stimulation utilize β2AR to activate ERKs. This activation shows an EC50 of roughly 1–3 μM, consistent with other actions of isoproterenol, and is rapid and transient (43). Its actions on ERKs are mimicked by forskolin and require
FIG. 8. ERK/Ras activation by β₂AR does not require EGF receptor phosphorylation. A, isoproterenol-mediated activation of ERKs is EGF receptor-independent. Serum-starved HEK293 cells were pretreated with 200 nM AG1478 for 20 min, followed by 10 μM isoproterenol stimulation for the indicated times. As a control, cells were also treated with 100 ng/ml EGF for 5 min. Lysates were subjected to Western blotting using pERK antibodies (upper panel) or ERK2 antibody (lower panel) to confirm equal protein amounts of cell lysate were utilized. B, isoproterenol stimulation of Ras is EGF receptor-independent. HEK293 cells were serum-starved and pretreated with 200 nM AG1478 for 20 min, followed by a 3-min stimulation with 10 μM isoproterenol. Stimulation with 100 ng/ml EGF for 5 min was used as a control for Ras activation. Equal amounts of cell lysate were incubated with pre-coupled GST-Raf1RBD and analyzed by Western blot for GTP-loaded Ras.

PKA, suggesting the involvement of Goαs and cAMP. Although signaling via Goα is classically thought to be insensitive to PTx, recent reports have demonstrated that β₂AR can couple to ERKs via PTx-sensitive pathways (41). These studies, which utilized transiently transfected cDNAs encoding β₂AR in HEK293 cells, proposed a PKA-dependent switch in β₂AR affinity from Gαs to Gαi. In our hands, PTx did not block β₂AR’s activation of ERKs, while blocking the action of known Gα-coupled agents, including carbachol, lysophosphatidic acid, and clomidine. It is possible that the ability of β₂AR to couple to PTx-sensitive pathways is dependent on elevated levels of β₂AR expression.

Both Ras-dependent and Rap1-dependent mechanisms of β₂AR’s activation of ERKs have been proposed (35, 40). Indeed, we show that both Ras and Rap1 were activated by isoproterenol. Ras is activated rapidly and transiently, whereas Rap1 activation is slower and is sustained. This is similar to the kinetics seen in other cell types, including PC12 cells (47) and in platelets (75). Interestingly, the activation of Rap1, but not Ras, required PKA. Forskolin, which acts downstream of Goαs to elevate cAMP, also activated Rap1 but did not activate Ras. These data suggest that β₂AR utilized distinct pathways to activate Ras and Rap1. We propose that Rap1 is activated by Goαs (via cAMP and PKA), and that Ras is activated independently of Goαs, possibly by a βγ-dependent pathway. For Rap1, PKA appears to act upstream of Rap1 itself, possibly through a mechanism involving the Rap1 guanine-nucleotide exchanger C3G (47). C3G is expressed in HEK293 cells and is distinct from recently proposed exchangers like cAMP-GEFs (Epacs) that appear to be activated by cAMP in a PKA-independent manner (37, 38).

Surprisingly, only Rap1, but not Ras, was required for β₂AR’s activation of ERKs. Two agents that interfere with Rap1 signaling, Rap1N17 and Rap1GAP1, were used. Overexpression of Rap1N17 is thought to sequester endogenous activators of Rap1, whereas Rap1GAP1 stimulates the GTPase activity of endogenous Rap1 to terminate Rap1 signaling (9, 18, 76). RasN17 is a well characterized selective interfering mutant of Ras (50, 77). These data suggest that, although both Ras and Rap1 are activated by β₂AR, only Rap1 is capable of transmitting a signal to ERKs. The signal to ERKs is likely to be B-Raf, since B-Raf is the only known MAP kinase kinase kinase that can be activated by Rap1. Indeed, HEK293 cells express the 96-kDa isoform of B-Raf that is activated by cAMP (9), and endogenous B-Raf is recruited to Rap1 upon isoproterenol stimulation, in a PKA-dependent manner. Both Raf-1 and B-Raf have been shown to be efficiently recruited to Ras under the appropriate conditions (54, 66, 78, 79). However, neither Raf-1 nor B-Raf were recruited to Ras by isoproterenol treatment, although Ras was GTP-loaded (activated) at the time point used for this study. The inability of Ras to couple to Raf explains why β₂AR’s activation of ERK was independent of Ras.

Isoproterenol not only did not induce Ras association with effectors, it reversed the ability of Ras to recruit both Raf-1 and B-Raf following EGF stimulation. For Raf-1, this may be due to the phosphorylation of Raf-1 at serine 43 by PKA, which dissociates Raf-1 from activated Ras. However, the ability of isoproterenol to block the recruitment of B-Raf to Ras cannot be explained by this mechanism and suggests that an additional action of PKA is antagonizing Ras function, in general. Indeed, cAMP can also block recruitment of B-Raf to Ras (data not shown). A potential mediator of this effect is Rap1 itself. We propose a model in which Rap1 activation by PKA has two opposing functions in B-Raf/Raf-1-expressing cells; the activation of B-Raf and the antagonism of Ras. The net effect of these two actions will depend on the relative levels of Rap1 as well as B-Raf and Raf-1 in each cell type.

Although we show that activated Ras cannot activate ERKs in these cells, the mechanism by which Ras was activated by β₂AR in these cells is not known. Recently, the ability of β₂AR to activate Ras-dependent signaling has been suggested by Leffkowitz and colleagues. In their model, transiently transfected β₂AR utilized a PTx-sensitive pathway to transactivate the endogenous EGF receptor. However, using cells expressing endogenous β₂AR, we show that isoproterenol’s ability to activate either ERK or Ras did not require EGF receptor kinase.
activity. In addition, Ras activation by isoproterenol was not blocked by PTXs. Since Ras activation by isoproterenol was not sensitive to H89, we propose that Ras activation by βAR is not mediated by PKA, Gαi, or EGF receptor. We suggest that βγ subunits, which have been shown to regulate ERKs via both α and Gαi, or EGF receptor. We suggest that the ability of Gs-coupled receptors to activate or inhibit ERKs (81) and deficits in this form of long term potentiation have recently been shown to require ERKs 82. B-Raf.

The Rap1/B-Raf pathway identified here may be an important mechanism by which βAR stimulates ERKs in multiple systems. This may be especially true in neurons and in prostate cells that express high levels of B-Raf and where cAMP signaling to ERKs has been shown to require Rap1 (9, 63, 80) For example, βAR-dependent models of long term potentiation in hippocampal neurons has recently been shown to require ERKs (81) and deficits in this form of long term potentiation have been identified in transgenic mice deficient in hippocampal Rap1 signaling (82). Taken together, these studies suggest that the ability of Gαi-coupled receptors to activate or inhibit ERKs may depend, in part, on the expression of B-Raf (51). Although the activation of Rap1 may have a significant positive effect on ERK signaling in B-Raf-expressing cells, one can speculate that the activation of Rap1 by Gαi-coupled receptors may antagonize Ras-dependent signaling to ERKs in cells that do not express B-Raf.

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