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B2-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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β_2-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 $\beta\gamma$ 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 β_2-adrenergic receptor (β_2AR), to stimulate extracellular signal-regulated kinases (ERKs). Using HEK293 cells, which express endogenous β_2AR, we show that isoproterenol stimulates ERKs via β_2AR. This action of isoproterenol requires cAMP-dependent protein kinase and is insensitive to pertussis toxin, suggesting that Gα_s activation of cAMP-dependent protein kinase is required. Interestingly, β_2AR activates both the small G proteins Rap1 and Ras, but only Rap1 is capable of coupling to Raf isoforms. β_2AR inhibits the Ras-dependent activation of both Raf isoforms Raf-1 and B-Raf, whereas Rap1 activation by isoproterenol recruits and activates B-Raf. β_2AR 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[1] kinase (or ERK) cascade triggers cellular proliferation through multiple mechanisms including inducing stimulation of progression through the G_1/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 serpentine (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 $\beta\gamma$ subunit. Both α and $\beta\gamma$ are released from hormone receptors upon ligand binding and can directly bind to and activate specific effectors. For α, one of these effectors is adenylate cyclase. Historically α subunits that stimulate adenylate cyclase are called αs for stimulatory, whereas those that inhibit adenylate 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 G_s, G_i, and G_q (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 $\beta\gamma$ subunit of heterotrimeric G proteins. Activation of both G_q- and G_i-coupled receptors releases $\beta\gamma$ 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 $\beta\gamma$ 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 G_i and G_o (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 G_s has also been implicated in MAP kinase activation. For example, constitutively activated mutants of Gα_s are oncogenic (22–25). These mutants encode an oncogene called *gsp* that can activate ERKs when expressed in transfected cells. Activated Gα_s triggers the synthesis of the second messenger cAMP through direct association with specific adenylate cyclases (26, 27). The major

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[1] The abbreviations used are: MAP, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; GEF, guanine nucleotide exchange factor; PKA, cAMP-dependent protein kinase; β_2AR, β_2-adrenergic receptor; EGF, epidermal growth factor; DMEM, Dulbecco's modified Eagle's medium; PTx, pertussis toxin; MBP, myelin basic protein; GST, glutathione *S*-transferase.

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 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-expressing cells, cAMP has at least two potential mechanisms to activate ERKs through Ras-independent pathways, one via PKA and another through direct activation of Rap1-GEFs.

The ability of hormones that couple to $G\alpha_s$ to activate Rap1 and ERKs has been examined in transfected cell lines overexpressing specific serpentine receptors. In Chinese hamster ovary cells overexpressing the adenosine A_{2A} receptor, adenosine has been shown to activate ERKs via Rap1 (39). In HEK293 cells, a well studied model of G protein coupling, overexpression of β_2-adrenergic receptor (β_2AR) was shown to couple to ERKs via a Ras-dependent pathway (40, 41). The best studied receptor system coupled to $G\alpha_s$ is the β_2AR 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 β_2AR.

EXPERIMENTAL PROCEDURES

Materials—Antibodies to Rap1, B-Raf, Raf-1, recombinant MEK-1 protein, and agarose-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-isoquinolinesulfonamide (H89) were purchased from Cal Biochem (Riverside, CA). Nickel-nitrilotriacetic acid-agarose was purchased from Qiagen Inc. (Chatswoth, CA). Radioisotopes were from NEN Life Science Products.

Cell Culture—HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal calf serum at 37 °C in 5% CO_2. Cells were maintained in serum-free DMEM for 16 h at 37 °C in 5% CO_2 prior to treatment with various reagents for both immune complex assays and Western blotting. Cells pre-treated with PTx (100 ng/ml) were incubated in serum-free media for 16 h prior to stimulations. All inhibitors, unless otherwise indicated, were added to cells 20 min prior to treatment.

Western Blotting—Cell lysates were prepared as described (9). Cell lysate protein concentrations were quantified using Bradford protein assay. For detection of B-Raf, ERK2, myc-ERK, Rap1, Flag, Ras, and phospho-ERK1/2 (pERK), equal protein amounts of cell lysate per treatment condition were resolved by SDS-polyacrylamide gel electrophoresis, blotted onto polyvinylidene difluoride (Millipore Corp., Bedford, MA) membranes and probed with the corresponding antibodies according to the manufacturer's guidelines.

Plasmids and Transfections—Seventy to 80% confluent HEK293 cells were co-transfected with the indicated cDNAs using a LipofectAMINE kit (Life Technologies, Inc.) according to the manufacturer's instructions. The control vector, pcDNA3 (Invitrogen Corp.), was included in each set of transfections to assure that each plate received the same amount of DNA. Following transfection, cells were allowed to recover in serum-containing media for 24 h. Cells were then starved overnight in serum-free DMEM before treatment and lysis.

Immune Complex Assays—For ERK assays, all cell treatments were lysed in ice-cold lysis buffer (50 mM Tris-HCl, pH 8.0, 10% glycerol, 1% Nonidet P-40, 200 mM NaCl, 2.5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 10 μg/ml soybean trypsin inhibitor, 10 mM NaF, 0.1 μM aprotinin, and 1 mM $NaVO_4$). The lysates were centrifuged at low speed to remove nuclei, and the supernatant was examined for ERK activity using myelin basic protein (MBP) as a substrate and [γ-^{32}P]ATP as a phosphate donor with equal protein amounts per assay condition as described (9). For B-Raf assays, untreated and treated cells were lysed in ice-cold 1% Nonidet P-40 buffer containing 10 mM Tris, pH 7.4, 5 mM EDTA, 50 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride. Immune complex kinase assays were performed as described (9) using MEK-1 as a substrate and [γ-^{32}P]ATP as a phosphate donor with equal protein amounts per assay. The reaction products of all kinase assays were resolved by 10% SDS-polyacrylamide gel and analyzed with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Nickel Affinity Chromatography—Experiments utilizing polyhistidine-tagged Rap1 (His-Rap1 and His-RapV12) and Ras (His-Ras), were performed by transfecting HEK293 cells using LipofectAMINE reagent. Cells were lysed in ice-cold buffer containing 1% Nonidet P-40, 10 mM Tris, pH 8.0, 20 mM NaCl, 30 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, and 0.5 mg/ml aprotinin. Supernatants were prepared by low speed centrifugation. Transfected His-tagged proteins were precipitated from supernatants containing equal amounts of protein using nickel-nitrilotriacetic acid-agarose and washed with 20 mM imidazole in lysis buffer and eluted with 500 mM imidazole and 5 mM EDTA in phosphate-buffered saline. One-half of the eluates containing His-tagged proteins were separated on SDS-polyacrylamide gel electrophoresis, and B-Raf or Raf-1 proteins were detected by Western blotting (9). The remaining His-Rap1 eluates, of equal amounts, were immunoprecipitated with B-Raf antisera, and B-Raf kinase activity was measured by immune complex assay. Equal amounts of His-Rap1 and His-Ras was confirmed by Western blotting.

Affinity Assay for Rap1 Activation in HEK293 Cells—GST fusion protein of the Rap1-binding domain of RalGDS was expressed in *Escherichia coli* following induction by isopropyl-1thio-β-D-galactopyranoside (GST-RalGDS was a gift from Dr. Bos (Utrecht University, Utrecht, The Netherlands) to P. J. S. S.). Bacterial lysates were prepared, and GST fusion proteins were immobilized by incubating lysates for 1 h at 4 °C with glutathione-Sepharose. Sepharose beads were washed three times in order to remove excess GST fusion protein. HEK293 cells were grown as described, and were stimulated at 37 °C for the indicated times and immediately lysed in ice-cold lysis buffer (50 mM Tris-HCl, pH 8.0, 10% glycerol, 1% Nonidet P-40, 200 mM NaCl, 2.5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 10 μg/ml soybean trypsin inhibitor, 10 mM NaF, 0.1 μM aprotinin, and 1 mM $NaVO_4$). Active Rap1 was isolated using methods as described by Franke *et al*. (42). Briefly, cell lysates were cleared by centrifugation, and equal amounts of supernatants were incubated with GST-RalGDS-Rap1 binding domain pre-coupled to glutathione beads. Following a 1-h incubation at 4 °C, beads were pelleted and rinsed threes times with ice-cold lysis buffer, protein was eluted from the beads using 2× Laemmli buffer and applied to a 12% SDS-polyacrylamide gel. Proteins were transferred to polyvinylidene difluoride membrane, blocked in 5% milk for 1 h, and probed with α-Rap1/Krev-1 or Flag (M2) antibody overnight at 4 °C, followed by a horseradish peroxidase-conjugated anti-rabbit secondary antibody. Proteins were detected using enhanced chemiluminescence.

Affinity Assay for Ras Activation in HEK293 Cells—HEK293 cells were grown as described, and were stimulated at 37 °C for the indicated times and immediately lysed in ice-cold lysis buffer (50 mM Tris-HCl, pH 8.0, 10% glycerol, 1% Nonidet P-40, 200 mM NaCl, 2.5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 10 μg/ml soybean trypsin inhibitor, 10 mM NaF, 0.1 μM aprotinin, and 1 mM $NaVO_4$). Following the manufacturer's recommended protocol, activated Ras was isolated from stimulated lysates using agarose-coupled GST-Raf1-RBD provided in the Ras activation assay kit (Upstate Biotechnology, Inc.). Proteins were eluted from the beads using 2× Laemmli buffer and applied to a 12% SDS-polyacrylamide gel. Proteins were transferred to polyvinylidene difluoride membrane, blocked in 5% milk for 1 h, and probed with α-Ras antibody overnight at 4 °C, followed by a horseradish peroxidase-conjugated anti-mouse secondary antibody. Proteins were detected using enhanced chemiluminescence.

RESULTS

Isoproterenol Activates ERK via Endogenous β_2ARs—Isoproterenol treatment of HEK293 cells with the β-adrenergic agonist, isoproterenol, induces phosphorylation of MAP kinase ERK in a dose-dependent manner (Fig. 1*A*). Three-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. 1*B*) (43). Isoproterenol-induced ERK kinase activation was completely blocked by pretreatment with

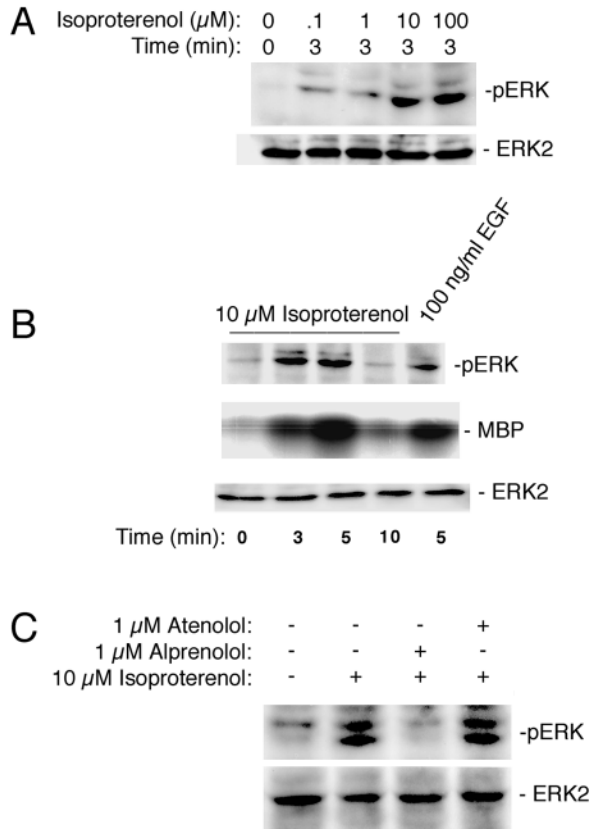

FIG. 1. **ERK activation by β_2AR.** *A*, isoproterenol dose response of phosphorylated ERKs (pERK). HEK293 cells were serum-starved and treated with increasing concentrations of isoproterenol for 3 min. Cell lysates were prepared as detailed under "Experimental Procedures." *B*, time course of endogenous ERK activation following isoproterenol stimulation in HEK293 cells. HEK293 cells were harvested for either immune complex kinase assay using MBP as a substrate or Western blotting, using phosphospecific ERK1/2 (pERK) antibodies. Cells were treated with isoproterenol or EGF, as indicated. *Upper panel*, a representative Western blot probed with pERK antibody. *Middle panel*, a representative autoradiogram with the position of MBP shown. *Lower panel*, Western blotting showing equal loading of protein amounts within cell lysate was performed using ERK2 antibody. *C*, blockade of isoproterenol stimulation of pERK. Serum-starved cells were treated with isoproterenol following a 10-min pretreatment with either atenolol or alprenolol. *Upper panel*, a representative Western blot probed with pERK antibody. *Bottom panel*, equal amounts of protein were utilized as evidenced by the Western blot probed with ERK2 antibody.

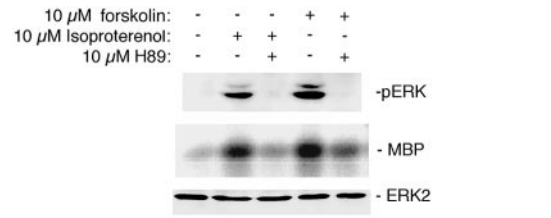

FIG. 2. **Endogenous β_2-adrenergic receptors in HEK293 cells activate ERKs via PKA.** Serum-starved HEK293 cells were treated with isoproterenol for 3 min or forskolin for 5 min in the absence or presence of the PKA inhibitor H89 (10 μM), as indicated. Cells were then lysed, and equal protein amounts 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 probed for ERK2.

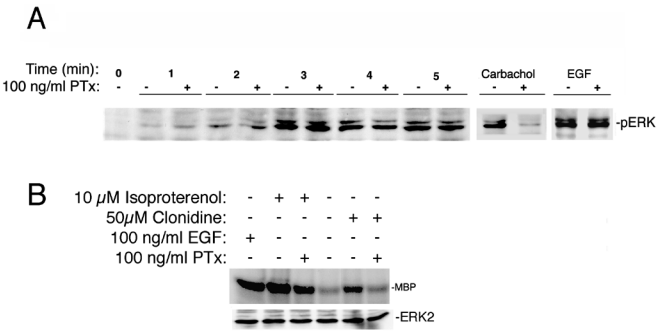

FIG. 3. **β_2AR-mediated activation of ERKs via endogenous receptors is insensitive to PTx.** *A*, HEK293 cells were serum-starved and received either no pretreatment or pretreatment with 100 ng/ml PTx for 16 h. Cells were then stimulated with 10 μM isoproterenol for the indicated times. As negative 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. 1*B*). 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.

the selective $\beta_{1,2}$-adrenergic antagonist alprenolol (Fig. 1*C*). Pretreatment with the selective β_1-adrenergic antagonist, atenolol, did not inhibit isoproterenol-mediated activation of MAP kinase. These results suggest that isoproterenol activates ERKs via endogenously expressed β_2ARs with maximal activation between 3 and 5 min.

β_2ARs mediate their intracellular signals via $G\alpha_s$, 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 β_2AR and the cAMP-dependent kinase PKA.

ERK Activation by Isoproterenol Is Insensitive to PTx Treatment —Recent reports using HEK293 cells transiently transfected with cDNA encoding the β_2AR have shown that isoproterenol-induced activation of ERK was blocked by PTx (41, 45). These data imply that ERK activation utilizes a $G\alpha_i$ (or $G\alpha_o$) pathway to stimulate ERK activity. To investigate whether β_2AR 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 cells and untreated cells were seen (Fig. 3*A*). ERK activation following treatment of HEK293 cells with both the muscarinic agonist carbachol (Fig. 3*A*) and lysophosphatidic acid (data not shown) was blocked by PTx, consistent with their ability to couple to $G\alpha_i$. 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. 3*B*, 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,

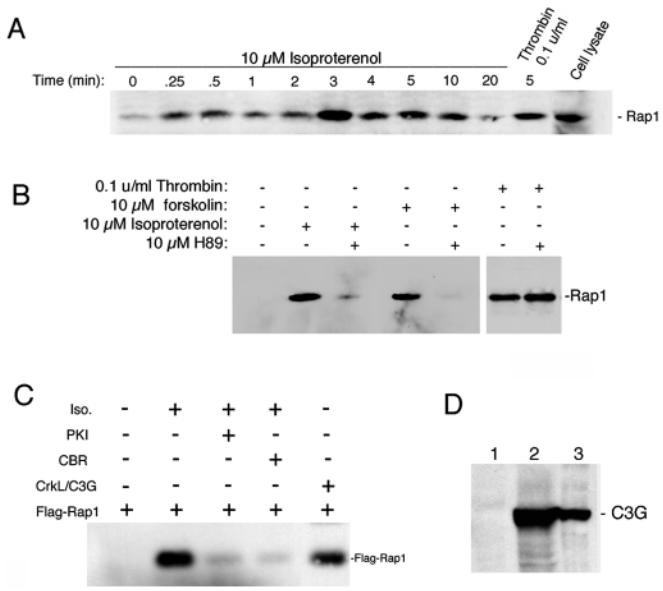

FIG. 4. **Isoproterenol activation of Rap1.** *A*, time course of activation of Rap1 by isoproterenol. Serum-starved HEK293 cells were treated with 10 μM isoproterenol or 0.1 unit/ml thrombin for the indicated times. Equal amounts of cell lysate were incubated with precoupled GST-RalGDS protein, and analyzed by Western blot for GTP-loaded Rap1. HEK293 cell lysate was used to indicate the position of Rap1. *B*, isoproterenol activation of Rap1 is sensitive to H89. Cells were stimulated 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 Rap is 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 to assay for GTP-loaded Rap1 using 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*).

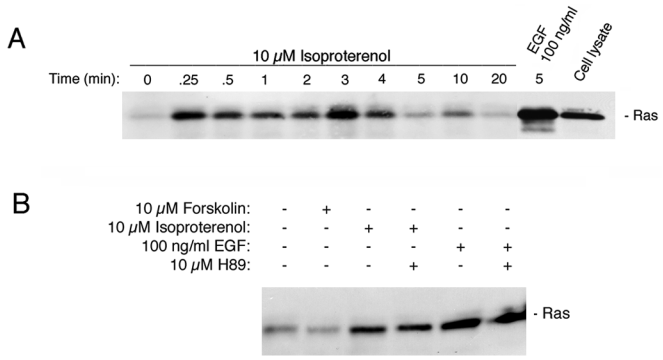

FIG. 5. **Isoproterenol activation of Ras.** *A*, time course of activation of Ras by isoproterenol. HEK293 cells were serum-starved and treated with 10 μM isoproterenol or 100 ng/ml EGF for the indicated times. Equal quantities of cell lysate were incubated with GST-Raf1RBD, and analyzed by Western blot for GTP-loaded Ras. HEK293 cell lysate was used to indicate the position of Ras. *B*, isoproterenol activation of Ras is insensitive to H89. Serum-starved HEK293 cells were treated with isoproterenol for 3 min, 10 μM forskolin for 5 min, or 100 ng/ml EGF for 5 min following a pretreatment with H89 (10 μM); equal amounts of cell lysate were used to assay for GTP-loaded Ras. EGF was used as a control for Ras activation.

we show that EGF-mediated activation of ERKs was not blocked by PTx (Fig. 3*A*). These results would indicate that β_2AR is able to activate endogenous ERKs via a $G\alpha_i/G\alpha_o$-independent pathway.

ERK Activation by β_2AR Requires Rap1—Recent studies have identified a role for Rap1 in signaling via G proteins (9, 18, 19). Therefore, we sought to determine whether endogenous β_2AR 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. 4*A*). 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. 4*B*). Taken together, these results would suggest that β_2AR 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. 4*C*, cotransfection of Flag-Rap1 along with Crk-L and C3G results 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. 4*C*). 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. 4*C*). These results would suggest that Rap activation in response to β_2AR stimulation is PKA-dependent and also utilizes the guanine-nucleotide exchange factor C3G. Indeed, HEK293 cells express endogenous levels of C3G (Fig. 4*D*) raising the possibility that the β_2AR 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 β_2AR (41, 48). To examine the ability of β_2AR 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. 5*A*). 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. 5*B*), 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 β_2AR did not require cAMP or PKA and suggests that $G\alpha_s$ 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).[2] Cells transfected

[2] K. D. Carey, J. M. Schmitt, A. M. Baird, T. J. Dillon, A. D. Holdorf, A. S. Shaw, and P. J. S. Stork, submitted for publication.

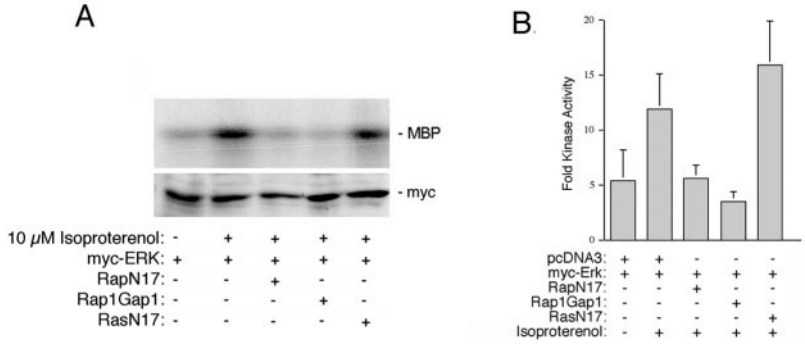

FIG. 6. **β_2AR-mediated activation of ERKs requires Rap1.** *A*, Rap is required for ERK activation by isoproterenol. HEK293 cells were transfected with the indicated cDNAs and treated with 10 μM isoproterenol for 3 min. Equivalent amounts of cell lysate were immunoprecipitated using an agarose-coupled Myc antibody followed by an immune complex kinase assay with the location and phosphorylation of MBP shown by autoradiography. A representative experiment can be seen in the *upper panel* ($n = 3$). *Lower panel*, equal amounts of Myc-tagged protein were loaded as evidenced by the Western blot probed with Myc antibody. *B*, data representing multiple myc-ERK immune complex kinase assays (from *A*) are shown as -fold activation over basal (untreated cells) ($n = 3 \pm$ S.D.).

with myc-ERK and stimulated with 10 μM isoproterenol for 3 min displayed robust ERK kinase activity (Fig. 6*A*). 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. 6*A*). The differences in kinase activity were not attributed to varying levels of myc-ERK expression (Fig. 6*A*, *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. 6*B*). These data indicate that endogenous Rap1, but not endogenous Ras, is required for β_2AR 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. 7*A*). 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. 7*B*). Both the association and kinase activity was blocked by the PKA inhibitor H89. Results from three independent experiments are shown in Fig. 7*C*.

Isoproterenol stimulation of HEK293 cells induced the activation of Ras (Fig. 5*A*). 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. 7*D* suggest that isoproterenol stimulation did not induce the association of endogenous Raf-1 with Ras. More importantly, pretreatment with isoproterenol inhibited the ability of EGF to induce the association of endogenous Raf-1 with Ras (Fig. 7*D*). 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. 7*E*). These results suggest that, although Ras is activated by β_2AR, it is unable to couple to either Raf-1 or B-Raf kinases.

ERK Activation by β_2AR Occurs Independently of EGF Receptor Phosphorylation —A recent study has suggested a role for the EGF receptor in mediating β_2AR-induced ERK activation (57). To address the requirement for the EGF receptor in β_2AR 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. 8*A*). The above results would suggest that Rap1-dependent activation of ERKs by β_2AR does not require EGF receptor transactivation.

Recent studies have also suggested that the activation of Ras by β_2AR may also utilize the EGF receptor, via non-classical coupling to $G\alpha_i$ (57). To further elucidate the mechanism by which Ras is activated by β_2AR, we determined whether endogenous Ras activation by isoproterenol was dependent on EGF receptor activation. Pretreatment of HEK293 cells with AG1478 did not block Ras activation by isoproterenol at 3 min (Fig. 8*B*). To investigate the possibility that $G\alpha_i$ 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. 8*C* 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. 8*C*). The above data as well as that presented in Fig. 5*A* indicate that Ras is activated by the endogenous β_2AR independently of either the EGF receptor or $G\alpha_i$.

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 $G\alpha_s$. The range of extracellular ligands that couple to $G\alpha s$ 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 G_s to stimulate adenylate cyclases, including dopamine (via the D1 receptor), adenosine (via the A_{2A} 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 $G\alpha_s$.

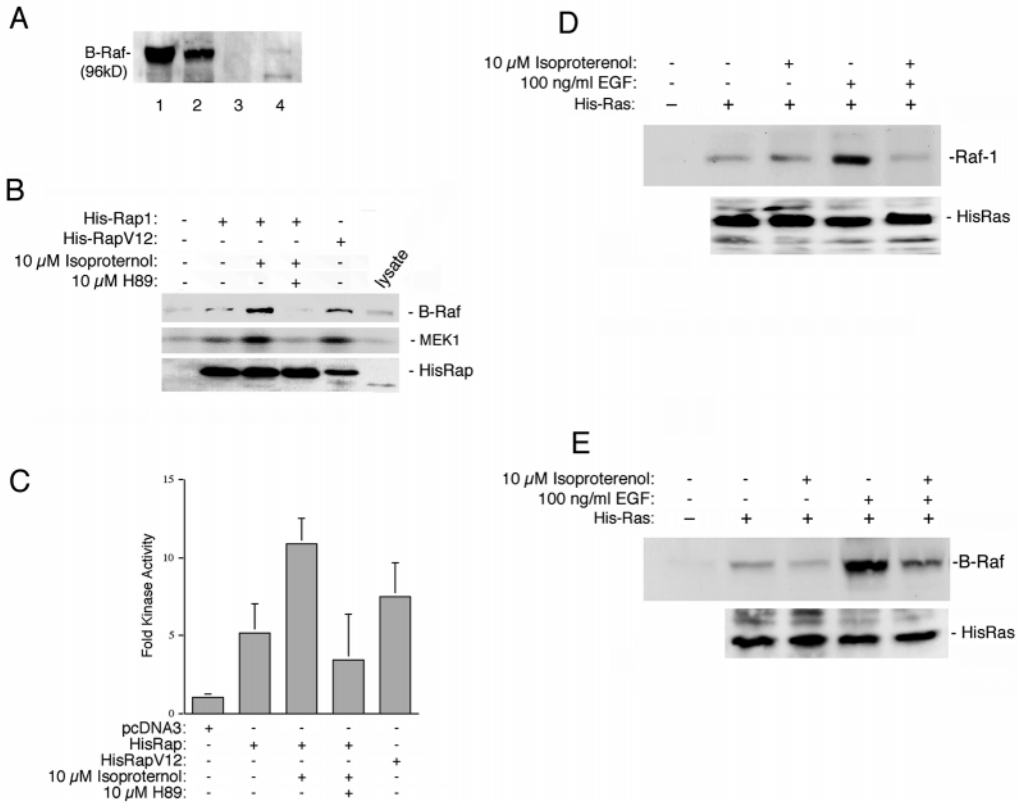

FIG. 7. **β_2AR activates B-Raf, but not Raf-1, via Rap1.** *A*, HEK293 cells express B-Raf. Western blotting of equal amounts of protein were used to represent cell lysates from various cell types: PC12 (*1*), HEK293 (*2*), Rat1 fibroblast (*3*), and PC3 (*4*). *B*, isoproterenol induces Rap1/B-Raf association and B-Raf kinase activity. HEK293 cells were transfected with His-tagged Rap1 (His-Rap) or His-RapV12 cDNAs, serum-starved, and treated with 10 μM isoproterenol for 3 min or left untreated, in the absence or presence of the PKA inhibitor, H89 as indicated. Equal amounts of protein were passed over a nickel column, and eluates were probed by Western blotting for B-Raf (*upper panel*) and kinase activity using MEK-1 as a substrate (*middle panel*). Representative results are shown (n = 3). The *bottom panel* indicates similar protein amounts of His-Rap per treatment as assayed by Western blot. *C*, data representing multiple B-Raf kinase assays. *Bars* indicate -fold activation over basal (n = 3 ± S.D.). *D*, isoproterenol inhibits Ras/Raf-1 association. HEK293 cells were transfected with His-Ras, serum-starved, and treated with either 10 μM isoproterenol for 3 min, 100 ng/ml EGF for 5 min, or pretreated with 10 μM isoproterenol for 5 min and then EGF. Equal amounts of protein lysate underwent nickel affinity purification, and eluates were probed by Western blotting for Raf-1 (*upper panel*). The *lower panel* demonstrates similar levels of His-Ras protein. *E*, isoproterenol inhibits Ras/B-Raf association. HEK293 cells were transfected with His-Ras, serum-starved, and stimulated identically to Fig. 6*D*. Following nickel affinity purification, eluates were probed by Western blotting for B-Raf (*upper panel*). The *lower panel* indicates similar levels of His-Ras.

In the unliganded, resting state, these receptors bind inactive GDP-bound $G\alpha_s$ subunits that are associated with specific $\beta\gamma$ 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 that $G\alpha_s$ dissociates from the receptor, $\beta\gamma$ is released from $G\alpha_s$ and can activate effectors independently of $G\alpha_s$. $\beta\gamma$ signaling from G_s-coupled receptors has not been reported. However, $\beta\gamma$ release from G_i and G_q 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 G_s-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 kinase Raf-1 on serine 43, which uncouples Raf-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 (52, 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 β_2AR to inhibit ERK signals has been demonstrated in adipocytes (32) and smooth muscle cells (31). Recently, β_2AR has been shown to activate ERKs in HEK293 cells (40, 41, 73). In this study, we show that β_2AR 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 β_2AR and upon isoproterenol stimulation utilize β_2AR to activate ERKs. This activation shows an EC_{50} 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

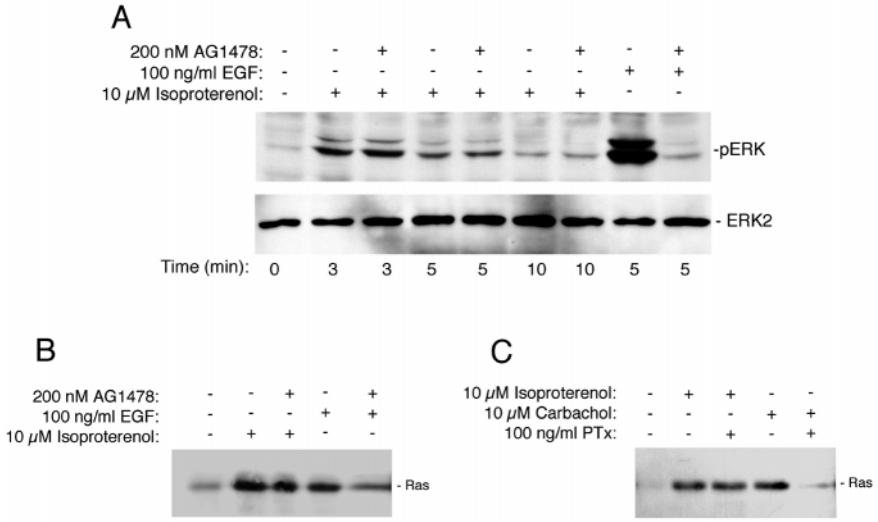

FIG. 8. **ERK/Ras activation by β_2AR does not require EGF receptor phosphorylation.** *A*, isoproterenol-mediated activation of ERKs is EGF receptor-independent. Serum-starved HEK293 cells were pretreated with 200 nM AG1478 for 20 min, followed by 10 μM isoproterenol stimulation for the indicated times. 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. *C*, isoproterenol-mediated activation of Ras is insensitive to PTx. HEK293 cells were pretreated with 100 ng/ml PTx for 16 h and then stimulated with either 10 μM isoproterenol for 3 min or 10 μM carbachol for 5 min, as indicated. 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 $G\alpha_s$ and cAMP. Although signaling via $G\alpha_s$ is classically thought to be insensitive to PTx, recent reports have demonstrated that β_2AR can couple to ERKs via PTx-sensitive pathways (41). These studies, which utilized transiently transfected cDNAs encoding β_2AR in HEK293 cells, proposed a PKA-dependent switch in β_2AR affinity from G_s to G_i. In our hands, PTx did not block β_2AR's activation of ERKs, while blocking the action of known G_i-coupled agents, including carbachol, lysophosphatidic acid, and clonidine. It is possible that the ability of β_2AR to couple to PTx-sensitive pathways is dependent on elevated levels of β_2AR expression.

Both Ras-dependent and Rap1-dependent mechanisms of β_2AR'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 $G\alpha_s$ to elevate cAMP, also activated Rap1 but did not activate Ras. These data suggest that β_2AR utilized distinct pathways to activate Ras and Rap1. We propose that Rap1 is activated by $G\alpha_s$ (via cAMP and PKA), and that Ras is activated independently of $G\alpha_s$, possibly by a $\beta\gamma$-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 β_2AR's activation of ERKs. Two agents that interfere with Rap1 signaling, RapN17 and Rap1GAP1, were used. Overexpression of RapN17 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 β_2AR, 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 β_2AR'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 β_2AR in these cells is not known. Recently, the ability of β_2AR to activate Ras-dependent signaling has been suggested by Lefkowitz and colleagues. In their model, transiently transfected β_2AR utilized a PTx-sensitive pathway to transactivate the endogenous EGF receptor. However, using cells expressing endogenous β_2AR, 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 PTx. Since Ras activation by isoproterenol was not sensitive to H89, we propose that Ras activation by β_2AR is not mediated by PKA, G_i, or EGF receptor. We suggest that $\beta\gamma$ subunits, which have been shown to activate Ras in many systems, may contribute to β_2AR's actions. The ability of both α and $\beta\gamma$ to regulate ERK signaling following receptor binding may be a common mechanism of coordinating signals to ERKs. For example, hormones that are able to activate G_i-coupled pathways have been shown to modulate ERKs via both $\beta\gamma$ and α subunits. $\beta\gamma$ activates Ras via phosphoinositol 3-kinase γ (21) and $G\alpha_i$ activates a Rap1GAPII to inactivate Rap1 (19). Here, we show a second mechanism of Rap1 regulation by α subunits, the activation of Rap1 via elevation of intracellular cAMP levels. Although PKA-independent regulation of Rap1 by cAMP has been proposed (37, 38), the data shown here demonstrate that cAMP requires PKA to activate Rap1 in HEK293 cells, as well as other cell types (9, 35, 39).

The Rap1/B-Raf pathway identified here may be an important mechanism by which β_2AR 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, β_2AR-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 G_s-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 G_s-coupled receptors may antagonize Ras-dependent signaling to ERKs in cells that do not express B-Raf.

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