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Conformational Rearrangements and Signaling Cascades Involved in Ligand-Biased Mitogen-Activated Protein Kinase Signaling through the β1-Adrenergic Receptor

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ABSTRACT

In recent years, several studies have demonstrated that different ligands can have distinct efficacy profiles toward various signaling pathways through a unique receptor. For example, β1-adrenergic compounds that are inverse agonists toward the adenylyl cyclase (AC) can display agonist activity for the mitogen-activated protein kinase (MAPK) pathway. Such a phenomenon, often termed functional selectivity, has now been clearly established for many G protein-coupled receptors when considering distinct signaling output. However, the possibility that ligands could selectively engage distinct effectors to activate a single signaling output by promoting specific receptor conformations has not been extensively examined. Here, we took advantage of the fact that isoproterenol, bucindolol and propranolol (full, partial, and inverse agonists for the AC pathway, respectively) all activate MAPK through the β1-adrenergic receptor (β1AR) to probe such conformational-biased signaling. Although the three compounds stimulated MAPK in a src-dependent manner, isoproterenol acted through both $G\alpha_i\beta\gamma$- and G protein-independent pathways, whereas bucindolol and propranolol promoted MAPK activation through the G protein-independent pathway only. The existence of such distinct signaling cascades linking β1AR to MAPK activation was correlated with ligand-specific conformational rearrangements of receptor/G protein complexes measured by bioluminescence resonance energy transfer. Taken together, our data indicate that discrete local conformational changes can selectively promote the recruitment of distinct proximal signaling partners that can engage distinct signaling outputs and/or converge on the same signaling output.

G protein-coupled receptors (GPCRs) represent the most prevalent class of transmembrane signaling proteins. They can modulate a large variety of signaling systems to ensure a fine regulation of cell function in response to external stimuli. This signaling diversity is achieved in part by the capacity of one receptor to couple to diverse G proteins and non-G protein effectors (Bockaert et al., 2004). Over the past decade, many studies have demonstrated that different subsets of these effector systems can be selectively modulated by distinct ligands through a unique receptor, a phenomenon often referred to as ligand-biased signaling (Galandrin et al., 2007; Kenakin, 2007). Hence, efficacy of GPCR ligands is increasingly considered as a pluridimensional parameter that should include in its definition, in addition to the ligand/receptor pair, the signaling pathways considered.

Characterizing a panel of β-adrenergic ligands for their efficacy profiles toward two of the β1-adrenergic receptor (β1AR)-stimulated signaling pathways, adenylyl cyclase (AC) and mitogen-activated protein kinase (MAPK) path-

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ABBREVIATIONS: GPCR, G protein-coupled receptor; βAR, β-adrenergic receptor; AC, adenylyl cyclase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; BRET, bioluminescence resonance energy transfer; AVP, arginine 8 vasopressin; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine; PD98059, 2′-amino-3′-methoxyflavone; EGF, epidermal growth factor; CTX, cholera toxin B; PTX, pertussis toxin; PKA, protein kinase A; HRP, horseradish peroxidase; GFP, green fluorescent protein; GFP10, blue-shifted mutant of green fluorescent protein; βARK, β-adrenergic receptor kinase; HEK, human embryonic kidney; δOR, δ opioid receptor; V2R, vasopressin type 2 receptor; PTH, parathyroid hormone; siRNA, small interfering RNA; PBS, phosphate-buffered saline; Rluc, *Renilla reniformis* luciferase; TBS-T, Tris-buffered saline/Tween 20; p-, phospho; MEK, mitogen-activated protein kinase kinase.

ways, we found that compounds displaying opposite efficacy toward AC shared common agonistic activity for MAPK. Indeed, isoproterenol, bucindolol, and propranolol that are, respectively, full agonist, partial agonist, and inverse agonists for AC were found to act as agonists for the extracellular signal-regulated kinase (ERK) 1/2 pathway (Galandrin and Bouvier, 2006). The inverse efficacy of propranolol compared with bucindolol and isoproterenol for β1AR-stimulated AC clearly reveals the ability of the compounds to promote distinct receptor conformations. Because the three compounds activate MAPK, the data indicate that distinct receptor conformations can converge on the stimulation of a single downstream effector system.

Multiple pathways have been shown to link GPCR to MAPK activation. Some of these involve the generation of second messengers resulting from classic G protein activation, whereas others rely on the scaffolding properties of proteins such as βarrestin (Luttrell, 2005). However, the correlation between the signaling cascades leading to MAPK and specific receptor conformation that can be promoted by different ligands has not been established yet. We therefore took advantage of the fact that isoproterenol-, bucindolol-, and propranolol-promoted receptor conformations converged on ERK1/2 stimulation to link receptor conformations to specific effector cascades. For this purpose, the pathways leading to the β1AR-mediated activation of ERK1/2 were investigated for the three ligands, whereas the conformations of the liganded receptor were assessed by bioluminescence resonance energy transfer (BRET) measurements monitoring structural rearrangements within receptor/G protein complexes. We report that isoproterenol stimulated ERK1/2 through both G_i-dependent and G protein-independent mechanisms, whereas bucindolol and propranolol engaged MAPK only via the G protein-independent pathway. Note that the distinct signaling pattern of isoproterenol was associated with a unique conformational signature of the receptor/G protein complex, confirming that distinct ligands can select different signaling cascades by promoting discrete conformational rearrangements.

Materials and Methods

Reagents. (−)-Isoproterenol, DL-propranolol, Leu-enkephalin, and arginine-8 vasopressin (AVP) were purchased from Sigma-Aldrich (St. Louis, MO), whereas bucindolol was a generous gift from Dr. Michael Bristow (University of Colorado Health Sciences Center, Aurora, CO). Recombinant human epidermal growth factor (EGF) was from PeproTech (Rocky Hill, NJ). Cholera toxin B subunit (CTX) and pertussis toxin (PTX) were from Sigma-Aldrich. Inhibitors PP2 and PD98059 were from Calbiochem (San Diego, CA). Mouse anti-phosphorylated ERK1/2, rabbit anti-ERK1/2, and anti-βarrestin2 (H9) antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibody recognizing the phosphor-(Ser/Thr) protein kinase A (PKA) substrate was purchased from Cell Signaling Technology Inc. (Danvers, MA). HRP-anti-mouse and HRP-anti-rabbit polyclonal antibodies were from GE Healthcare (Baie d'Urfé, QC, Canada). All other reagents were of analytical grade, and they were obtained from various suppliers.

Expression Vectors. The plasmid encoding rat ERK2-GFP (DeFea et al., 2000b) and the β-adrenergic receptor kinase (βARK) carboxyl-terminal (C)-tail conjugated to the extracellular and transmembrane domain of the CD8 protein (T8βARKctail) (Crespo et al., 1995) were generous gifts from K. DeFea (University of California, Riverside, CA) and J. S. Gutkind (National Institutes of Health, Bethesda, MD), respectively. Plasmid encoding βarrestin1 deleted in its C-tail from amino acid 319 to amino acid 418 (β-Arr Δ318-419) was generously provided by Jeffrey L. Benovic (Thomas Jefferson University, Philadelphia, PA). Plasmids encoding the β1-adrenergic receptor fused to its C terminus with humanized *Renilla reniformis* luciferase (β1AR-h*R*Luc) or the blue variant of GFP (β1AR-GFP10) were described previously (Mercier et al., 2002). The expression vectors containing human G protein subunits ($G\alpha_{i1}$, $G\gamma_2$, and $G\beta_1$) were obtained from Missouri University of Science and Technology (Rolla, MO). Plasmids encoding $G\gamma_2$ fused at its N terminus to GFP10 (GFP10-$G\gamma_2$) or $G\alpha_{i1}$ tagged with h*R*Luc inserted between residues L91 and K92 ($G\alpha_{i1}$-91h*R*Luc) were described previously (Galés et al., 2006).

Stables Cell Lines and Transfections. HEK293S cells (Reeves et al., 1996) stably expressing the hemagglutinin-tagged human β1AR, the human δ-opioid receptor tagged with FLAG (δOR), or the myc-tagged V2 vasopressin receptor (V2R) were described previously (Petaja-Repo et al., 2000; Galandrin and Bouvier, 2006; Charest et al., 2007). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum, 100 U/ml penicillin and streptomycin, 2 mM L-glutamine, and 200 μg/ml G418, in a 37°C humidified 5% CO_2 atmosphere. For all BRET experiments, HEK293 cells were transiently transfected with the indicated BRET partners (tagged receptor and/or tagged G protein subunits) along with the complementary untagged G protein subunits, so to maintain the stoichiometric expression of the $G\alpha_{i1}\beta_1\gamma_2$ heterotrimer. For ERK phosphorylation assays, cells were transfected in six-wells plates, and they were harvested 48 h after transfection. In all cases, transient transfections were performed using the FuGENE 6 transfection reagent (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's protocol. The previously described siRNAs for βarrestin 1 and 2 (Ahn et al., 2003) were purchased from QIAGEN and transfected at 400 nM final concentration (300 nM βarrestin 1 siRNA and 100 nM βarrestin 2 siRNA) using the RNAiFect transfection Reagent (QIAGEN, Mississauga, ON, Canada), according to the manufacturer's protocol.

Quantification of cAMP Accumulation. Agonist induced cAMP accumulation was measured as described previously (Galandrin and Bouvier, 2006). Cells were grown in 60-mm dishes, and they were incubated for 16 h in Dulbecco's modified Eagle's medium, with or without 300 ng/ml CTX when indicated. The day of the experiment, cells were resuspended in PBS/0.1% glucose/1 mM 3-isobutyl-1-methylxanthine and treated for 20 min at 37°C with the indicated drugs. Propranolol was tested in the presence of 0.3 μM forskolin to increase the signal-to-noise ratio for detecting inhibition, because it has been described as an inverse agonist. After drug treatment, cells were immediately lysed, and cAMP levels were measured using the Catch Point cAMP kit (Molecular Devices, Sunnyvale, CA), according to the manufacturer's recommendations. In brief, cells lysates were incubated in 384-well plates coated with anti-cAMP antibodies in the presence of known amounts of HRP-cAMP. cAMP from cell lysates was allowed to compete with the HRP-cAMP for 2 h, and the remaining peroxidase activity was measured after three washes. The cAMP generated under the different conditions was interpolated from a cAMP standard curve generated in parallel for each experiment. Triplicates were used for each condition, and all experiments were repeated at least three times.

Western Blotting. Cells expressing β1AR, δOR, or V2R were seeded in poly-D-lysine-coated six-well plates. The next day, cells were washed once with PBS, and they were rendered quiescent by serum starvation for 16 h. Cells (~80% confluence) were then stimulated at 37°C with conditions corresponding to the maximal response obtained from time course and dose-response experiments: 4 min with 10 μM isoproterenol, 2 min with 10 μM bucindolol or propranolol, 5 min with 1 μM AVP or Leu-enkephalin, or 10 min with 1 ng/ml EGF. To terminate the stimulation, the media were rapidly removed, and cells were placed on ice and washed with ice-cold PBS before being lysed using 100 μl/well Laemmli sample buffer (62.5

mM Tris-HCl, 2% SDS, 10% glycerol, 50 mM dithiothreitol, and 0.1% bromphenol blue, pH 6.8). Whole cell lysates were sonicated, resolved by SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulose. The blots were then blocked at room temperature for 1 h with TBS-T buffer [50 mM Tris, pH 7.4, 150 mM NaCl, and 0.1% (v/v) Tween 20] containing 5% fat-free milk. Phospho-ERK1/2 or -ERK2-GFP were detected using mouse polyclonal anti-phospho p42/p44 ERK-specific antibody (1:3000, overnight at 4°C in TBS-T/5% fat-free milk). The immunoreactivity was revealed using a secondary HRP-conjugated anti-mouse antibody (1:10,000, 1 h at room temperature in TBS-T/5% fat-free milk), and the peroxidase activity was detected by chemiluminescence (PerkinElmer Life and Analytical Sciences, Waltham, MA). Blots were stripped and reprobed for total ERK using rabbit polyclonal anti-ERK1/2 antibody (1:25,000, 1 h at room temperature in TBS-T/5% fat-free milk) followed by HRP-anti-rabbit antibody (1:20,000, 1 h at room temperature in TBS-T/5% fat-free milk). Films were scanned, and band intensities were quantified using Quantity One software (Bio-Rad, Hercules, CA). ERK1/2 or ERK2-GFP phosphorylation was normalized according to the loading of proteins by expressing the data as a percentage of P-ERK1/2/ERK1/2 total (or P-ERK2-GFP/ERK2-GFP total) of the level observed in agonist-stimulated condition. When using P-ERK2-GFP, because a basal activity was detectable, it was not subtracted from the ligand-promoted ERK activity. Phosphorylation of PKA substrates was revealed using a rabbit polyclonal anti-P-SPKA antibody, detecting proteins containing a phosphor-Ser/Thr residue with arginine at the −3 position (1:1000, overnight at 4°C in TBS-T/5% bovine serum albumin), followed by anti-rabbit HRP-conjugated IgG (1:10,000, 1 h at room temperature in TBS-T/5% fat-free milk). Detection of βarrestin1 and -2 was achieved using the mouse monoclonal anti-βarrestin2 (H9) antibody, which recognizes both βarrestin isoforms (1:1000, overnight at 4°C in TBS-T/0.5% fat-free milk), followed by anti-mouse HRP-conjugated IgG (1:5000, 1 h at room temperature in TBS-T/0.5% fat-free milk).

Bioluminescence Resonance Energy Transfer Measurement. *R*luc- and GFP10-tagged receptor or G protein constructs were transiently transfected into HEK293 cells. Forty-eight hours after transfection, cells were washed twice with PBS, detached with PBS/5 mM EDTA, and resuspended in PBS/0.1% (w/v) glucose at room temperature. Cells were then distributed (50 μg of protein per well) in a 96-well microplate (Optiplate; PerkinElmer Life and Analytical Sciences) and incubated in the presence or absence of the different ligands for 1 min. BRET between *R*luc and GFP10 was measured after the addition of the *R*luc substrate DeepBlueC coelenterazine (5 μM) (PerkinElmer Life and Analytical Sciences). BRET^2 readings were collected using a modified TopCount NXT apparatus (customized version purchased from BioSignal Packard, Inc., now; PerkinElmer Life and Analytical Sciences) that allows sequential integration of signals detected in the 370- to 450- and 500- to 530-nm windows, using filters with the appropriate band pass (Chroma Technology Corp., Brattleboro, VT). The BRET signal was calculated as the ratio of the light emitted by GFP10 (510–550 nm) over the light emitted by *R*luc (460–500 nm).

Statistical Analysis. Statistical analysis and curve fitting were done using Prism 3.01 (GraphPad Software, San Diego, CA). Statistical significance of the differences was assessed using one-way analysis of variance and post hoc Bonferroni's test. In some cases, one-way analysis of variance followed by Dunnett's test was used to determine statistically significant differences from basal.

Results

Ligand Profiles. HEK293 cells stably expressing the human β1AR were generated as described previously (Galandrin and Bouvier, 2006). The ability of the three selected compounds to modulate the AC and ERK1/2 pathways was first assessed to confirm their reported activity toward these two signaling pathways. As reported previously (Galandrin and Bouvier, 2006), isoproterenol is an efficacious agonist toward AC, whereas bucindolol behaves as a partial agonist, reaching approximately one third of the maximal isoproterenol-promoted response (Fig. 1A). Propranolol for its part acted as an inverse agonist, leading to an inhibition of the forskolin-induced cAMP accumulation (Fig. 1B). These compounds were next evaluated in the same cells on the ERK1/2 pathway. As shown in Fig. 1C, the three compounds promoted ERK1/2 activation, with isoproterenol being the most efficacious ligand and bucindolol and propranolol acting as equivalent partial agonists. Thus, two efficacy profiles could be distinguished. Isoproterenol and bucindolol behaved as

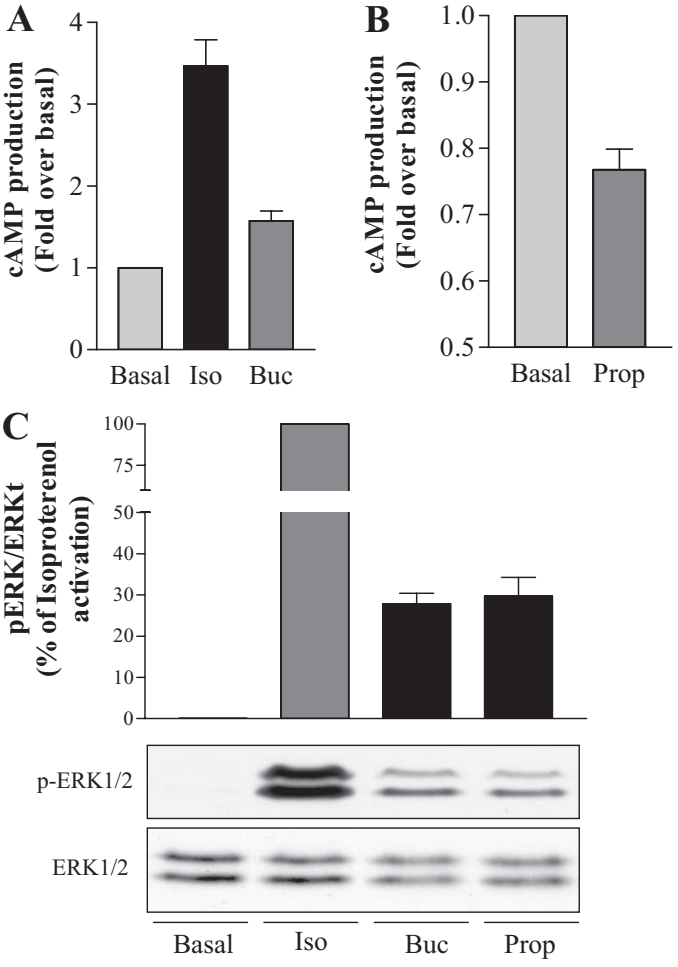


Fig. 1. Profiles of the different ligands on the β1AR adenylate cyclase and ERK1/2 pathways. cAMP accumulation experiments were performed in HEK293 cells stably expressing β1AR. E_{max} values were tested using a 10 μM concentration of the indicated ligands, without (A) or with (B) 0.3 μM forskolin. Data are expressed as -fold increase or decrease of cAMP accumulation over basal conditions. cAMP content under basal conditions were 3.94 ± 0.56 pmol/10^6 cells (A) and 8.02 ± 0.48 pmol/10^6 cells (B). Data represent the mean ± S.E. of at least three experiments performed in triplicates. C, serum-starved HEK293 cells stably expressing β1AR were stimulated at 37°C with 10 μM isoproterenol (4 min), 10 μM propranolol, or 10 μM bucindolol (2 min). Cells were then lysed in Laemmli sample buffer and subjected to SDS-polyacrylamide gel electrophoresis. MAPK activity was detected by Western blot using a phospho-specific anti-ERK1/2 antibody (p-ERK1/2). Expression level of ERK1/2 was controlled using an antibody directed against the total kinase population (ERK1/2), and data are expressed as a percentage of pERK/ERK of the level observed in isoproterenol-stimulated conditions. Data represent the mean ± S.E. of at least three independent experiments.

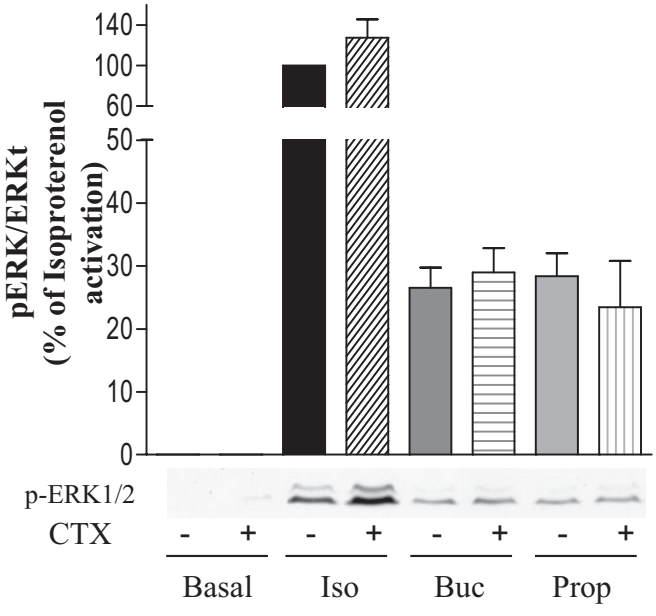
agonists for the two signaling pathways, whereas propranolol displayed an opposite efficacy profile, being an inverse agonist for the AC pathway but a partial agonist in stimulating ERK1/2 phosphorylation. No response was observed for any of the three compounds in untransfected HEK293 cells, confirming that the ligand-promoted AC and ERK modulation are β1AR-mediated (data not shown). The distinct efficacy profiles observed for the two pathways are not an idiosyncrasy of the cell line selected because similar results were obtained in cells transiently expressing the β1AR.

Involvement of Heterotrimeric G Proteins. To determine whether three compounds displaying distinct efficacies toward AC (full agonist, isoproterenol; partial agonist, bucindolol; and inverse agonist, propranolol) can engage distinct ERK1/2 activation mechanisms, the effector systems specifically stimulated by the ligand-bound receptor for the activation of the MAPK pathway were investigated. The potential implication of Gα proteins was first examined. To test the role of $G\alpha_s$, we subjected HEK293 cells stably expressing the human β1AR to a sustained treatment with CTX, an approach that was described previously to down-regulate $G\alpha_s$ in HEK293 cells (Seidel et al., 1999; Charest et al., 2007). The CTX-promoted knockdown of $G\alpha_s$ was confirmed by the observed inhibition of isoproterenol-induced cAMP accumulation and PKA substrate phosphorylation (Fig. 2, inset). ERK1/2 phosphorylation stimulated by isoproterenol, propranolol, and bucindolol remained unaffected by the CTX-promoted knockdown of $G\alpha_s$ (Fig. 2), indicating that $G\alpha_s$ engagement is not necessary for ERK1/2 activation by any of the ligands.

Because several $G\alpha_s$-coupled receptors, notably the β1- and β2AR, were shown to promote ERK1/2 activation through a switch of coupling from $G\alpha_s$ to $G\alpha_i$ (Daaka et al., 1997; Martin et al., 2004), the contribution of $G\alpha_i$ to the isoproterenol-, bucindolol- and propanolol-promoted ERK1/2 activation was evaluated. The selective inhibition of $G_{i/o}$ signaling by PTX treatment partially blocked the isoproterenol-mediated ERK1/2 activation, but it did not affect either bucindolol- or propranolol-stimulated ERK1/2 activity (Fig. 3A). Note that the inhibition of isoproterenol-stimulated ERK1/2 phosphorylation by the PTX treatment was not complete, leaving 30% of the response unaffected (Fig. 3A). This residual response did not result from an incomplete inhibition of G_i because the same PTX treatment fully blocked Leu-enkephalin-promoted MAPK stimulation through the $G\alpha_i$-coupled δOR (Fig. 3A, inset). These results suggest that isoproterenol relies on $G\alpha_i$ protein to promote ERK1/2 activation, whereas bucindolol and propanolol do not.

The $G\alpha_i$ contribution to isoproterenol- but not bucindolol- or propranolol-stimulated ERK1/2 activation was confirmed by directly assessing the ability of the compounds to activate $G\alpha_i$. For that purpose, we took advantage of a BRET-based assay monitoring the interaction between $G\alpha_{i1}$-h*R*luc and GFP10-$G\gamma_2$ fusion proteins, a BRET pair that was previously shown to reflect G protein activation (Galés et al., 2005, 2006). As shown in Fig. 3B, stimulation of β1AR with isoproterenol led to a decrease in BRET between $G\alpha_{i1}$-h*R*luc and GFP10-$G\gamma_2$, reflecting a separation between $G\alpha_{i1}$ and Gβγ subunits that is characteristic of G protein activation. In contrast, no BRET signal modulation was observed when cells were stimulated with bucindolol or propranolol, suggesting that these two compounds are unable to promote $G\alpha_{i1}$ activation.

Because Gα and Gβγ protein subunits are known to engage distinct effectors to activate MAPK (Crespo et al., 1994; Neves et al., 2002), the potential role of Gβγ in the isoproterenol-, bucindolol-, and propanolol-mediated ERK1/2 activation was also evaluated. For this purpose, we took advantage of the previously described Gβγ scavenger consisting of the C-tail of GRK2 (βARK) conjugated to the extracellular and transmembrane domains of the CD8 protein that provides a membrane anchor for the C-tail of βARK (T8βARKctail) (Crespo et al., 1995). Coexpression of this scavenger of Gβγ with ERK2-GFP significantly inhibited iso-



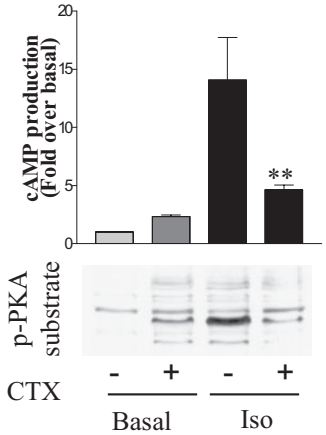


Fig. 2. $G\alpha_s$ is not involved in the ERK1/2 signaling cascades promoted by the three ligands. Serum-starved HEK293 cells stably expressing β1AR were pretreated or not with 300 ng/ml CTX for 16 h before stimulation at 37°C with 10 μM isoproterenol (4 min), 10 μM propranolol, or 10 μM bucindolol (2 min). ERK1/2 activation was detected and quantified as described in Fig. 1C. Inset, isoproterenol-induced cAMP accumulation (top) and PKA substrate phosphorylation (bottom). Absolute value for cAMP content under control conditions was 4.96 ± 1.18 pmol/10^6 cells. Data represent the mean ± S.E. of at least three independent experiments. Statistical significance of the differences was assessed using one-way analysis of variance and post hoc Bonferroni test, **, $p < 0.01$.

proterenol-stimulated ERK2-GFP activity (Fig. 4A). In contrast, the G$\beta\gamma$ scavenger did not inhibit the bucindolol- and propranolol-stimulated ERK1/2, but rather it increased this response (Fig. 4A), demonstrating that G$\beta\gamma$ activation is not required for these two ligand-promoted responses. Such transient cotransfection protocol tended to increase the detected basal ERK activity differentially in the presence and absence of the dominant-negative mutants. This precluded us from subtracting the basal ERK activity as was done in other experimental conditions in which it was found to be marginal. The basal are shown as percentage of isoproterenol-stimulated activity. This rise in MAPK basal activity upon G$\beta\gamma$ sequestration may be due to increased receptor surface expression resulting from a reduction in GRK-promoted phosphorylation and endocytosis, thus leading to detectable spontaneous activity. Similar effects were also seen after βarrestin knockdown (Fig. 5). The potentialization of propanolol- and bucindolol-stimulated ERK1/2 activation upon T8-βARKctail expression is consistent with this notion.

Note that the G$\beta\gamma$ sequestration led to a partial blockade of the isoproterenol-stimulated ERK1/2 as was observed after PTX treatment, suggesting a major contribution of the G$\beta\gamma$ subunits originating from G_i in the isoproterenol-stimulated MAPK activation. The persistence of some ERK1/2 activity after Gα_i/G$\beta\gamma$ inactivation indicates the contribution of a G_i-independent component to the isoproterenol-stimulated response. As shown in Fig. 4B, the maximal ERK1/2 stimulation induced by isoproterenol in the absence of PTX was reached after 4 min, whereas the maximal response of bucindolol and propanolol peaked at 2 min. Removal of the G_i component by PTX shifted the time course of the isoproterenol-stimulated ERK1/2 response so that it became similar to the kinetic profiles observed for bucindolol and propranolol. These data indicate that two kinetically distinct components contribute to the isoproterenol-stimulated response: a late component (peaking at 4 min) that most likely reflects the G_i-dependent response, and a more rapid G_i-independent phase (peaking at 2 min) that may also be shared by bucindolol and propranolol.

Involvement of the Scaffolding Protein βArrestin. In addition to their role in receptor desensitization and internalization, βarrestins are now known to scaffold members of

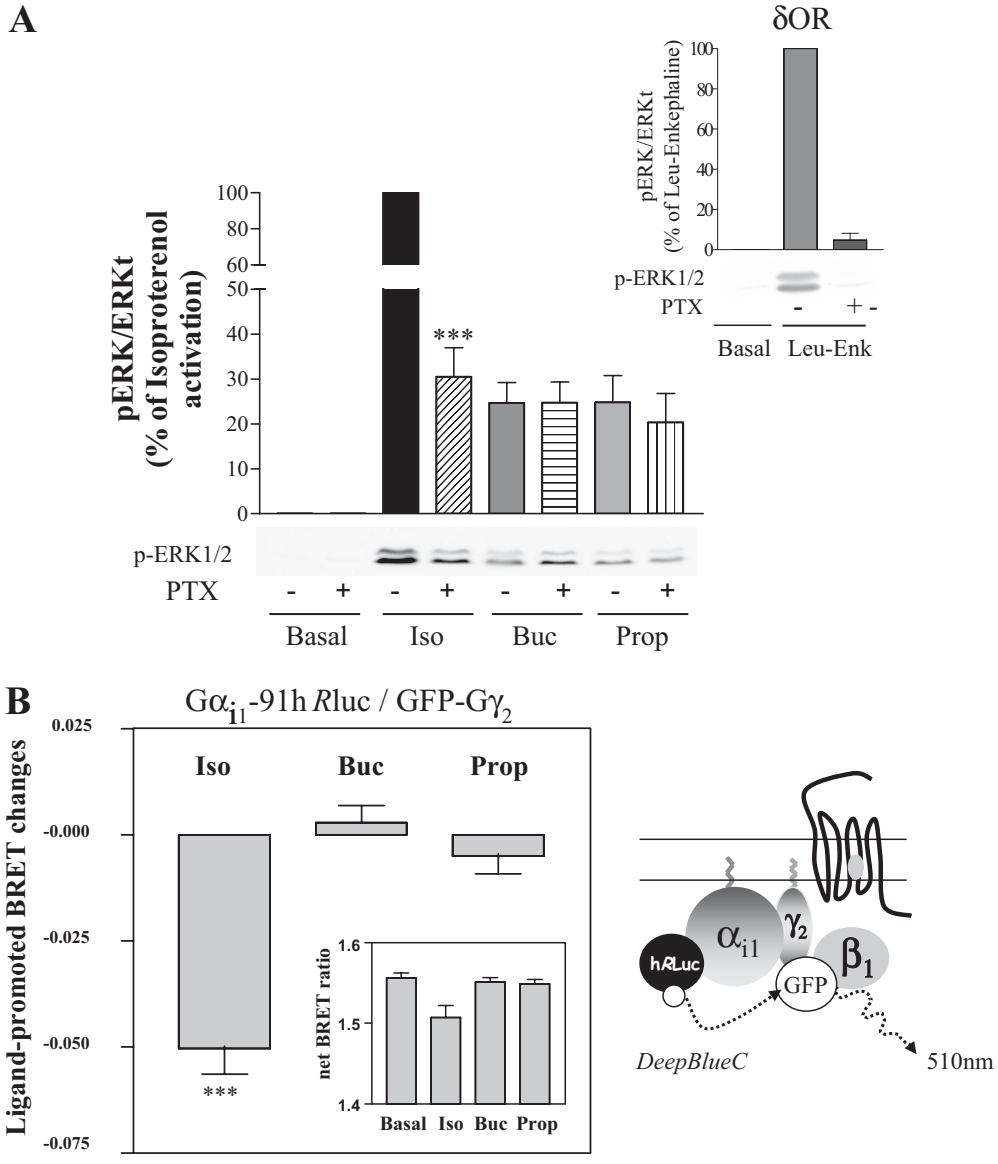


Fig. 3. G_i engagement is required for isoproterenol- but not bucindolol- and propranolol-stimulated ERK1/2 activity. A, serum-starved HEK293 cells stably expressing β1AR or the δOR (inset) were pretreated or not with 100 ng/m PTX for 16 h before stimulation at 37°C with 10 μM isoproterenol (4 min), 10 μM propranolol, or 10 μM bucindolol (2 min), or 1 μM Leu-enkephalin (5 min). ERK1/2 activation was detected and quantified as described in Fig. 1C. Statistical significance of the differences was assessed using one-way analysis of variance and post hoc Bonferroni's test; ***, $p < 0.001$. B, BRET was measured in cells coexpressing Gα_{i1}-91h*R*luc and GFP10-Gγ_2, in the presence of untagged β1AR and stimulated or not with 10 μM isoproterenol (Iso), 10 μM bucindolol (Buc), or 10 μM propranol (Prop). Results are expressed as the difference in the BRET signal measured in the presence and the absence of the indicated ligands. Inset, net BRET values obtained between Gα_{i1}-91h*R*luc and GFP10-Gγ_2 (bar graph) and schematic representation of the proteins used in the assay. Data represent the mean ± S.E. of at least three independent experiments. One-way analysis of variance followed by Dunnett's test was used to determine statistically significant differences from basal; ***, $p < 0.001$.

the MAPK signaling cascade and to contribute to some GPCR-mediated MAPK activation (Luttrell et al., 1999; DeFea et al., 2000a; McDonald et al., 2000; Miller et al., 2000; Luttrell et al., 2001). Hence, to test their involvement in isoproterenol-, bucindolol-, and propranolol-induced ERK1/2 stimulation, we used a C-tail truncated form of βarrestin 1 (βarr Δ318-419) as a dominant-negative for βarrestin. Overexpression of this construct in cells stably expressing V2R, a receptor described previously to activate ERK1/2 via βarrestin (Charest et al., 2007), significantly decreased the AVP-induced ERK1/2 stimulation (Fig. 5A, inset), confirming the dominant-negative activity of βarr Δ318-419. However, using the same conditions, ERK1/2 response induced by isoproterenol, bucindolol, and propranolol was not affected by the βarrestin dominant-negative mutant (Fig. 5A). Of notice, as was the case in the T8-βARKctail cotransfection experiments, the basal ERK1/2 activity observed in the experiments testing the role of βarrestin tended to be elevated. This, most likely, results from the fact that, to properly assess the effects of the dominant-negative constructs, ERK2-GFP needed to be cotransfected, leading to an increased basal signal resulting from its intrinsic activity. As a consequence, the bucindolol- and propranolol-stimulated responses were smaller than those observed in the absence of ERK2-GFP expression.

The lack of involvement of βarrestin in the β1AR-stimulated ERK1/2 response was further confirmed by the observation that intracellular depletion of both βarrestin isoforms (Fig. 5B, inset), using siRNAs, did not inhibit ERK1/2 phosphorylation promoted by the three ligands (Fig. 5B). Of notice, both siRNA and dominant-negative approaches targeting βarrestins led to a potentiation of the β1AR-promoted MAPK response, most likely resulting from an increased receptor cell surface density as a consequence of a blunted βarrestin-mediated endocytosis. Note that the increases observed in ERK1/2 activity upon βarrestin depletion were not proportional to the responses elicited by each ligand in normal conditions; propranolol and bucindolol stimulation led to a level of ERK1/2 activation similar to that of isoproterenol upon knockdown of βarrestins. The smaller relative potentiating effect on the isoproterenol response may result from a saturation of the response for the full agonist as a consequence of limited signaling partners in the context of the increased cell surface receptor.

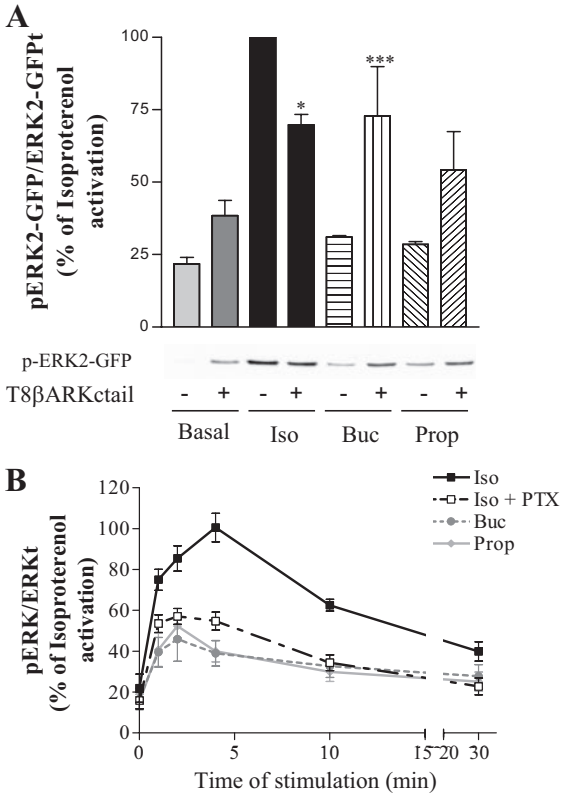


Fig. 4. Gβγ engagement is required for isoproterenol- but not bucindolol- and propranolol-stimulated ERK1/2 activity. A, cells stably expressing β1AR were cotransfected with ERK2-GFP and either T8βARKctail (the dominant negative of Gβγ, +) or pcDNA3.1 (−), and serum-starved before activation at 37°C with 10 μM isoproterenol (4 min), 10 μM propranolol, or 10 μM bucindolol (2 min). Expression of total and phosphorylated ERK2-GFP was detected and quantified as described in Fig. 1C. Statistical significance of the differences was assessed using one-way analysis of variance and post hoc Bonferroni's test; *, $p < 0.05$; ***, $p < 0.001$. B, serum-starved HEK293 cells stably expressing β1AR were pretreated or not with 100 ng/ml PTX for 16 h before stimulation at 37°C with 10 μM isoproterenol, 10 μM propranolol, or 10 μM bucindolol for the indicated time. ERK1/2 activation was detected and quantified as described in Fig. 1C. Data represent the mean ± S.E. of at least three independent experiments.

Involvement of the Src and MEK Kinases. Because several studies documented the role of Src tyrosine kinase proteins in GPCR-mediated MAPK activation (Luttrell et al., 1999; Cao et al., 2000; DeFea et al., 2000a; Charest et al., 2007), we sought to determine whether this cytosolic kinase family was involved in β1AR-mediated ERK1/2 activation induced by isoproterenol, bucindolol, and propanolol. For that purpose, we assessed the effect of Src family proteins tyrosine kinase inhibition using the selective inhibitor PP2. As shown in Fig. 6A, such treatment almost completely blocked isoproterenol-, bucindolol-, and propranolol-stimulated ERK1/2 activity, suggesting that Src tyrosine kinase proteins play a key role in the MAPK signaling cascades promoted by the three compounds. For isoproterenol, the extensive inhibition of ERK1/2 activation upon PP2 treatment indicates that Src is involved in both G_i-dependent and -independent β1AR-mediated MAPK stimulation. Likewise, the inhibition of the penultimate kinase involved in the prototypical ERK1/2 signaling cascade, MEK, completely blocked the response promoted by the three ligands (Fig. 6B), suggesting the convergence of the G_i-dependent and -independent pathways.

Linking Receptor Conformational Changes to Signaling Efficacy. The above-mentioned data indicate that, apart from having different efficacy profiles toward AC and MAPK pathways, isoproterenol, bucindolol, and propranolol also differ in their ability to engage G_i, the MAPK activation being partially G_i-dependent for isoproterenol but G_i-independent for bucindolol and propranolol. Such ligand-selective behavior, variously referred to as "ligand-directed trafficking of receptor stimulus," "collateral efficacy," "ligand-biased signaling," or "functional selectivity," is believed to originate from distinct ligand-promoted conformational reorganization of the receptor (Galandrin et al., 2007). To directly probe the link between the conformations and the functional selectivity promoted by the three ligands, we took advantage of a BRET-based assay to monitor structural rearrangement within receptor/G protein complex (Galés et al., 2006). This receptor/G protein

BRET sensor was previously demonstrated to reflect ligand-induced conformational rearrangements leading to changes in the distance between the receptor carboxyl tail and either Gα or Gγ. As shown in Fig. 7A, stimulation of the β1AR with isoproterenol increased the BRET between β1AR-h*R*Luc and GFP10-$G\gamma_2$ in the presence of untagged $G\alpha_i$, which was selected rather than $G\alpha_s$ because of its differential involvement in the β1AR-mediated MAPK activation. In the same conditions, bucindolol and propranolol both decreased the BRET signal between the two partners. Similar results were obtained when BRET was measured between $G\alpha_{i1}$-h*R*Luc and β1AR-GFP10 (Fig. 7B), indicating that isoproterenol promoted a conformational state of the β1AR that is distinct from those induced by bucindolol and propranolol. However, it was not possible to distinguish the specific conformational rearrangements promoted by bucindolol and propranolol, even when BRET was measured between β1AR-h*R*Luc and GFP10-$G\gamma_2$ in the presence of untagged $G\alpha_s$ or between $G\alpha_s$-h*R*Luc and β1AR-GFP10 (data not shown).

Discussion

The three βAR ligands tested in the present study displayed distinct efficacy profiles toward AC and MAPK, confirming the existence of functional selectivity for β1AR ligands (Galandrin and Bouvier, 2006). Whereas isoproterenol, bucindolol, and propranolol behaved as full, partial, and inverse agonists, respectively, on AC, the three compounds are agonists on MAPK. An additional level of selectivity was revealed when the signaling cascades leading to ERK1/2 activation were examined. The partial agonists bucindolol and propranolol activated ERK1/2 in a G_i-independent manner, whereas both G_i-dependent and -independent pathways contributed to the isoproterenol-stimulated response, indicating that different ligands can recruit distinct subsets of signaling effectors to activate a single pathway. This is reminiscent of few other cases where distinct effectors were selectively engaged by different ligand/receptor pairs to stimulate a common downstream signaling integrator. For example, whereas PTH1-34 was found to activate MAPK through both G_s and βarrestin, PTH7-34 and PTHrp1-36 en-

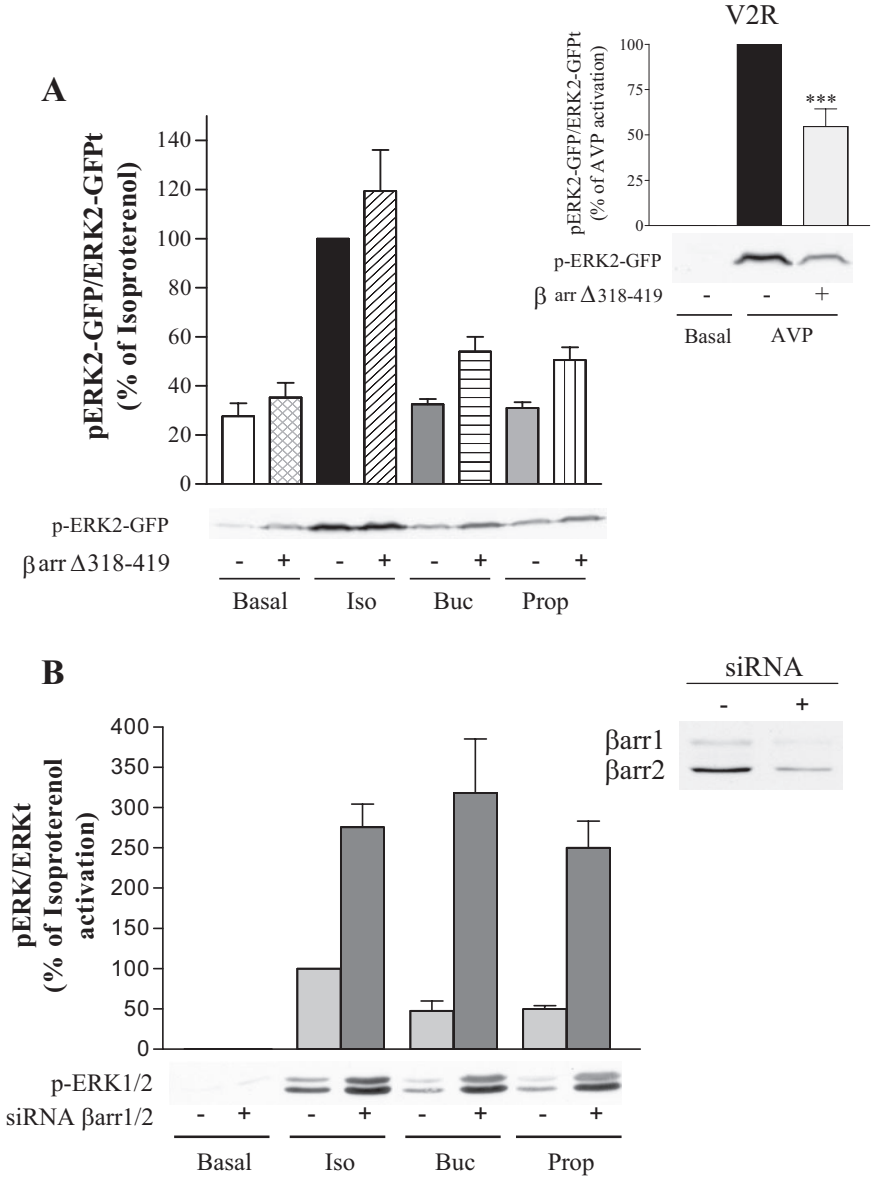


Fig. 5. βArrestin is not involved in the ERK1/2 signaling cascades promoted by the three ligands. A, cells stably expressing the β1AR or the V2R (inset) were cotransfected with ERK2-GFP and either βarrestin1 Δ318-419 (+) or pcDNA3.1 (−), and serum-starved before stimulation at 37°C with 10 μM isoproterenol (4 min), 10 μM propranolol, or 10 μM bucindolol (2 min), or 1 μM AVP (5 min). Expression of total and phosphorylated ERK2-GFP was detected and quantified as described in Fig. 1C. Statistical significance of the differences was assessed using one-way analysis of variance and post hoc Bonferroni's test; ***, $p < 0.001$. B, HEK293 cells stably expressing β1AR were transfected with siRNAs targeting both βarrestin isoforms or nonspecific siRNA (−), and they were serum-starved before stimulation at 37°C with 10 μM isoproterenol, 10 μM propranolol, or 10 μM bucindolol. βArrestin knockdown was assessed by subjecting cell lysates to Western blot using the anti-βarrestin2 (H9) antibody recognizing both βarrestin isoforms (inset). ERK1/2 activation was detected and quantified as described in Fig. 1C. Data represent the mean ± S.E. of at least three independent experiments.

gaged either βarrestin or the G protein, respectively (Gesty-Palmer et al., 2006). Likewise, although binding of angiotensin-II to its type 1 receptor AT1R engaged both G_q and βarrestin, a synthetic angiotensin-II analog stimulated MAPK in an exclusively βarrestin-dependent manner (Wei et al., 2003). In addition, in a recent study, two β-adrenergic ligands, cyclopentylbutanephrine and isoproterenol, were found to rely differentially on βarrestin to activate ERK1/2 (Drake et al., 2008). The assessment in the present study of two signaling responses (i.e., AC and MAPK) allowed to further refine the level of selectivity by distinguishing two compounds that share the same G_i-independent MAPK signaling signature (bucindolol and propranolol), but they have opposite efficacies (i.e., agonist versus inverse agonist) when considering AC activity.

Although ERK1/2 activation can lie downstream of AC stimulation (Vossler et al., 1997; Grewal et al., 2000), our results clearly indicate that the two pathways can be independently regulated in HEK293 cells, as demonstrated previously for the A2-adenosine receptor (Seidel et al., 1999). Indeed, even though isoproterenol and bucindolol are agonists for the two signaling systems, their ability to activate ERK1/2 was found to be independent of their positive coupling to cAMP generation (Fig. 2). This independence of the ERK1/2 activation from cAMP production is further substan-

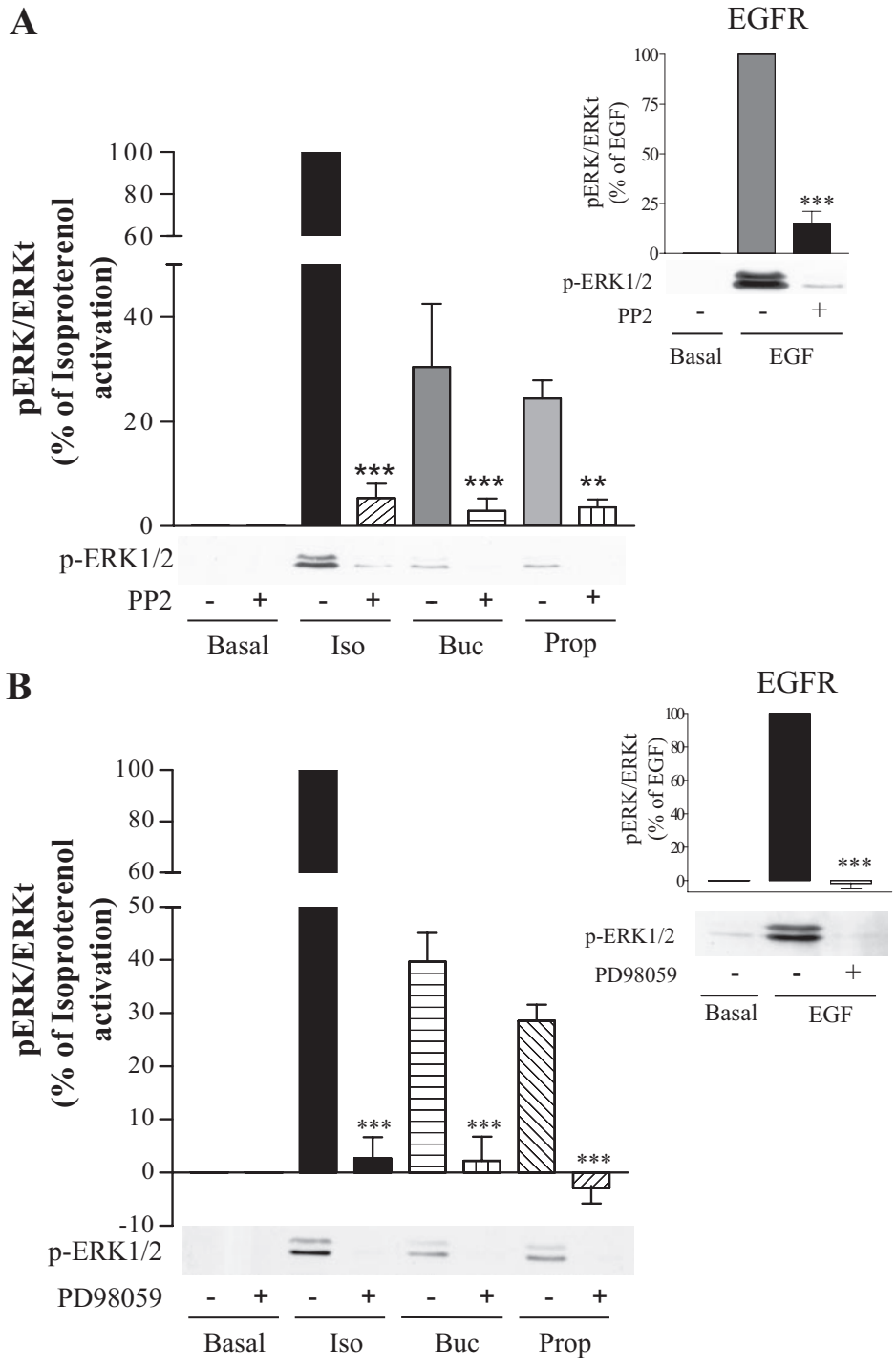


Fig. 6. Src and MEK kinases are involved in the ERK1/2 signaling cascades promoted by the three ligands. Serum-starved HEK293 cells stably expressing the β1AR were pretreated or not for 1 h at 37°C with the Src inhibitor PP2 (10 μM) (A) or with the MEK inhibitor PD98059 (50 μM) (B) before stimulation with 10 μM isoproterenol (4 min), 10 μM propranolol, or 10 μM bucindolol (2 min), or 1 ng/ml EGF (10 min; see insets). ERK1/2 activation was detected and quantified as described in Fig. 1C. Data represent the mean ± S.E. of at least three independent experiments. For A and B, statistical significance of the differences was assessed using one-way analysis of variance and post hoc Bonferroni test; **, $p < 0.01$ and ***, $p < 0.001$.

tiated by the fact that propranolol, which acts as an inverse agonist for AC, stimulated the ERK1/2 activity to the same extent than bucindolol. The observation that propranolol acts as an inverse agonist for AC but as a partial agonist for ERK1/2 activities clearly demonstrates the occurrence of functional ligand selectivity at the β1AR.

Functional selectivity was also revealed by the distinct ERK1/2 cascades promoted by the three β-adrenergic ligands. Indeed, inactivation of G_i by PTX treatment partially inhibited isoproterenol- but not bucindolol- or propranolol-stimulated ERK1/2. Such ligand-specific sensitivity to chemical G_i knockdown suggests that only isoproterenol can induce β1AR coupling to G_i. This differential involvement of G_i in the β1AR-stimulated ERK1/2 cascades was further confirmed by the observation that isoproterenol but not bucindolol or propranolol promoted G_i activation, as reflected by the decrease in the BRET signal detected between $G\alpha_{i1}$ and $G\gamma_2$. The involvement of G_i in the isoproterenol-stimulated ERK1/2 activation has been proposed previously for the β1AR in Chinese hamster ovary cells (Martin et al., 2004). As is often the case for G_i-dependent activation of ERK1/2 (Crespo et al., 1994; Koch et al., 1994; Della Rocca et al., 1997), the isoproterenol-stimulated MAPK activation involves $G\beta\gamma$, as indicated by the inhibitory action of the $G\beta\gamma$ scavenger T8βARKctail. Given that chronic CTX treatment should inhibit $G\beta\gamma$ activation arising from $G\alpha_s$ stimulation, the lack of CTX inhibitory effect on the isoproterenol-stimulated ERK1/2 activity suggests that $G\beta\gamma$ originating from G_i but not G_s account for the MAPK activation.

Among the three ligands tested, only isoproterenol led to ERK1/2 activation through the engagement of both G_i-dependent and -independent pathways. Note that these two pathways require the activation of Src tyrosine kinases for the ERK1/2 response, indicating a convergence of all cascades on a single integrator. Having several distinct cascades converging on the MAPK could have many important biological implications. For one thing, the existence of more than one pathway regulating a common cell signal generates additional intermediates that offer more control and check points to fine tune the response. Obvious consequences of such signaling convergence could include changes in the intensity and spatiotemporal characteristic of the response. For example, the greater maximal ERK1/2 response observed for isoproterenol compared with bucindolol and propranolol could reflect the convergence of the G_i-dependent and -independent pathways. This hypothesis is supported by the observation that PTX treatment blunted the isoproterenol-stimulated response to a level equivalent to that reached upon bucindolol and propranolol stimulation. Removing the G_i component also affected the kinetics of the isoproterenol-mediated response, consistently shifting the peak stimulation from 4 to 2 min. It follows that the time course and amplitude of the isoproterenol-stimulated response upon G_i inactivation becomes almost identical to those of bucindolol and propranolol,

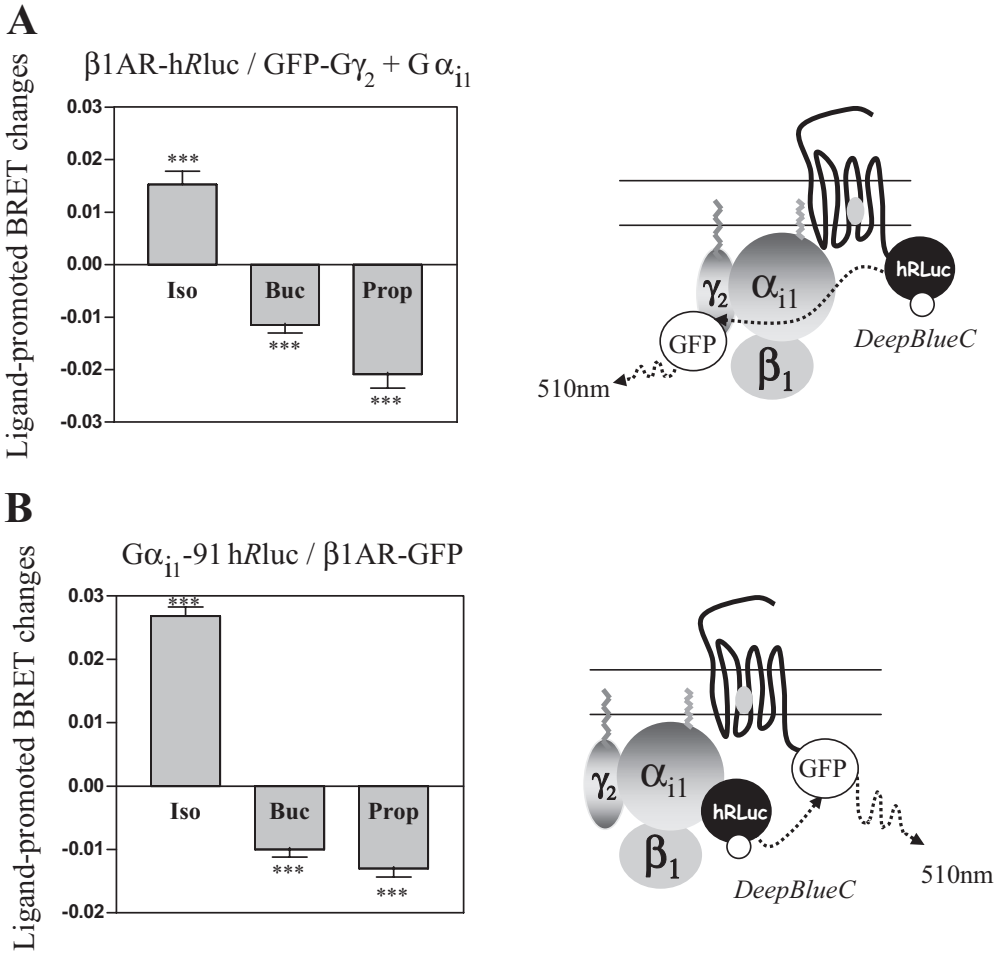


Fig. 7. Linking receptor conformational changes to signaling efficacy. BRET was measured in cells coexpressing β1AR-h*R*luc and GFP10-$G\gamma_2$ in the presence of untagged $G\alpha_{i1}$ (A) or β1AR-GFP10 and $G\alpha_{i1}$-91h*R*luc (B), and stimulated or not with 10 μM isoproterenol (Iso), 10 μM bucindolol (Buc), or 10 μM propranolol (Prop). Results are expressed as the difference in the BRET signal measured in the presence or absence of the indicated ligands. A schematic representation of the proteins used is shown (insets). Data represent the mean ± S.E. of at least three independent experiments. For A and B, one-way analysis of variance followed by Dunnett's test was used to determine statistically significant differences from basal; ***, $p < 0.001$.

indicating a common G_i-independent pathway for the three ligands. Although the downstream consequences of the differences in MAPK activation kinetics were not investigated in the present study, different temporal patterns of ERK1/2 activation have previously been suggested to have distinct consequences on gene regulation and cell proliferation (Murphy et al., 2004; Santos et al., 2007). Multicomponent kinetics of GPCR-promoted ERK1/2 activation have been reported previously for several receptors, including parathyroid hormone receptor, β2-adrenergic receptor, and angiotensin II receptor (Ahn et al., 2004; Gesty-Palmer et al., 2006; Shenoy et al., 2006). In these studies, the early activation phase has been attributed to G protein-dependent pathways, whereas βarrestins contribution has been linked to the late phase. However, the kinetics observed for those receptors were different from the kinetics detected herein for the β1AR, with the late phase peaking at much longer times than 4 min. Consistent with this difference, a βarrestin dominant-negative mutant and cellular depletion of βarrestins by siRNAs failed to inhibit the isoproterenol-, bucindolol-, and propranolol-stimulated ERK1/2, indicating that βarrestin is not involved in the β1AR-promoted MAPK activation by these three compounds. This may not be surprising given the low affinity of βarrestins for the β1AR (Shiina et al., 2000). The phase peaking at 4 min in our study was found to be G_i-dependent, and it most likely corresponds to the G protein-dependent early phase observed in previous studies. Our results thus reveal the existence of an even earlier phase (peaking at 2 min), which is independent of both G proteins and βarrestin. This does not rule out the possibility that β1AR could promote βarrestin-dependent ERK1/2 activity upon stimulation by different ligands. Indeed, in a recent study, Noma et al. (2007) reported that the selective β1AR agonist dobutamine can activate ERK1/2 in a βarrestin-dependent manner, further supporting the notion of ligand directed signaling. The existence of a G protein and βarrestin independent, but Src-dependent ERK1/2 activation pathway promoted by bucindolol and propranolol in our study, has also been recently revealed for the 5-hydroxytryptamine 4 receptors (Barthet et al., 2007)

Taken together, our results clearly demonstrate the occurrence of functional selectivity of the β1AR/ligand pairs not only toward the two signaling outputs studied (i.e., AC and MAPK) but also between two distinct pathways converging on the same output (i.e., MAPK). This implies that several distinct receptor conformations can be selectively promoted by different ligands. In line with this notion, BRET measured between the carboxyl tail of the β1AR and either $G\alpha_{i1}$ or $G\gamma_2$ revealed distinct conformational rearrangements of the receptor/G protein complex upon activation with the different ligands. Conformational rearrangements induced by isoproterenol could be readily distinguished from those promoted by either bucindolol or propranolol, the orientation of the BRET changes being opposite. Previous biophysical studies monitoring the fluorescent properties of intramolecular probes within purified receptors demonstrated that different ligands can induce distinct conformations of the receptor (Ghanouni et al., 2001; Swaminath et al., 2005). Our findings extend such observation to living cells and start establishing correlation between specific conformational changes promoted by ligands and their ability to selectively engage distinct signaling pathways. However, not all distinct conformational changes could be detected by the BRET biosensor used. Indeed, given that bucindolol and propranolol have opposite efficacy toward AC, they should promote distinct structural rearrangements. Yet, this difference could not be detected, with both ligands promoting a decrease in BRET between the receptor and the G protein subunits. This is not surprising when considering that BRET can only monitor the changes in distance between two specific points in the complex. Thus, the lack of difference in ligand-promoted BRET only indicates that local conformational changes maybe shared by the ligands, but it does not allow to rule out the occurrence of different conformational rearrangement in spatially distinct domains of the receptor. Additional biosensors monitoring structural rearrangements from multiple view points should, in the future, allow to precisely link specific local receptor conformations to distinct signaling pathways.

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