Effect of Different G Protein-coupled Receptor Kinases on Phosphorylation and Desensitization of the \( \alpha_{1B} \)-Adrenergic Receptor*

Dario Diviani, Anne-Laure Lattion, Nadia Larbi, Priya Kunapuli, Alexey Pronin, J effrey L. Benovic, and Susanna Cotechia

From the Institut de Pharmacologie et Toxicologie, Faculte de Medicine, 1005 Lausanne, Switzerland and the

Department of Pharmacology, Jefferson University, Philadelphia, Pennsylvania 19107-5541

The \( \alpha_{1B} \)-adrenergic receptor (\( \alpha_{1B} \)AR), its truncated mutant T368, different G protein-coupled receptor kinases (GRK) and arrestin proteins were transiently expressed in COS-7 or HEK293 cells alone and/or in various combinations. Coexpression of \( \beta \)-adrenergic receptor kinase (\( \beta \)ARK) 1 (GRK2) or 2 (GRK3) could increase epinephrine-induced phosphorylation of the wild type \( \alpha_{1B} \)AR above basal as compared to that of the receptor expressed alone. On the other hand, overexpression of the dominant negative \( \beta \)ARK (K220R) mutant impaired agonist-induced phosphorylation of the receptor. Overexpression of GRK6 could also increase epinephrine-induced phosphorylation of the receptor, whereas GRK5 enhanced basal but not agonist-induced phosphorylation of the \( \alpha_{1B} \)AR. Increasing coexpression of \( \beta \)ARK1 or \( \beta \)ARK2 resulted in the progressive attenuation of the \( \alpha_{1B} \)AR-mediated response on polyphosphoinositide (PI) hydrolysis. However, coexpression of \( \beta \)ARK1 or 2 at low levels did not significantly impair the PI response mediated by the truncated \( \alpha_{1B} \)AR mutant T368, lacking the C terminus, which is involved in agonist-induced desensitization and phosphorylation of the receptor. Similar attenuation of the receptor-mediated PI response was also observed for the wild type \( \alpha_{1B} \)AR, but not for its truncated mutant, when the receptor was coexpressed with \( \beta \)-arrestin 1 or \( \beta \)-arrestin 2. Despite their pronounced effect on phosphorylation of the \( \alpha_{1B} \)AR, overexpression of GRK5 or GRK6 did not affect the receptor-mediated response. In conclusion, our results provide the first evidence that \( \beta \)ARK1 and 2 as well as arrestin proteins might be involved in agonist-induced regulation of the \( \alpha_{1B} \)AR. They also identify the \( \alpha_{1B} \)AR as a potential phosphorylation substrate of GRK5 and GRK6. However, the physiological implications of GRK5- and GRK6-mediated phosphorylation of the \( \alpha_{1B} \)AR remain to be elucidated.

Homologous desensitization to the effects of hormones and neurotransmitters is a ubiquitous regulatory mechanism of receptor function defined by a rapid and specific loss of responsiveness for receptors which have been repeatedly stimulated by an agonist (1). In the G protein-coupled receptor family (2), receptor desensitization has been extensively characterized for rhodopsin mediating phototransduction in retinal rod cells and for the \( \beta_{2} \)-adrenergic receptor (\( \beta_{2} \)AR), which mediates catecholamine-induced stimulation of adenylly cyclase. The second messenger-dependent cAMP-dependent protein kinase can phosphorylate and desensitize the \( \beta_{2} \)AR both in response to its agonist as well as to other agents increasing the cellular content of cAMP. On the other hand, a prominent role in homologous desensitization of rhodopsin and \( \beta_{2} \)AR is played by the two second messenger-independent kinases rhodopsin kinase (3) and the \( \beta \)-adrenergic receptor kinase (\( \beta \)ARK) (4), respectively. Once the receptor is occupied by the agonist, it is recognized by the kinase and becomes phosphorylated. The subsequent uncoupling of the receptor and G protein is then mediated by arrestin proteins which specifically bind to the phosphorylated receptor (5, 6). Rhodopsin kinase and \( \beta \)ARK are members of the newly discovered family of G protein-coupled receptor kinases (GRKs) (7). These protein kinases have the unique ability to recognize and phosphorylate their G protein-coupled receptor substrates only in their active (i.e. agonist-occupied) conformations. Molecular cloning techniques have revealed that the current members of the GRK family include rhodopsin kinase (GRK1) (8), \( \beta \)ARK 1 and 2 (GRK2 and 3, respectively) (9, 10), the human gene ITI1 (GRK4) (11), GRK5 (12), GRK6 (13) and several homologs from Drosophila (14). \( \beta \)ARK isozymes can phosphorylate a variety of receptors in vitro including the \( \beta_{2} \)AR (9, 10, the \( \alpha_{2A} \)AR (15), the M2 muscarinic cholinerger receptor (M2-AchR) (16) and, at lower stoichiometry, rhodopsin (9, 10). The lack of proportion between the small number of GRKs and the large number of G protein-coupled receptors suggests that different GRKs can recognize several receptors in vivo.

Despite the large amount of information about the adenyly cyclase-linked \( \beta_{2} \)AR, much less is known about the molecular mechanisms involved in desensitization of G protein-coupled receptors which activate polyphosphoinositide (PI) hydrolysis via phospholipase C (PLC). Protein kinase C inhibitors or phorbol ester-induced depletion of cellular protein kinase C do not alter homologous desensitization of various receptors including the receptors for thrombin (17), bombesin (18), histamine and ATP (19). We have recently shown that agonist-induced phosphorylation and desensitzation of the cloned \( \alpha_{1B} \)AR expressed

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§ To whom correspondence should be addressed: Institut de Pharmacologie et Toxicologie, Rue du Bugnon 27, 1005 Lausanne, Switzerland. Fax: 41-21-692-5355.

1 The abbreviations used are: AR, adrenergic receptor(s); G protein, guanylyl nucleotide-binding regulatory protein; PI, phosphatidylinositol; PIP2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; GRK, G protein-coupled receptor kinase; JARK, \( \beta \)-adrenergic receptor kinase; HEK 293, human embryonic kidney 293 cells; PBS, phosphate-buffered saline; [\(^{125}\)I]HEAT, \( \beta-(4\text{-hydroxy-}[\^{125}\text{I}]\text{iodophenyl})\text{ethylamino}-\text{methyl} \) tetralone; AchR, cholinergic receptor; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; ROS, rod outer segments; DMEM, Dulbecco’s modified Eagle’s medium.

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in Rat-1 fibroblasts as well as in COS-7 cells does not primarily involve phorbol ester-sensitive protein kinase C (20). However, the protein kinases and, in more general terms, the biochemical mechanisms involved in agonist-induced regulation of G protein-coupled receptors linked to the PLC signaling pathway remain to be assessed. Potential protein kinase candidates might belong to the GRK family. A role of βARK in the regulation of PLC-linked receptors has been suggested by few studies. Both the receptor for substance P (21) and the M3 muscarinic cholinergic (M3-AChR) (22) have been shown to be substrates for βARK-mediated phosphorylation in vitro. A more recent study has shown that the thrombin receptor-mediated response on intracellular calcium was impaired after coexpression of the receptor with βARK2 in Xenopus oocytes (23).

In the present study, we wished to investigate whether protein kinases belonging to the GRK family play a role in the regulation of the α1BAR. For this purpose, we transiently coexpressed different GRKs with the α1BAR and its truncated mutant T368 (20) in COS-7 or HEK293 cells to assess their effects on agonist-induced phosphorylation and desensitization of the receptors. We also investigated the effect of two arrestin proteins on receptor-mediated response on PI hydrolysis. Our findings indicate that distinct GRKs might be differentially involved in agonist-induced phosphorylation and regulation of the α1BAR.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—COS-7 cells were transfected with different DNAs by DEAE-dextran method. The DNAs encoding the hamster α1BAR (24) and its truncated mutant (20) were subcloned in pRK5 (25); those coding for rat or bovine GRK1 and GRK2 (26, 9, 10) and their K220R mutants (27) were subcloned in pCMV5 (28); those encoding bovine β-arrestin 1 and 2 (29) were subcloned in pRK5, and those encoding human GRK5 (12) and GRK6 (13) were subcloned both in pRK5 and pcMV. For phosphorylation experiments, COS-7 cell (1 × 10⁶) were grown in 55-mm dishes and the transfected DNA was 1 µg/1,000,000 cells for the α1BARs, whereas the DNA for different GRKs could vary as indicated in the figures. The total amount of DNA transfected was kept constant (4 µg/1,000,000 cells) under different conditions adding pRK5 or pcMV. For inositol phosphate determination, COS-7 cell (0.5 × 10⁶) grown in 35-mm dishes were transfected with 0.2 µg of DNA/1,000,000 cells for the α1BARs, whereas the DNA for different GRKs or arrestins could vary as indicated in the figures. The total amount of DNA transfected was kept constant (2 µg/1,000,000 cells) under different conditions adding pRK5 or pcMV. COS-7 cells were harvested 48 h after their transfection.

HEK293 cells were transfected with different DNAs by calcium phosphate precipitation. HEK293 cells (5 × 10⁶) grown in 100-mm dishes were transfected with 1 and 3 µg of DNA/1,000,000 cells for the α1BAR and different GRKs, respectively. The total amount of DNA transfected was kept constant (4 µg/1,000,000 cells) under different conditions adding pRK5 or pcMV. 24 h after the transfection, cells were trypsinized and seeded in 55- or 35-mm dishes for phosphorylation experiments and inositol phosphate determination, respectively. HEK293 cells were harvested 72 h after their transfection.

Antibodies Against the α1BAR—Peptides corresponding to the first 22 amino acids (residues 1–22), the last 24 amino acids (residues 492–515), and a middle region of the third intracellular loop of the α1BAR (residues 246–261) were synthesized by Dr. R. R. Randall (Howard Hughes Medical Institute, Duke University, Durham, NC) using standard methodology. Peptide coupling to keyhole limpet hemocyanin, immobilization of the rabbits with the receptor peptide-keyhole limpet hemocyanin conjugates, and the characterization of the crude antisera were carried on as described previously (20). All the antisera were used at a 1:100 dilution and could immunoprecipitate about 75% of the photoaffinity labeled α1BAR.

Western Blot Analysis of GRKs and β-Arrestin—Cell monolayers (2–4 × 10⁶) transfected with the DNA encoding βARK1 or 2 or β-arrestin 1 were homogenized in 0.2–0.5 ml of ice-cold lysis buffer “A” (20 mM Tris-HCl, pH 7.4, 5 mM EDTA) with protease inhibitors. For GRK5 and GRK6, transfected cell monolayers were homogenized in 0.2–0.5 ml of ice-cold lysis buffer “B” (20 mM Hepes, pH 7.4, 1% Triton X-100, 150 mM NaCl, 10 mM EDTA) with protease inhibitors. The protease inhibitors in the lysis buffer were: leupeptin, 10 µg/ml; bovine pancreatic trypsin inhibitor, 10 µg/ml; aprotinin, 10 µg/ml; pepstatin A, 10 µg/ml; benzamidine, 100 µg/ml; and phenylmethylsulfonyl fluoride, 200 µM. The supernatant fractions were obtained by centrifugation at 100,000 × g for 10 min at 4°C. The pellets were washed with 3% glycerol in TBS (100 mM Tris-HCl, pH 7.4, 0.9% NaCl) for 2 h at room temperature and washed three times with the “washing” buffer (0.05% Tween in TBS). The blots were incubated with the specific antisera diluted in the “antibody” buffer (0.05% Tween in 1% glycerol in TBS) for 2 h at room temperature. After washing the antiserum, the membranes were incubated with the secondary antibody coupled to horseradish peroxidase in the “antibody” buffer, subsequently washed with the “washing” buffer, and developed using the enhanced chemiluminescence method according to the manufacturer’s protocol (ECL, Amersham Corp.). For immunodetection of βARK, the antiserum (used at 1:100 dilution) was raised against a glutathione S-transferase fusion protein encoding residues 467–688 of rat βARK2, as described previously (26). For immunodetection of GRK5 and GRK6, the antiserum (used at 1:500 dilution) was raised against a glutathione S-transferase fusion protein encoding residues 488–590 of human GRK5 (12). For immunodetection of β-arrestin 1, the antiserum (used at 1:200 dilution) was raised against a glutathione S-transferase fusion protein encoding residues 172–268 of bovine β-arrestin 1. The immunoreactivity of different cytosols was compared to that of bovine βARK1 and 2 purified from SF9 cells (4, 6), GRK5 and GRK6 purified from SF9 cells (30, 31) and in vitro translated bovine β-arrestin 1 (29).

Bovine Rod Outer Segments (ROS) Phosphorylation Assay—Bovine urea-treated ROS were prepared as described previously (32). To assess the cytosolic kinase activity, COS-7 cells were lysed as described previously for Western blot analysis of βARK. For the phosphorylation assay (20 µl volume), 20 µg of cytosolic proteins were incubated with urea-treated ROS (150 pmd of rhodopsin) in 20 µl Tris-HCl, pH 7.4, 2 mM EDTA, 10 mM MgCl₂, 0.1 mM [γ-32P]ATP (1000 cpm/pmol). The incubation was carried out for 30 min at 30°C in the absence or presence of 32P. The samples were terminated with SDS-PAGE loading buffer and electrophoresed on 10% SDS-PAGE. After autoradiography, the 32P content of gel slices containing rhodopsin was quantified by liquid scintillation spectroscopy.

Ligand Binding—Membrane preparations derived from cells expressing the α1BAR or its truncated mutant and ligand binding assays using [125I]HEAT were performed as described (20). Prazosin (10 µM) was used to determine nonspecific binding. [125I]HEAT concentration was 300 pm for saturation binding and 80 pm for competition binding analysis of epinephrine. Intact cell receptor binding assays were performed as described (20) by incubating cell monolayers grown in 35-mm dishes with [1H]Prazosin (2 nM) in 2.5 ml of DMEM at 4°C for 10–15 h. After incubation, three times with 0.1% bovine serum albumin, scraped in water, and counted. Phentolamine (10−4 M) was used to determine nonspecific binding, which was 30% of total binding. Data were analyzed by nonlinear least-square regression analysis (33).

P Labeling and Immunoprecipitation of the Receptors—COS-7 or HEK293 cells transfected with the DNAs encoding the α1BARs and GRKs in different combinations were grown in 55-mm dishes, equilibrated in phosphate-free DMEM for 2 h, and then incubated in the same buffer containing 32P (0.2 µCi/ml) for 2 h at 37°C. The incubation was then continued in the presence of epinephrine as indicated. A separate set of dishes was incubated under similar conditions, but in the absence of 32P, to measure receptor binding. Following incubation, cells were washed three times with ice-cold PBS, resuspended in lysis buffer (10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 5 mM EGTA, 10 mM sodium pyrophosphate, 10 mM NaF, 10 µg/ml leupeptin, 10 µg/ml bovine pancreatic trypsin inhibitor, 10 µg/ml aprotinin), and centrifuged at 40,000 × g for 15 min. Membranes were resuspended in binding buffer for ligand binding or in solubilization buffer (PBS, 1% Triton X-100, 0.05% SDS, 1 mM EDTA, 1 mM EGTA, 10 mM sodium pyrophosphate, 10 mM NaF, and protease inhibitors as in lysis buffer) for receptor immunoprecipitation performed as described previously (20). The different antisera raised against the α1BAR were used at a dilution of 1:100. The immune complexes isolated on protein A-Sepharose beads were resuspended in SDS-sample buffer and resolved by 10% SDS-PAGE. After autoradiography, the 32P content of gel slices con-
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RESULTS

Expression of the α1bARs, GRKs and β-Arrestin—COX-7 and HEK293 cells were transfected with the DNA encoding the α1bAR or its truncated mutant T368 in the absence or presence of the expression vectors coding for different GRKs or β-arrestin. Ligand binding of [125I]HEAT on membranes from transfected cells indicated that the expression levels of the α1bARs expressed alone were similar to those of the receptors coexpressed with different GRKs or β-arrestin. The levels of receptor expression in cell membranes ranged 1–2 pmol/mg of protein for both the α1bAR and T368 (corresponding to approximately 200–400 fmo/1,000,000,000 cells). The Kd of [125I]HEAT binding was ~80 pm for both receptors. As previously shown (20), epinephrine could bind to the truncated mutant with slightly higher affinity (Kd = 10–5 M) as compared to the wild type receptor (Kd = 3 × 10–5 M). The Ks of epinephrine binding in membranes were similar for the receptors expressed alone or in the presence of different GRKs or arrestins (results not shown).

The expression of rat βARK1 and 2 in COS-7 and HEK293 cells was assessed by Western blot analysis of the cytosolic proteins from COS-7 cells with an antiserum against βARKs purified from Sf9 cells and of cytosolic proteins from COS-7 cells transfected with the DNA encoding the α1bAR alone (0.2 μg/1,000,000,000 cells) or in combination with increasing amounts of DNA (μg/1,000,000,000 cells) encoding rat βARK1 (lanes 1–3), its K220R mutant (lanes 4 and 5), bovine βARK2 (lanes 6 and 7), or its K220R mutant (lanes 8 and 9). Each purified kinase was 150 ng. B, Western blot analysis of cytosolic proteins from COS-7 cells transfected with the DNA encoding the α1bAR alone (0.2 μg/1,000,000,000 cells) (lane 1) or in combination with increasing amounts of DNA (μg/1,000,000,000 cells) encoding rat βARK1 (lanes 2 and 3), its K220R mutant (lanes 4 and 5), bovine βARK2 (lanes 6 and 7), or its K220R mutant (lanes 8 and 9). C, Western blot analysis of GRK5 (lane 1) and GRK6 (lane 2) purified from Sf9 cells and of cytosolic proteins from COS-7 cells transfected with the DNA encoding the α1bAR alone (0.2 μg/1,000,000,000 cells) (lane 3) or in combination with the DNA (2 μg/1,000,000,000 cells) encoding GRK5 (lane 4) or GRK6 (lane 5). Each purified kinase was 20 ng. D, Western blot analysis of in vitro translated β-arrestin 1 was 100 ng. In all the experiments the cytosolic proteins were 25 μg (derived from 0.3–0.5, 1,000,000,000 cells). The results are representative of several experiments.

We also tested the cytosolic kinase activity of COS-7 cells expressing different βARKs on urea-treated ROS. Despite the similarity of their protein expression detected by Western blot analysis, the cytosolic kinase activity on ROS of rat βARK2 was ~5-fold greater as compared to that of rat βARK1 (results not shown). Our results are in agreement with those of a previous study in which the cytosolic activity on ROS of rat βARK2 expressed in Xenopus oocytes was greater than that of rat βARK1 (23). On the other hand, the bovine βARK1 and 2 did not differ in their cytosolic kinase activity on ROS (results not shown).

The expression of GRK 5 and 6 was assessed by Western blot analysis on cytosolic proteins from COS-7 cells with an antiserum raised against the C-terminal portion of GRK5. This antiserum seems to recognize about 2–3 times better purified GRK5 than GRK6 (Fig. 1). These experiments indicated that the expression of GRK6 in COS-7 cells was higher than that of GRK5. Some immunoreactivity was also detected in the crude membrane fractions, and it was also higher for GRK6 than GRK5 (results not shown).

Effect of GRKs on Phosphorylation of the α1bAR—We have previously shown a close relationship between agonist-induced phosphorylation and desensitization of the α1bAR (20). To assess whether a specific GRK could play a role in the agonist-dependent regulation of the α1bAR, the wild type receptor as well as its truncated mutant T368 were coexpressed with dif-
different GRKs in COS-7 or in HEK23 cells and receptor phosphorylation was measured. Cells transfected with the DNA encoding the α₁βAR in the absence or presence of the expression vectors coding for different GRKs were labeled with [32P]γATP and the α₁βAR was immunoprecipitated using antibodies raised against different regions of the receptor. As previously reported (20), the α₁βAR could be immunoprecipitated by specific antibodies as a phosphorylated polypeptide migrating at ~80 kDa (Fig. 2A). On the other hand, no major phosphopeptide of ~80 kDa was immunoprecipitated from untransfected cells or cells expressing the GRKs alone (results not shown). In most of the phosphorylation experiments, the α₁βAR was immunoprecipitated with antibodies raised against a peptide derived from its C terminus. However, the results of these experiments were similar to those obtained immunoprecipitating the α₁βAR with two different antibodies raised against peptides of its third intracellular loop or N terminus, respectively (results not shown).

Treatment of COS-7 cells expressing the α₁βAR alone with epinephrine (10⁻⁴ M) resulted in a time-dependent increase of receptor phosphorylation which reached its maximal increase of about 55% above basal after 15 min (Fig. 4). In cells overexpressing rat βARK1, agonist-induced phosphorylation of the α₁βAR above basal was about 180% greater than that of cells expressing the receptor alone (Fig. 2). Also overexpression of GRK6 could enhance agonist-induced phosphorylation of the α₁βAR. Following 5 min of stimulation with epinephrine, agonist-induced phosphorylation of the receptor above basal was 230% greater than that of the receptor expressed alone (Fig. 2). On the other hand, coexpression of GRK5 resulted in a 60% increase of basal phosphorylation of the α₁βAR, as compared to the basal level of the receptor expressed alone, without significantly affecting the maximal level of epinephrine-induced phosphorylation (Fig. 2).

To further characterize the GRK-mediated phosphorylation of the α₁βAR, the kinases were coexpressed with the truncated receptor mutant T368. Coexpression of different GRKs in COS-7 cell was not able to increase either basal or epinephrine-induced phosphorylation of the T368 mutant (results not shown). This observation is in agreement with our previous findings indicating that phosphorylation of the α₁βAR requires the integrity of its C-terminal portion (20).

The effect of different GRKs on agonist-induced phosphorylation of the α₁βAR was also investigated in HEK293 cells. As observed in COS-7 cells, coexpression of rat βARK1 or GRK6 could also increase epinephrine-induced phosphorylation of the α₁βAR expressed in HEK293 cells, whereas coexpression of GRK5 enhanced its basal phosphorylation, but not that induced by the agonist (results not shown). The increasing effect of rat βARK2 on agonist-induced phosphorylation of the α₁βAR could only be observed using low amounts of transfected DNA (0.1–0.3 μg/1,000,000 cells) (Fig. 3). These amounts of DNA were lower than those used for rat βARK1. We then discovered that overexpression of rat βARK2 obtained using 2 μg of transfected DNA/1,000,000 cells induced a 45% decrease of the cell surface α₁βARs measured by [3H]Prazosin binding at 4 °C on intact COS-7 cells (Fig. 3), whereas the receptor number measured on cell membranes was similar to that of cells expressing the receptor alone. A decrease of cell surface receptors was also observed for the truncated mutant T368 in cells overexpressing rat βARK2 (results not shown). Similar results were obtained for both the wild type and truncated α₁βAR in HEK293 cells overexpressing rat βARK2 (results not shown). However, no change in cell surface receptors was observed in cells coexpressing the α₁βAR with any of the other GRKs tested (results not shown).

We are currently unable to understand the biochemical mechanisms underlying the effect of rat βARK2 on the expression of cell surface receptors. However, our findings indicate that βARK2-mediated increase of receptor phosphorylation could be observed only when the cell surface α₁βARs were similar to those of the control cells expressing the receptors alone. Our hypothesis is that a loss of cell surface receptors resulting from the overexpression of rat βARK2 might decrease the availability of the receptors for agonist binding. This might result in the underestimation of agonist-induced phosphorylation of the α₁βAR coexpressed with βARK2.

To better assess the role of βARK in agonist-induced phosphorylation of the α₁βAR, we performed a new series of experiments using the βARK 1 and 2 from the bovine species, which have been more extensively characterized in a variety of G protein-coupled receptor systems. The effect of bovine βARK 1 and 2 on receptor phosphorylation was also compared with that of their dominant negative mutants K220R lacking their kinase activity (27). The expression of the α₁βAR both in cell membranes and at the cell surface was similar in cells overex-
pressing bovine βARKs or their mutants as compared to that of cells expressing the receptor alone (results not shown). Over-expression of both bovine βARK 1 and 2 resulted in a pronounced increase of epinephrine-induced phosphorylation of the α1B AR (Fig. 4). On the other hand, in cells overexpressing the dominant negative kinase mutants K220R agonist-induced phosphorylation of the receptor was greatly impaired (Fig. 4). Our hypothesis is that the dominant negative kinase mutants can inhibit the effect of the endogenous kinases involved in agonist-induced phosphorylation of the α1B AR in COS-7 cells. These findings have two main implications. First, they indicate that both βARK1 and 2 can increase agonist-induced phosphorylation of the α1B AR with an apparently similar affinity for the receptor. Second, they strongly suggest that agonist-induced phosphorylation and regulation of the α1B AR occurring in a variety of cells is mediated, at least in part, by βARK.

Effect of GRKs on α1B AR-mediated PI Response—To assess whether GRK-induced increase in the phosphorylation of the α1B AR could also result in receptor desensitization, the wild type receptor as well as its truncated mutant T368 were coexpressed with different GRKs in COS-7 or HEK293 cells and receptor-mediated PI response was measured. Transfection of COS-7 cells with increasing amounts of DNA encoding rat βARK1 or 2 resulted in their progressive expression (Fig. 1). Increasing expression of both rat βARK1 and 2 caused a progressive impairment of α1B AR-mediated PI response as compared to that of the receptor expressed alone (Fig. 5). The inhibitory effect on the α1B AR-mediated PI response was similar for rat βARK1 and 2, and it seemed to correlate well with their expression at the protein level both in COS-7 cells (Fig. 5) and in HEK293 cells (results not shown). Surprisingly, despite their effect on receptor phosphorylation, overexpression of GRK5 or GRK6 did not have any effect on the α1B AR-mediated PI response either when they were coexpressed alone with the receptor or in combination with β-arrestin. The lack of effect of GRK5 and GRK6 on the α1B AR-mediated response was observed both in COS-7 and HEK293 cells (results not shown).

Fig. 6 shows that coexpression of rat βARK1 with the α1B AR induced both a decrease of the maximal effect of epinephrine and 100-fold increase of its EC50 to stimulate receptor-mediated PI response. Similar results were obtained when the α1B AR was coexpressed with rat βARK2 (results not shown). Decreased sensitivity to the agonist and reduced ability to mediate the maximal response are two properties of G protein-
coupled receptors following agonist-induced desensitization. Thus, overexpression of βARK1 and 2 seems to result in biochemical modifications similar to those occurring during homologous desensitization of the α1BAR. To further test this hypothesis, rat βARK1 and 2 were coexpressed with the truncated α1BAR mutant T368. We had previously shown that the T368 receptor was impaired in its ability to undergo agonist-induced desensitization and phosphorylation. In agreement with these findings, the PI response mediated by the T368 mutant was only slightly impaired by overexpression of rat βARK1 or 2 (Figs. 5 and 6).

This strongly suggests that for both rat βARK1 or 2 the most probable mechanism underlying their facilitating effect on desensitization of the α1BAR is receptor phosphorylation. As shown in Fig. 3, overexpression of rat βARK2 (but not that of rat βARK1) achieved using 2 μg of DNA/1,000,000 cells resulted in a 45% decrease of cell surface receptors. However, two observations seem to rule out that rat βARK2-induced decrease of cell surface receptors is responsible for the desensitization of the α1BAR. First, overexpression of rat βARK2 could also decrease the cell surface expression of the truncated mutant T368 without impairing its response. In addition, in separate experiments using smaller amounts of transfected DNA encoding the α1BAR, we observed that a 40–50% lower expression of cell surface receptors did not result in a reduction of the receptor-mediated response because a large portion of receptor in COS-7 cells are spare (results not shown).

In conclusion, our results suggest that coexpression of βARK1 and 2 can promote desensitization of the wild type α1BAR. On the other hand, GRK5 and GRK6 can increase the phosphorylation of the receptor without inducing any desensitization.

Because of this apparently conflicting result concerning βARK1 and 2 versus GRK5 and GRK6, we wished to further assess whether βARK-induced desensitization of the α1BAR was truly mediated by the phosphorylation of the receptor. Thus, the α1BAR and its truncated mutant T368 were cotransfected with the dominant negative mutants K220R of βARKs lacking their kinase activity. For these experiments both the wild type and mutant βARK 1 and 2 were from the bovine species. Transfection of COS-7 cells with increasing amounts of DNA encoding bovine βARK1 or 2 or their mutants resulted in their progressive expression, which was comparable for all the kinases (Fig. 1). Low expression of bovine βARK1 (obtained

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**Fig. 5. Overexpression of rat βARK1 and 2 attenuates α1BAR-mediated PI hydrolysis.** COS-7 cells (500,000–1,000,000) grown in 35-mm dishes were transfected with the DNAs (0.2 μg/1,000,000 cells) encoding the α1BAR or the T368 receptor alone or in combination with increasing amounts of DNA (μg/1,000,000 cells) encoding rat βARK1 or 2. Total inositol phosphates were measured as described under “Experimental Procedures.” Receptor numbers measured in membrane preparations were similar under the different conditions for both receptors and in the range of 1–2 pmol of protein (200–300 fmol/1,000,000 cells). Control indicates the increase of inositol phosphates induced by 20 min of stimulation with epinephrine (10−6 M) in cells expressing the receptor alone (CON), which was 273 ± 48 and 321 ± 47% over basal (mean ± S.E. of eight independent experiments) for the α1BAR and the T368, respectively. The results are the mean ± S.E. of three independent experiments done in triplicate.

**Fig. 6. Dose response of epinephrine-stimulated PI hydrolysis.** COS-7 cells (500,000–1,000,000) grown in 35-mm dishes were transfected with the DNAs (0.2 μg/1,000,000 cells) encoding the α1BAR or the T368 receptor alone or in combination with the DNA (1 μg/1,000,000 cells) encoding rat βARK1. The experimental conditions are as in Fig. 5. %o fm a x indicates the response induced by 10−3 M epinephrine (EPI) for each dose response. The results are representative of two experiments.
mediated PI response. On the other hand, coexpression of arrestin proteins, tors. Thus we attempted to assess the effect of two different phosphorylated receptor (1). Much less is known about the role of these receptors expressed alone which were 92 ± 8 and 120 ± 15 fmol/1,000,000 cells (mean ± S.E. of three independent experiments) for the wild type and T368 receptor, respectively. The results are the mean ± S.E. of three independent experiments done in triplicate.

using 0.2 μg of DNA/1,000,000 cells) resulted in about 40% impairment of α1BAR-mediated PI response, without any significant effect on the response mediated by the T368 mutant (Fig. 7). However, higher expression of βARK1 could also impair the T368-mediated response even if at a smaller extent as compared to the wild type receptor (35% versus 75% for the T368 and wild type receptor, respectively). For both the wild type and truncated α1BAR, a low expression of the dominant negative mutant βARK1 did not impair the PI response, whereas its high expression could impair about 40% of both receptor-mediated response. Similar results were obtained when both receptors were expressed with the wild type βARK2 or its dominant negative mutant (results not shown). These results indicate that overexpression of βARK can impair the α1BAR-mediated response by at least two mechanisms depending on the expression level of the kinase. The first, occurring at lower level of expression, might be mediated by receptor phosphorylation because it is not observed with similar expression levels of the kinase-deficient mutant K220R. The second, occurring at higher expression of βARK, seems independent from receptor phosphorylation. This is supported by the fact that at higher expression both the wild type (βARK and its dominant negative mutant) exert similar effects on the phosphorylation-deficient T368 mutant.

Altogether, these results provide strong evidence that both βARK1 and 2 can promote desensitization of the α1BAR and that this is mediated by their ability to phosphorylate the receptor. Effect of β-Arrestins on the α1BAR-Mediated PI Response—For the βAR and rhodopsin the uncoupling between the receptor and the G protein occurring during desensitization seems to be mediated by arrestin proteins which specifically bind to the phosphorylated receptor (1). Much less is known about the role of arrestins in desensitization of other G protein-coupled receptors. Thus we attempted to assess the effect of two different arrestin proteins, β-arrestin 1 and 2 (29), on α1BAR-mediated signaling. Fig. 8 shows that coexpression of β-arrestin 1 with the α1BAR in COS-7 cells induced a 50% decrease of receptor-mediated PI response. On the other hand, coexpression of β-arrestin 1 could only modestly impair the functional response of the T368 receptor. Similar results were obtained when both receptors were coexpressed with β-arrestin 2 (results not shown). Coexpression of β-arrestin 1 or 2 caused both a downward and a 10-fold rightward shift of the dose-response curve of epinephrine-induced PI hydrolysis (results not shown). These findings provide the first evidence that arrestin proteins can play a role in the regulation of the α1BAR-mediated response. The modest effect of arrestins on the T368 mutant strongly suggests that the C-terminal portion of the receptor is crucially involved in the interaction of arrestin proteins with the α1BAR.

DISCUSSION

This study provides the first evidence that members of the GRK family as well as arrestin proteins might play a role in agonist-induced regulation of the α1BAR. This is mainly supported by the finding that cellular overexpression of βARK1 or 2 can both increase agonist-induced phosphorylation of the α1BAR and promote desensitization of the receptor-mediated PI response. Coexpression of β-arrestin 1 or 2 could also desensitize the α1BAR-mediated activation of PLC. On the other hand, the truncated α1BAR mutant T368, which was unable to undergo homologous desensitization in Rat1 cells, was largely insensitive to the effect of both βARKs and arrestins. βARK 1 and 2 Can Promote Agonist-induced Phosphorylation and Desensitization of the α1BAR—Our findings extend the notion that protein kinases belonging to the GRK family play a
general role in the homologous desensitization of a variety of G protein-coupled receptors (7). Rhodopsin mediating phototransduction in retinal rod cells, the β2-AR coupled to Gs, mediated stimulation of adenyl cyclase, and the M2 muscarinic cholinergic receptor (M2-AChR) coupled to Gq mediated inhibition of adenyl cyclase represent the three G protein-coupled receptor systems for which homologous desensitization has been more extensively characterized. The biochemical mechanisms underlying homologous desensitization have been elegantly elucidated by in vitro studies reconstituting purified receptors, G protein subunits, GRKs, and arrestin proteins (3–6, 16). In fewer studies, the interactions among these different proteins and their role in receptor desensitization have been explored in intact cells. In particular, overexpression of βARK1 or β-arrestin 1 with the β2-AR in Chinese hamster ovary cells could enhance the desensitization of the receptor-mediated cAMP response (34). On the other hand, overexpression of a dominant negative mutant of βARK1 in bronchial epithelial cells attenuated the desensitization of the endogenous β2-AR-mediated response (27). More recently, it was shown that overexpression of rhodopsin kinase, βARK1, βARK2, or GRK5 could concomitantly increase agonist-induced phosphorylation of the β2-AR and promote its desensitization (35). Finally, coexpression of βARK2 with the thrombin receptor in Xenopus oocytes could desensitize the receptor-mediated response on intracellular calcium (23).

The experimental approach undertaken in our present study consisted in coexpressing different GRKs or arrestin proteins with the α1AR or its truncated mutant T368 in two different cell systems, COS7 and HEK293 cells. Overexpression of both βARK1 and 2 with the α1AR resulted in two of the most common biochemical modifications occurring during homologous desensitization of G protein-coupled receptors, namely a decreased sensitivity of the receptor to the agonist and its reduced ability to mediate the maximal response (Fig. 6). In addition, overexpression of both βARK1 and 2 could increase the agonist-induced phosphorylation of the α1AR above basal of almost 2-fold as compared to that of the receptor expressed alone (Figs. 2–4). Two lines of evidence support the notion that βARK-induced desensitization of the α1AR is, at least in part, mediated by phosphorylation of the receptor. First, the phosphorylation-deficient T368 receptor mutant, which could mediate the activation of PI response as well as the wild type α1AR, was largely insensitive to both βARK1 and 2 (Fig. 5). Second, when the kinases were overexpressed at low level, the α1AR-mediated response could be inhibited by wild type βARK1 or 2, but not by their dominant negative mutants lacking the kinase activity (Fig. 7).

In cotransfection experiments, to assess the role of a single biochemical component, such as a specific receptor kinase or arrestin, this latter must be overexpressed to overcome the different endogenous mechanisms involved in receptor function and regulation. Thus, in our experiments the α1AR was expressed at a constant level of 200–400 fmol/1,000,000 cells, whereas the cytosolic expression of βARK was about 10-fold higher of that of the receptor (see “Results”). These experimental conditions might be considered far from being physiological. However, our findings provide also the evidence that βARK might play a general role in homologous desensitization of the α1AR. This is supported by the finding that overexpression of the dominant negative βARK mutants could inhibit the agonist-induced phosphorylation of the α1AR mediated by the endogenous kinases in COS-7 (Fig. 4). Thus, agonist-induced phosphorylation and regulation of the α1AR occurring in a variety of cells might be mediated, at least in part, by βARK. In a previous study (15), we were unsuccessful at phosphor-

A Dominant Negative Mutant of βARK Can Impair the Receptor-mediated PI Response—Our results indicate that βARK-induced impairment of the α1AR-mediated PI response can be clearly distinguished in two phases, which depend on the level of overexpression of the kinase (Fig. 7). At lower expression, only βARK (and not its dominant negative mutant K220R) can desensitize the α1AR and this effect seems truly mediated by phosphorylation of the receptor. On the other hand, at higher expression also the dominant negative mutant K220R can induce a 30–40% impairment of the response mediated by both the wild type α1AR and its phosphorylation-defective T368 mutant (Fig. 7). Thus, this effect is independent from phosphorylation of the receptor, and it might reflect the interaction of βARK with other signaling molecules downstream the receptor. This hypothesis is supported by our recent knowledge about the ability of βARK to bind βγ subunits of heterotrimeric G proteins (Gβγ) as well as phosphatidylinositol 4,5-bisphosphate (PIP2) via its pleckstrin homology domain (37). One possibility is that the inhibitory effect of βARK on the receptor-mediated PI response is due to the inhibition of Gβγ-induced activation of PLC. However, previous findings (38) seem to rule out that Gβγ is involved in the α1AR-mediated activation of PI hydrolysis. Another possible explanation of our findings is that the dominant negative βARK overexpressed in COS-7 cells can bind to PIP2, thus inhibiting its hydrolysis by the endogenous PLC. This hypothesis is strongly supported by recent findings indicating that overexpression of pleckstrin in COS cells can inhibit the PI response mediated by a variety of receptors linked to the PLC signaling pathway (39). Thus, both pleckstrin and βARK might compete with different PLC for their access to PIP2. Our findings represent the first evidence that βARK in intact cells can interfere with the PLC signaling pathway beyond its direct effect on the receptor. This suggests a growing complexity in the biochemical mechanisms involved in the regulation of PLC-linked G protein coupled receptors as well as in the potential roles played by βARK in different cell systems.

β-Arrestin Can Desensitize the α1AR-mediated Response—Uncoupling of the receptor and G protein occurring during homologous desensitization of G protein coupled receptors is mediated by arrestin proteins, which preferentially bind to the phosphorylated receptor (3, 4). In agreement with this notion, we have found that overexpression of β-arrestin 1 or 2 could also attenuate the α1AR-mediated PI response suggesting that arrestin can play a role also in the desensitization process of the α1AR (Fig. 8). Our results are in agreement with previous studies showing that overexpression of arrestin could impair the cAMP response mediated by the β2-AR (34) and, more recently, by the

3 S. Catecchia, unpublished observations.
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\( \beta_2AR \) (35). However, these findings are not simple to interpret from a mechanistic point of view. For non-visual arrestin, very little is known about the molecular mechanisms underlying its interaction with the receptors. An important contribution has been provided by a recent study (36), which has proposed a kinetic model of non-visual arrestin interaction with receptors based on a detailed binding analysis of the different purified components. The model of arrestin-receptor interaction proposed is analogous to the current model of G protein-receptor interaction (33) in several aspects. Thus, similarly to the G protein, an excess of arrestin can drive all the agonist-receptor complexes to bind arrestin. Cotransfection experiments in intact cells cannot provide precise mechanistic information because it is difficult to assess the precise stoichiometry of the expressed proteins as well as to which extent they are functionally active. Our hypothesis is that, in cotransfection experiments with different receptors (34, 35), arrestin can induce receptor desensitization independently of the phosphorylation state of the receptor because of its stochiometric excess over the G protein.

Another important finding of our study is that the integrity of the C-terminal portion of the \( \alpha_{1B}AR \) is required for its interaction with arrestin. This was demonstrated by the fact that overexpression of \( \beta \)-arrestin could impair the wild type receptor-mediated response, but not that of its truncated mutant T368 (Fig. 8). Virtually nothing is known about the structural domains of G protein-coupled receptors interacting with non-visual arrestins. It is well documented that arrestin preferentially binds to the phosphorylated receptor (36). This cannot be demonstrated by our study because the amount of phosphorylated versus non-phosphorylated \( \alpha_{1B}AR \) is not known and cannot be unequivocally established in cells overexpressing the receptors. However, our findings indicate that, independently of the phosphorylation state of the receptor, a main structural determinant of the receptor binding site for arrestin is located in the C terminus of the \( \alpha_{1B}AR \), which is also an important phosphorylation domain of the receptor.

GRK5 and GRK6 Can Increase Phosphorylation of the \( \alpha_{1B}AR \)—GRK5 and GRK6 are the most recently identified members of the receptor kinase family. In vitro studies have elucidated that GRK5 can phosphorylate the \( \beta_2AR \), the M2-AChR, and rhodopsin in an agonist-dependent fashion (30). GRK6 can also phosphorylate the same substrates, but with stochiometries significantly lower than those achieved by GRK5 or \( \beta ARK \) (31). More recently, it has been shown that GRK5 can increase agonist-induced phosphorylation of the \( \beta_2AR \) both in membrane preparations and when it is overexpressed in cells (35). Thus, very little is known about the potential role of GRK5 and GRK6 in the regulation of receptor function in intact cells. Here we report that in both COS-7 and HEK293 cells coexpression of GRK5 or GRK6 have an effect on the phosphorylation of the \( \alpha_{1B}AR \), but not of its truncated mutant T368. Whereas coexpression of GRK6 could clearly increase epinephrine-induced phosphorylation of the \( \alpha_{1B}AR \) above its basal level, coexpression of GRK5 increased the basal level without any important effect on agonist-stimulated phosphorylation. These findings identify the \( \alpha_{1B}AR \) as a potential substrate for GRK5 and GRK6 in intact cells. However, it cannot be ruled out that the effects of GRK5 and GRK6 on the phosphorylation of the \( \alpha_{1B}AR \) in intact cells is indirectly mediated by other or more endogenous kinases and intracellular components. Interestingly, recent in vitro studies reveal that GRK5 can induce a small increase of the basal phosphorylation of the purified \( \beta_2AR \) (40) as well as of the M3-AChR expressed in Sf9 cell membranes (22). Thus, the increased basal phosphorylation of the \( \alpha_{1B}AR \) in intact cells might be truly mediated by the GRK5 overexpressed in the cells. Recently, it has been shown that GRK5 can undergo autoprophosphorylation, which is stimulated by phospholipids in vitro and might regulate its kinase activity (41). Thus, the effect of GRK5 on the \( \alpha_{1B}AR \) might reflect its constitutive activation following phospholipid-stimulated autoprophosphorylation in intact cells.

However, despite their effect on phosphorylation of the \( \alpha_{1B}AR \), coexpression of GRK5 or GRK6 either with or without \( \beta \)-arrestin did not induce any change of the receptor-mediated response. Thus, the functional correlates of GRK5 and GRK6-mediated phosphorylation of the \( \alpha_{1B}AR \) remain unknown. One possibility is that COS-7 or HEK293 cells are missing a yet unidentified component essential for the full regulatory activity of GRK5 and GRK6. Alternatively, our measurement of the \( \alpha_{1B}AR \)-mediated response is not sufficiently sensitive to assess more rapid or subtle regulatory events.

Conclusions—The implications of this study are twofold. First, they provide strong evidence that \( \beta ARK1 \) and 2 as well as \( \beta \)-arrestin 1 and 2 can play a role in agonist-dependent regulation of the \( \alpha_{1B}AR \). Second, they contribute to the elucidation of the receptor substrate specificity of different GRKs identifying the \( \alpha_{1B}AR \) as a potential phosphorylation substrate for \( \beta ARK \) and 2 as well as for GRK5 and GRK6. In addition, they indicate that cotransfection experiments can be an useful tool to explore the potential interactions among signaling and regulatory proteins in intact cells. Our findings provide the groundwork for further investigation of the biochemical mechanisms underlying the regulation of the \( \alpha_{1B}AR \) in vitro possibly reconstituting purified components.

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Dario Diviani, Anne-Laure Lattion, Nadia Larbi, Priya Kunapuli, Alexey Pronin, Jeffrey L. Benovic and Susanna Cotecchia

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