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

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The α_{1B}-adrenergic receptor (α_{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 β-adrenergic receptor kinase (βARK) 1 (GRK2) or 2 (GRK3) could increase epinephrine-induced phosphorylation of the wild type α_{1B}AR above basal as compared to that of the receptor expressed alone. On the other hand, overexpression of the dominant negative βARK (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 α_{1B}AR. Increasing coexpression of βARK1 or βARK2 resulted in the progressive attenuation of the α_{1B}AR-mediated response on polyphosphoinositide (PI) hydrolysis. However, coexpression of βARK1 or 2 at low levels did not significantly impair the PI response mediated by the truncated α_{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 α_{1B}AR, but *not* for its truncated mutant, when the receptor was coexpressed with β-arrestin 1 or β-arrestin 2. Despite their pronounced effect on phosphorylation of the α_{1B}AR, overexpression of GRK5 or GRK6 did not affect the receptor-mediated response. In conclusion, our results provide the first evidence that βARK1 and 2 as well as arrestin proteins might be involved in agonist-induced regulation of the α_{1B}AR. They also identify the α_{1B}AR as a potential phosphorylation substrate of GRK5 and GRK6. However, the physiological implications of GRK5- and GRK6-mediated phosphorylation of the α_{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 β_2-adrenergic receptor (β_2AR),[1] which mediates catecholamine-induced stimulation of adenylyl cyclase. The second messenger-dependent cAMP-dependent protein kinase can phosphorylate and desensitize the β_2AR 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 β_2AR is played by the two second messenger-independent kinases rhodopsin kinase (3) and the β-adrenergic receptor kinase (βARK) (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 βARK 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), βARK 1 and 2 (GRK2 and 3, respectively) (9, 10), the human gene *IT11* (GRK4) (11), GRK5 (12), GRK6 (13) and several homologs from *Drosophila* (14). βARK isozymes can phosphorylate a variety of receptors *in vitro* including the β_2AR (9, 10), the α_{2A}AR (15), the M2 muscarinic cholinergic 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 adenylyl cyclase-linked β_2AR, 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 desensitization of the cloned α_{1B}AR expressed

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[1] The abbreviations used are: AR, adrenergic receptor(s); G protein, guanyl nucleotide-binding regulatory protein; PI, polyphosphoinositide; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; GRK, G protein-coupled receptor kinase; βARK, β-adrenergic receptor kinase; HEK293, human embryonic kidney 293 cells; PBS, phosphate-buffered saline; [^{125}I]HEAT, (β-(4-hydroxy-[^{125}I]iodophenyl)ethylaminomethyl)tetralone; AchR, cholinergic receptor; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; ROS, rod outer segments; DMEM, Dulbecco's modified Eagle's medium.

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 the α_{1B}AR. For this purpose, we transiently coexpressed different GRKs with the α_{1B}AR 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 differently involved in agonist-induced phosphorylation and regulation of the α_{1B}AR.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—COS-7 cells were transfected with different DNAs by DEAE-dextran method. The cDNAs encoding the hamster α_{1B}AR (24) and its truncated mutant (20) were subcloned in pRK5 (25); those coding for rat or bovine βARK1 and 2 (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 (1×10^6) were grown in 55-mm dishes and the transfected DNA was 1 μg/1,000,000 cells for the α_{1B}ARs, whereas the DNA for different GRKs could vary as indicated in the figures. The total amount of DNA transfected was kept constant (5 μg/1,000,000 cells) under different conditions adding pRK5 or pCMV. For inositol phosphate determination, COS-7 cell (0.5×10^6) grown in 35-mm dishes were transfected with 0.2 μg of DNA/1,000,000 cells for the α_{1B}ARs, 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 (5×10^6) grown in 100-mm dishes were transfected with 1 and 3 μg of DNA/1,000,000 cells for the α_{1B}ARs 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 α_{1B}AR—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 α_{1B}AR (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, immunization of the rabbits with the receptor peptide-keyhole limpet hemocyanin conjugates, and the characterization of the crude antiserum 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 α_{1B}AR.

Western Blot Analysis of GRKs and β-Arrestin—Cell monolayers ($2–4 \times 10^6$) 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" (10 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*. 25 μg of proteins of the supernatant fractions (corresponding to the cytosolic proteins of 300,000–500,000 cells) were subjected to SDS-PAGE followed by electroblotting onto nitrocellulose membranes. The blots were blocked with 3% gelatin 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 antiserum diluted in the "antibody" buffer (0.05% Tween and 1% gelatin in TBS) for 2 h at room temperature. After washing the antiserum, the membranes were incubated with the secondary antiserum 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 1:1000 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 1:300 dilution) was raised against a glutathione *S*-transferase fusion protein encoding residues 172–268 of bovine β-arrestin 1.[2] 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 (50 μl volume), 20 μg of cytosolic proteins were incubated with urea-treated ROS (150 pmol of rhodopsin) in 20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 10 mM $MgCl_2$, 0.1 mM [γ-^{32}P]ATP (1000 cpm/pmol). The incubation was carried out for 30 min at 30 °C in the absence or presence of light. The reactions were terminated with SDS-PAGE loading buffer and electrophoresed on 10% SDS-PAGE. After autoradiography, the ^{32}P content of gel slices containing rhodopsin was quantified by liquid scintillation spectroscopy.

Ligand Binding—Membrane preparations derived from cells expressing the α_{1B}AR or its truncated mutant and ligand binding assays using [^{125}I]HEAT were performed as described (20). Prazosin (10^{-6} M) was used to determine nonspecific binding. [^{125}I]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 [^{3}H]Prazosin (2 nM) in 2.5 ml of DMEM at 4 °C for 10–15 h. After binding, cells were washed three times with ice-cold PBS containing 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).

^{32}P Labeling and Immunoprecipitation of the Receptors—COS-7 or HEK293 cells transfected with the DNAs encoding the α_{1B}ARs 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 $^{32}P_i$ (0.2 mCi/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 $^{32}P_i$ 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), scraped 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 α_{1B}AR 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 ^{32}P content of gel slices con-

[2] F. Mayor, Jr., unpublished results.

taining the receptor was quantified by liquid scintillation spectroscopy and normalized to the receptor amount loaded on the gel.

Inositol Phosphate Determination—For the determination of labeled total inositol phosphates, cells expressing the α_{1B}ARs and different GRKs or arrestins were grown in 35-mm dishes and labeled with [^{3}H]inositol at 5–10 μCi/ml for 15–18 h in inositol-free DMEM supplemented with 1% fetal bovine serum. After labeling, cells were stimulated for 20 min with epinephrine in the presence of 20 mM LiCl. Inositol phosphates were extracted as described previously (24) and separated by Dowex AG1-X8 columns. Total inositol phosphates were eluted with 1 M ammonium formate, 0.1 M formic acid.

Materials—Materials used are as follows: DMEM, minimal essential medium, gentamicin, fetal bovine serum, and horse serum (Life Technologies, Inc.); [^{125}I]HEAT (DuPont NEN); [^{3}H]Prazosin and [^{3}H]inositol (Anawa); Dowex AG1-X8 (Bio-Rad); epinephrine (Sigma); Prazosin (Research Biochemical International); and COS-7 and HEK293 cells (American Tissue Culture Collection).

RESULTS

Expression of the α_{1B}ARs, GRKs and β-Arrestin—COS-7 and HEK293 cells were transfected with the DNAs encoding the α_{1B}AR or its truncated mutant T368 in the absence or presence of the expression vectors coding for different GRKs or β-arrestin. Ligand binding of [^{125}I]HEAT on membranes from transfected cells indicated that the expression levels of the α_{1B}ARs 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 α_{1B}AR and T368 (corresponding to approximately 200–400 fmol/1,000,000 cells). The K_d of [^{125}I]HEAT binding was ~80 pM for both receptors. As previously shown (20), epinephrine could bind to the truncated mutant with slightly higher affinity (K_i= 10^{-5} M) as compared to the wild type receptor (K_i= 3×10^{-5} M). The K_i values 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 with an antiserum which could equally recognize βARK1 and 2 (26). These experiments indicated that equal amounts of transfected DNA resulted in similar expression of rat βARK1 and 2 both in COS-7 (Fig. 1, *panel A*) and HEK293 cells (results not shown). The expression of both kinases was similar in COS-7 cells coexpressing the wild type α_{1B}AR or its truncated mutant (results not shown). Similarly to what observed with the rat βARKs, equal amounts of DNA resulted in similar expression of bovine βARKs as well as of their dominant negative mutants K220R (Fig. 1, *panel B*). Some βARK immunoreactivity was also detected in the crude membrane fractions of transfected cells, and it was similar for both kinases (results not shown). The comparison of the signals corresponding to the cytosolic rat βARKs expressed in COS-7 cells with those of known amounts of βARKs purified from Sf9 cells (Fig. 1, *panel A*) suggests that the expression of cytosolic rat βARK1 or 2 is about 3.5 pmol/1,000,000 cells. This level of expression is about 10–15-fold higher than that of the α_{1B}AR as assessed by ligand binding on cell membranes (200–300 fmol/1,000,000 cells) when both values are normalized/1,000,000 cells.

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 nor 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).

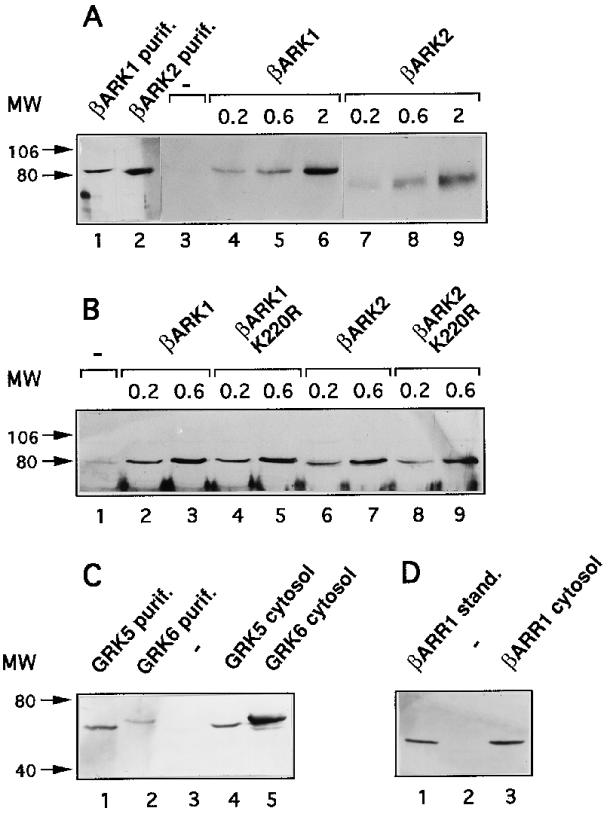

FIG. 1. **Expression of GRKs and β-arrestin in Cos-7 cells.** *A*, Western blot analysis of bovine βARK1 and 2 (*lanes 1* and *2*) purified from Sf9 cells and of cytosolic proteins from COS-7 cells transfected with the DNAs encoding the α_{1B}AR alone (0.2 μg/1,000,000 cells) (*lane 3*) or in combination with increasing amounts of DNA (μg/1,000,000 cells) encoding rat βARK1 (*lanes 4–6*) or rat βARK2 (*lanes 7–9*). Each purified kinase was 150 ng. *B*, Western blot analysis of cytosolic proteins from COS-7 cells transfected with the DNAs encoding the α_{1B}AR alone (0.2 μg/1,000,000 cells) (*lane 1*) or in combination with increasing amounts of DNA (μg/1,000,000 cells) encoding bovine 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 from Sf9 cells and of cytosolic proteins from COS-7 cells transfected with the DNAs encoding the α_{1B}AR alone (0.2 μg/1,000,000 cells) (*lane 3*) or in combination with the DNA (2 μg/1,000,000 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 (*lane 1*) and cytosolic proteins from COS-7 cells transfected with the DNAs encoding the α_{1B}AR alone (0.2 μg/1,000,000 cells) (*lane 2*) or in combination with the DNA (2 μg/1,000,000 cells) encoding β-arrestin 1 (*lane 3*). The *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 cells). The results are representative of several experiments.

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 α_{1B}AR—We have previously shown a close relationship between agonist-induced phosphorylation and desensitization of the α_{1B}AR (20). To assess whether a specific GRK could play a role in the agonist-dependent regulation of the α_{1B}AR, the wild type receptor as well as its truncated mutant T368 were coexpressed with dif-

ferent GRKs in COS-7 or in HEK23 cells and receptor phosphorylation was measured. Cells transfected with the DNA encoding the α_{1B}AR in the absence or presence of the expression vectors coding for different GRKs were labeled with $^{32}P_i$ and the α_{1B}AR was immunoprecipitated using antibodies raised against different regions of the receptor. As previously reported (20), the α_{1B}AR could be immunoprecipitated by specific antibodies as a phosphorylated polypeptide migrating at ~80 kDa (Fig. 2*A*). 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 α_{1B}AR 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 α_{1B}AR 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 α_{1B}AR 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 α_{1B}AR 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 α_{1B}AR. 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 α_{1B}AR, 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 α_{1B}AR, 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 α_{1B}AR requires the integrity of its C-terminal portion (20).

The effect of different GRKs on agonist-induced phosphorylation of the α_{1B}AR 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 α_{1B}AR 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 α_{1B}AR 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 α_{1B}ARs measured by [^{3}H]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 α_{1B}AR in HEK293 cells overexpressing rat βARK2 (results not shown). However, no change in cell surface receptors was observed in cells coexpressing the α_{1B}AR with any of the other GRKs tested (results not shown).

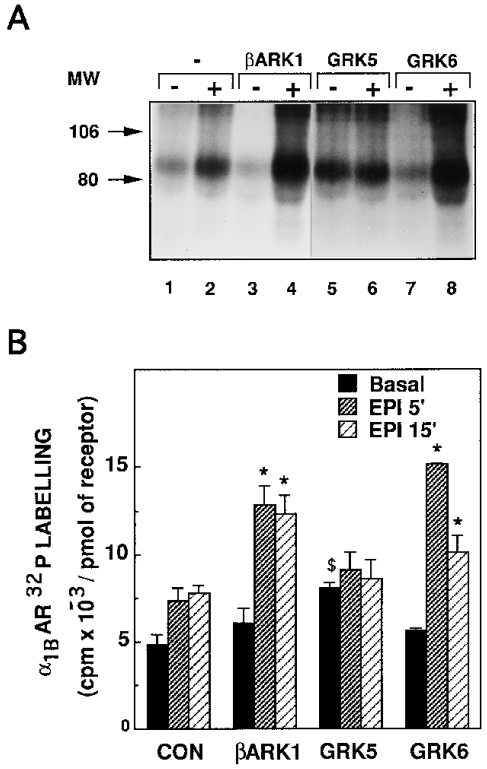

FIG. 2. **Phosphorylation of the α_{1B}AR in COS-7 cells.** *A*, COS-7 cells expressed the α_{1B}AR alone (*lanes 1* and *2*) or in the presence of rat βARK1 (*lanes 3* and *4*), GRK5 (*lanes 5* and *6*), and GRK6 (*lanes 7* and *8*). The transfected DNA was 1 and 5 μg/1,000,000 cells for the α_{1B}AR and the GRKs, respectively. After labeling with $^{32}P_i$, cells were treated for 5 min in the absence (−) or presence (+) of 10^{-4} M epinephrine. The phosphorylated receptors were immunoprecipitated with the antiserum against the C terminus of the α_{1B}AR, as described under "Experimental Procedures." 0.25 pmol of receptors were loaded in each lane. Receptors were resolved by 10% SDS-PAGE. Positions of prestained molecular mass markers are indicated in kDa. Results are from a representative experiment in which the effects of different GRKs were tested in parallel. *B*, COS-7 cells expressing the α_{1B}AR in the absence (*CON*) or presence of different GRKs were labeled with $^{32}P_i$ and treated with 10^{-4} M epinephrine (*EPI*) for different times. The ^{32}P content of the phosphorylated receptors was quantified as described under "Experimental Procedures." *Basal* indicates the receptor phosphorylation of untreated cells. Results are the mean ± S.E. of five or three independent experiments for the control (CON) and for the other conditions, respectively. *, $p < 0.05$ as compared to epinephrine-induced phosphorylation of control (*CON*); $, $p < 0.05$ as compared to the basal phosphorylation of control (*CON*).

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 α_{1B}ARs 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 α_{1B}AR coexpressed with βARK2.

To better assess the role of βARK in agonist-induced phosphorylation of the α_{1B}AR, 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 α_{1B}AR both in cell membranes and at the cell surface was similar in cells overex-

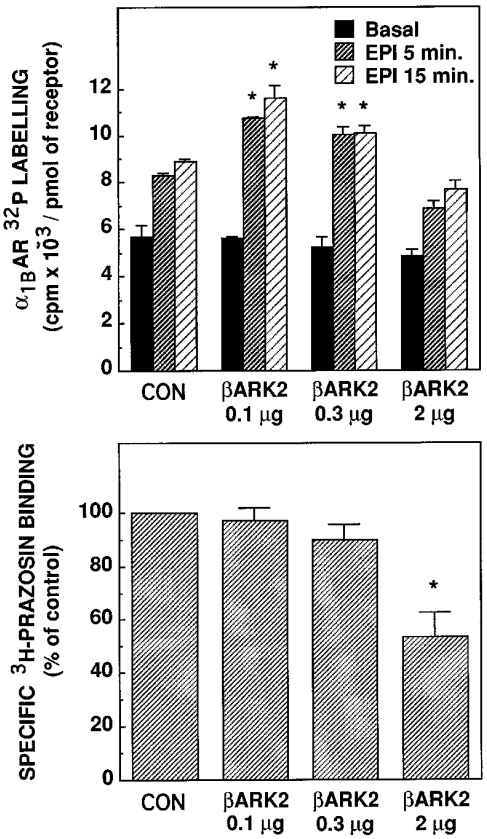

FIG. 3. **Effect of rat βARK2 on phosphorylation of the α_{1B}AR and cell surface receptors.** COS-7 cells were transfected with the DNA encoding the α_{1B}AR alone (0.5 μg/1,000,000 cells) or in combination with increasing amounts of DNA (μg/1,000,000 cells) encoding rat βARK2. *A*, phosphorylation of the α_{1B}AR was measured as in Fig. 2. Results are the mean ± S.E. of three independent experiments. *, $p <$ 0.05 as compared to epinephrine-induced phosphorylation of control (*CON*). *B*, [^{3}H]Prazosin binding to intact cell monolayers was measured as described under "Experimental Procedures." Control indicates the B_{max} in cells expressing the α_{1B}AR alone (*CON*), which was 133 ± 30 fmol/1,000,000 cells (means ± S.E. of three experiments). Receptor numbers measured in membrane preparations were similar under the different conditions and were in the range of 1–2 pmol/mg of protein (200–300 fmol/1,000,000 cells). *, $p < 0.05$ as compared to control (*CON*).

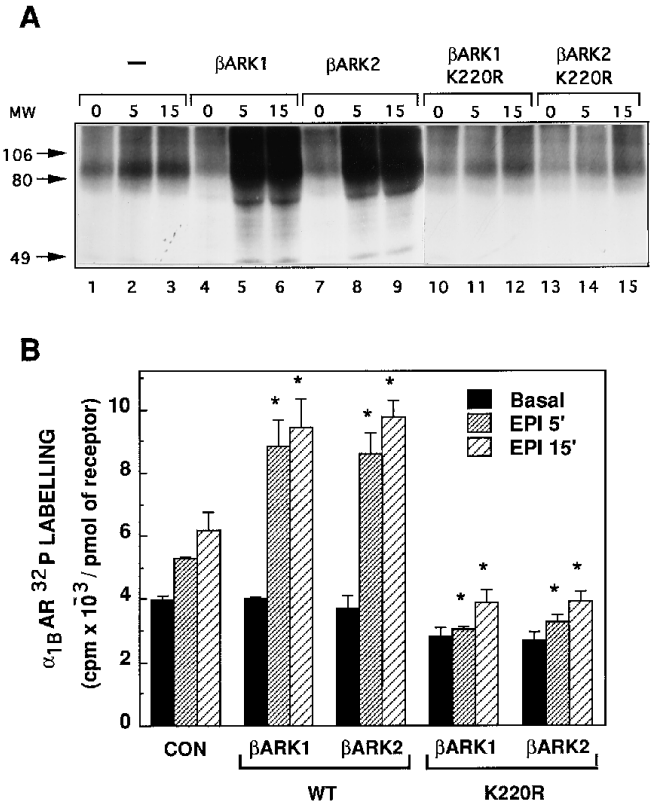

FIG. 4. **Effect of bovine βARKs and their dominant negative mutants on phosphorylation of the α_{1B}AR.** *A*, COS-7 cells expressed the α_{1B}AR alone (*lanes 1–3*) or in the presence of bovine βARK1 (*lanes 4–6*), bovine βARK2 (*lanes 7–9*), the βARK1 mutant K220R (*lanes 10–12*), or the βARK2 mutant K220R (*lanes 13–15*). The transfected DNA was 1 and 5 μg/1,000,000 cells for the α_{1B}AR and the βARKs, respectively. After labeling with $^{32}P_i$, cells were not treated (*0*) or stimulated with 10^{-4} M epinephrine for 5 and 15 min. The other experimental conditions are as in Fig. 2. *B*, COS-7 cells expressing the α_{1B}AR in the absence (*CON*) or presence of different βARKs were labeled with $^{32}P_i$ and stimulated with 10^{-4} M epinephrine (*EPI*) for different times as indicated. Receptor numbers measured in membrane preparations were similar under the different conditions and in the range of 1–2 pmol/mg of protein (200–300 fmol/1,000,000 cells). Receptor numbers measured in intact cell monolayers were also similar under the different conditions and in the range of 110–125 fmol/1,000,000 cells. Results are the mean ± S.E. of three independent experiments. *, $p < 0.05$ as compared to epinephrine-induced phosphorylation of control (*CON*) for each time of stimulation.

pressing bovine βARKs or their mutants as compared to that of cells expressing the receptor alone (results not shown). Overexpression of both bovine βARK 1 and 2 resulted in a pronounced increase of epinephrine-induced phosphorylation of the α_{1B}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 α_{1B}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 α_{1B}AR with an apparently similar affinity for the receptor. Second, they strongly suggest that agonist-induced phosphorylation and regulation of the α_{1B}AR occurring in a variety of cells is mediated, at least in part, by βARK.

Effect of GRKs on α_{1B}AR-mediated PI Response—To assess whether GRK-induced increase in the phosphorylation of the α_{1B}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 α_{1B}AR-mediated PI response as compared to that of the receptor expressed alone (Fig. 5). The inhibitory effect on the α_{1B}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 α_{1B}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 α_{1B}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 α_{1B}AR induced both a decrease of the maximal effect of epinephrine and 100-fold increase of its EC_{50} to stimulate receptor-mediated PI response. Similar results were obtained when the α_{1B}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-

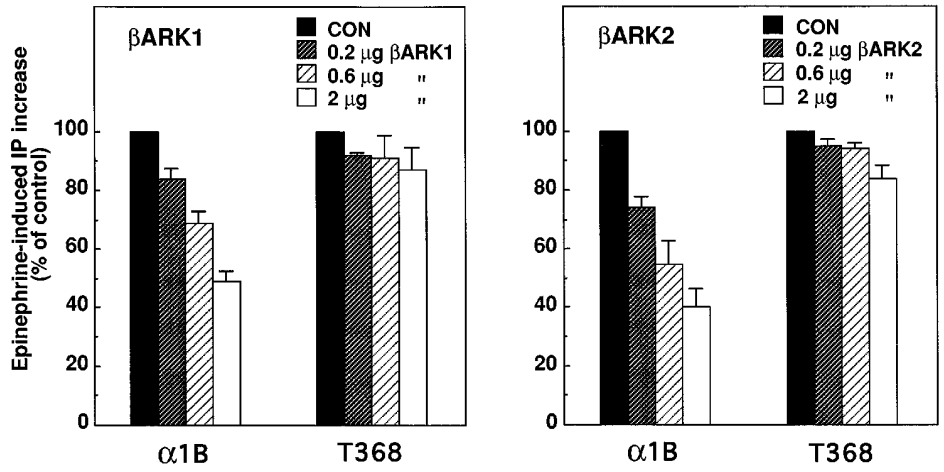

FIG. 5. **Overexpression of rat βARK1 and 2 attenuates α_{1B}AR-mediated PI hydrolysis.** COS-7 cells (500,000–1,000,000) grown in 35-mm dishes were transfected with the DNAs (0.2 μg/1,000,000 cells) encoding the α_{1B}AR or the T368 receptor alone or in combination with increasing amounts of DNA (μg/1,000,000 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/mg 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^{-4} 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 α_{1B}AR and the T368, respectively. The results are the mean ± S.E. of three independent experiments done in triplicate.

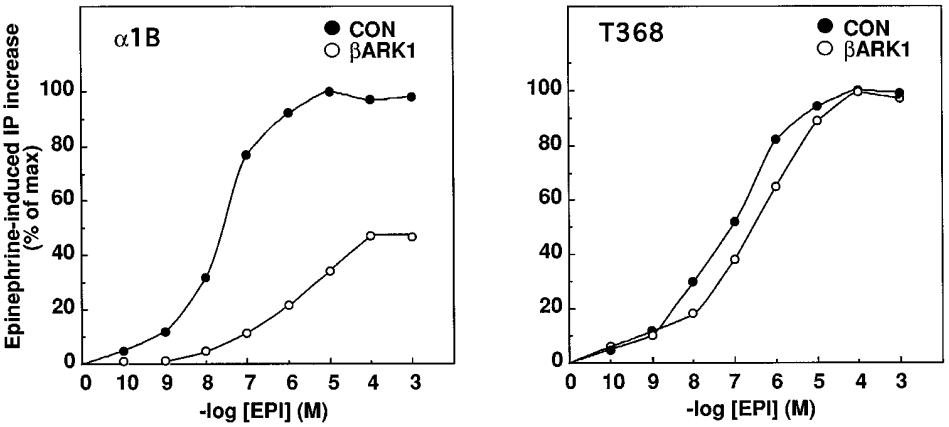

FIG. 6. **Dose response of epinephrine-stimulated PI hydrolysis.** COS-7 cells (500,000–1,000,000) grown in 35-mm dishes were transfected with the DNAs (0.2 μg/1,000,000 cells) encoding the α_{1B}AR or the T368 receptor alone or in combination with the DNA (1 μg/1,000,000 cells) encoding rat βARK1. The experimental conditions are as in Fig. 5. *% of max* indicates the response induced by 10^{-3} M epinephrine (*EPI*) for each dose response. The results are representative of two experiments.

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 α_{1B}AR. To further test this hypothesis, rat βARK1 and 2 were coexpressed with the truncated α_{1B}AR 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 α_{1B}AR-mediated response 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 α_{1B}AR. 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 α_{1B}AR, 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 α_{1B}AR. 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 α_{1B}AR was truly mediated by the phosphorylation of the receptor. Thus, the α_{1B}AR 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

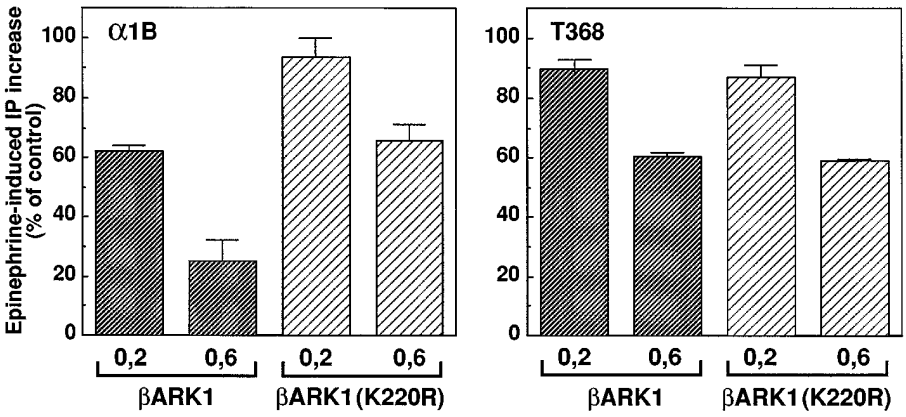

FIG. 7. **Effect of bovine βARK1 and its dominant negative mutant on α_{1B}AR-mediated PI hydrolysis.** COS-7 cells were transfected with the DNAs encoding the α_{1B}AR or the T368 receptor alone or in combination with increasing amounts of DNA (μg/1,000,000 cells) encoding bovine βARK1 or its dominant negative mutant K220R. The experimental conditions are as in Fig. 5. Receptor numbers measured in membrane preparations were similar under the different conditions for both receptors and in the range of 1–2 pmol/mg of protein (200–300 fmol/1,000,000 cells). [^{3}H]Prazosin binding to intact cell monolayers indicated that the expression levels of the receptors coexpressed with different βARKs were similar to those of the 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 α_{1B}AR-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% of impairment for the T368 and wild type receptor, respectively). For both the wild type and truncated α_{1B}AR, 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 α_{1B}AR-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 truly 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 α_{1B}AR and that this is mediated by their ability to phosphorylate the receptor.

Effect of β-Arrestins on the α_{1B}AR-mediated PI Response—For the β_2AR 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 α_{1B}AR-mediated signaling. Fig. 8 shows that coexpression of β-arrestin 1 with the α_{1B}AR 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

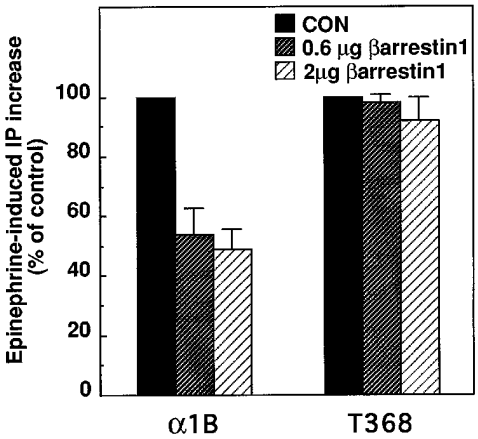

FIG. 8. **Overexpression of β-arrestin 1 attenuates α_{1B}AR-mediated PI hydrolysis.** COS-7 cells were transfected with the DNAs encoding the α_{1B}AR or the T368 receptor alone or in combination with increasing amounts of DNA (μg/1,000,000 cells) encoding β-arrestin 1. The experimental conditions are as in Fig. 5. The results are the mean ± S.E. of three independent experiments done in triplicate.

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 α_{1B}AR-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 α_{1B}AR.

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 α_{1B}AR. This is mainly supported by the finding that cellular overexpression of βARK1 or 2 can both increase agonist-induced phosphorylation of the α_{1B}AR and promote desensitization of the receptor-mediated PI response. Coexpression of β-arrestin 1 or 2 could also desensitize the α_{1B}AR-mediated activation of PLC. On the other hand, the truncated α_{1B}AR 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 α_{1B}AR—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 β_2-AR coupled to G_s-mediated stimulation of adenylyl cyclase, and the M2 muscarinic cholinergic receptor (M2-AchR) coupled to G_i-mediated inhibition of adenylyl 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 β_2-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 β_2-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 β_1-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 α_{1B}AR or its truncated mutant T368 in two different cell systems, COS7 and HEK293 cells. Overexpression of both βARK1 and 2 with the α_{1B}AR 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 α_{1B}AR 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 α_{1B}AR 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 α_{1B}AR, was largely insensitive to both βARK1 and 2 (Fig. 5). Second, when the kinases were overexpressed at low level, the α_{1B}AR-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 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 α_{1B}AR 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 α_{1B}AR. This is supported by the finding that overexpression of the dominant negative βARK mutants could inhibit the agonist-induced phosphorylation of the α_{1B}AR mediated by the endogenous kinases in COS-7 (Fig. 4). Thus, agonist-induced phosphorylation and regulation of the α_{1B}AR occurring in a variety of cells might be mediated, at least in part, by βARK.

In a previous study (15), we were unsuccessful at phosphorylation of the α_{1B}AR purified from DDT1 MF-2 smooth muscle cells reconstituted *in vitro* with βARK purified from bovine brain. An explanation of our previous lack of success might be that the reconstitution procedure impaired the receptor's ability to bind the agonist or to be stabilized in its "active" conformation, which is the substrate conformation required by βARK. This hypothesis can be supported by the fact that the efficiency of reconstitution in phospholipid vesicles (assessed as the ratio between ligand binding activity and the amount of reconstituted protein) was always much lower for the α_{1B}AR as compared to other receptors like the β_2AR suggesting that only a very small fraction of the reconstituted α_{1B}AR was functional.[3] Thus, future *in vitro* studies should first attempt to optimize the experimental conditions which can preserve the functional properties of the purified α_{1B}AR.

A Dominant Negative Mutant of βARK Can Impair the Receptor-mediated PI Response—Our results indicate that βARK-induced impairment of the α_{1B}AR-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 α_{1B}AR 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 α_{1B}AR 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 (PIP_2) 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 α_{1B}AR-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 PIP_2, 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 PIP_2. 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 α_{1B}AR-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 α_{1B}AR-mediated PI response suggesting that arrestin can play a role also in the desensitization process of the α_{1B}AR (Fig. 8).

Our results are in agreement with previous studies showing that overexpression of arrestin could impair the cAMP response mediated by the β_2AR (34) and, more recently, by the

[3] S. Cotecchia, unpublished observations.

β_1AR (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 stoichiometric excess over the G protein.

Another important finding of our study is that the integrity of the C-terminal portion of the α_{1B}AR is required for its interaction with arrestin. This was demonstrated by the fact that overexpression of β-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 α_{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 α_{1B}AR, which is also an important phosphorylation domain of the receptor.

GRK5 and GRK6 Can Increase Phosphorylation of the α_{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 β_2AR, the M2-AchR, and rhodopsin in an agonist-dependent fashion (30). GRK6 can also phosphorylate the same substrates, but with stoichiometries significantly lower than those achieved by GRK5 or βARK (31). More recently, it has been shown that GRK5 can increase agonist-induced phosphorylation of the β_1AR 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 α_{1B}AR, but *not* of its truncated mutant T368. Whereas coexpression of GRK6 could clearly increase epinephrine-induced phosphorylation of the α_{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 α_{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 α_{1B}AR in intact cells is indirectly mediated by one 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 β_2AR (40) as well as of the M3-AchR expressed in Sf9 cell membranes (22). Thus, the increased basal phosphorylation of the α_{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 autophosphorylation, which is stimulated by phospholipids *in vitro* and might regulate its kinase activity (41). Thus, the effect of GRK5 on the α_{1B}AR might reflect its constitutive activation following phospholipid-stimulated autophosphorylation in intact cells.

However, despite their effect on phosphorylation of the α_{1B}AR, coexpression of GRK5 or GRK6 either with or without β-arrestin did not induce any change of the receptor-mediated response. Thus, the functional correlates of GRK5 and GRK6-mediated phosphorylation of the α_{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 α_{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 βARK1 and 2 as well as β-arrestin 1 and 2 can play a role in agonist-dependent regulation of the α_{1B}AR. Second, they contribute to the elucidation of the receptor substrate specificity of different GRKs identifying the α_{1B}AR as a potential phosphorylation substrate for βARK1 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 ground for further investigation of the biochemical mechanisms underlying the regulation of the α_{1B}AR *in vitro* possibly reconstituting purified components.

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