Receptor-specific Desensitization with Purified Proteins

KINASE DEPENDENCE AND RECEPTOR SPECIFICITY OF β-ARRESTIN AND ARRESTIN IN THE β2-ADRENERGIC RECEPTOR AND RHODOPSIN SYSTEMS

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Martin J. Lohse‡, Sabine Andexinger‡, Julie Pitcher‡, Susan Trukawinski‡, Juan Codina†, Jean-Pierre Faure†, Marc G. Caron‡, and Robert J. Lefkowitz‡

From the ‡Laboratory of Molecular Biology, University of Munich, Max-Planck-Institute of Biochemistry, 8033 Martinsried, Germany, the §Howard Hughes Medical Institute, Duke University Medical Center, Durham, North Carolina 27710, the †Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030, and the ¶Institut National de la Santé et de la Recherche Médicale U 86, Université Pierre et Marie Curie, 75270 Paris 06, France

Homologous desensitization of β-adrenergic receptors, as well as adaptation of rhodopsin, are thought to be triggered by specific phosphorylation of the receptors. However, phosphorylation alone seems insufficient to inhibit receptor function, and it has been proposed that the inhibition is mediated, following receptor phosphorylation, by the additional proteins β-arrestin in the case of β-adrenergic receptors and arrestin in the case of rhodopsin.

In order to test this hypothesis with isolated proteins, β-arrestin and arrestin were produced by transient overexpression of their cDNAs in COS7 cells and purified to apparent homogeneity. Their functional effects were assessed in reconstituted receptor/G protein systems using either β2-adrenergic receptors with overexpression of their cDNAs in COS7 cells and purified arrestin and B-arrestin are similar proteins, they display marked specificity for their respective receptors, and that phosphorylation of the receptors by the receptor-specific kinases serves to permit the inhibitory effects of the "arresting" proteins by allowing them to bind to the receptors and thereby inhibit their signal-transducing properties. Furthermore, it is shown that this mechanism of receptor inhibition can be reproduced with isolated purified proteins.

Specific mechanisms have evolved to dampen the cellular responses that are mediated via different G protein-coupled receptors. One of these mechanisms is thought to be triggered by phosphorylation of the receptors by specific kinases (Kühn, 1984; Dohlman et al., 1991). Two such systems have been described. In light perception, the "receptor" rhodopsin is phosphorylated by rhodopsin kinase, and this appears to lead to inhibition of rhodopsin function (Wilden et al., 1986). An analogous system appears to exist in the case of β-adrenergic receptors, which can be phosphorylated by their own specific kinase, termed β-arrestin receptor kinase (βARK) (Benovic et al., 1986a). In both cases, the triggering factor is the activation of the receptors by their respective agonists, since the kinases phosphorylate only the active form of the receptors (Kühn and Dreyer, 1972; Benovic et al., 1986a).

However, it has become clear that phosphorylation of the receptors alone is insufficient to cause inhibition of receptor function. This is particularly evident from attempts to reproduce inhibition of receptor function with the purified protein components. Both in the case of β-adrenergic receptors (Benovic et al., 1987a) and in that of rhodopsin (Wilden et al., 1986), phosphorylation of the receptors by the specific kinase results at most in a modest reduction of receptor function. Using a system consisting of partially purified retinal proteins, Wilden et al. (1986) showed that addition of the retinal 48-kDa protein arrestin markedly enhanced the inhibitory effects of phosphorylation. Arrestin was later also shown to potentiate the inhibitory effects of βARK-mediated phosphorylation on β2-receptor function (Benovic et al., 1987a), but high concentrations of arrestin were required. Subsequently, the cDNA for β-arrestin, a potentially more specific inhibitor protein for β-receptors, was found in bovine brain; its sequence is almost 60% identical with that of arrestin. Expression of β-arrestin cDNA resulted in the appearance of an activity in the cytosol of transfected cells that was indeed capable of inhibiting the function of β2-receptors (Lohse et al., 1990a).

These observations suggested that receptor desensitization

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† The abbreviations used are: βARK, β-adrenergic receptor kinase; BKAR, β2-adrenergic receptor; PKA, protein kinase A; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; App(NH)p, adenylyl-5'-yl β,γ-imidodiphosphate; SDS, sodium dodecyl sulfate.
might proceed in two steps. Agonist binding triggers phosphorylation of the receptors by a specific kinase. This phosphorylation then permits the binding of β-arrestin (or arrestin, respectively), resulting in inhibition of receptor function. This hypothesis leaves open several questions. First, are these two proteins (the kinase and the “arresting” protein) sufficient to produce inhibition of receptor function, or are additional proteins required? To answer this question, it is necessary to reproduce the phenomenon with the isolated protein components, i.e. receptors, G proteins, receptor-specific kinases and arresting proteins.

Second, what is the role of receptor phosphorylation for the inhibitory effects of β-arrestin? β2-Adrenergic receptors can be phosphorylated not only by βARK but also by protein kinase A (PKA) (Benovic et al., 1985), and PKA-mediated receptor phosphorylation is thought to be responsible for the heterologous, i.e. generalized, not the receptor-specific form of desensitization (Sibley et al., 1987; Clark et al., 1988; Hausdorff et al., 1988; Lohse et al., 1990b). It is unknown, however, whether PKA-mediated receptor desensitization also might involve β-arrestin. The goal was to set out to determine the effects of receptor phosphorylation by these two kinases on the postulated inhibitory action of β-arrestin.

Third, what are the specificities of these receptor regulatory systems? At the level of the kinases, there is some evidence that these systems are not entirely selective. For example, βARK can phosphorylate purified β2- and α2-adrenergic, as well as muscarinic receptors (Benovic et al., 1987b; Kwatra et al., 1989), and βARK also phosphorylates rhodopsin, although only to a very low level (Benovic et al., 1986b). Only for β2-receptors it has been shown, however, that βARK-mediated phosphorylation has effects on receptor function. Of the arresting proteins, arrestin has been shown to be capable of interacting with β2-adrenergic receptors (Benovic et al., 1987a), but the relative specificities of arrestin and β-arrestin for rhodopsin and β2-receptors have not been investigated.

Since the cDNAs for arrestin (Shinohara et al., 1987) and β-arrestin (Lohse et al., 1990a) have now been cloned, we undertook the purification of the two proteins from overexpressing cells. The functional activities of the purified proteins were then characterized in terms of their ability to impair the interaction of purified β2-receptors or purified rhodopsin with their respective G proteins in order to elucidate their mechanism of action and their receptor specificities.

**EXPERIMENTAL PROCEDURES**

**Materials**—³²P-Labeled-cyanopindolol was obtained from Amer sham Corp. [γ-³²P]GTP was purchased from ICN and was purified by anion exchange chromatography on Dowex 1-X2-CI (Walsh and Johnson, 1979). 11-cis-Retinal was a gift from Hoffmann-La Roche. Cell culture media, fetal calf serum, and antibiotics were from GIBCO. Mono Q and Superose-12 columns were from Pharmacia LKB Biotechnology Inc.; HA-Ultrogel was from IBF. Nitrocellulose membranes for Western blotting and peroxidase-coupled goat anti-mouse antibodies were from Bio-Rad. Diaminobenzidine was from Kodak. Other chemicals were from standard sources and were of the highest quality available.

**Expression of β-Arrestin and Arrestin in COS7 Cells**—Expression vectors for β-arrestin and arrestin were constructed on the basis of the plasmid pBC/CMV/IL2 (Cullen, 1987) and were called pBC-β-arrestin and pBC-arrestin (Lohse et al., 1990a). COS7 cells were grown to 50% confluence in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum plus streptomycin and penicillin. They were transfected with the appropriate vectors at 5 µg/ml by the DEAE-dextran method as described by Cullen (1987). Two days later, the cells were harvested by scraping in a small volume (2 ml/10 cm²) of lysis buffer (5 mM Tris-HCl, 2 mM EDTA, pH 7.4, containing as protease inhibitors 10 µg/ml soybean trypsin inhibitor, 15 µg/ml benzamidine, and 100 µM phenylmethylsulfonyl fluoride). Cells were disrupted with a Polytron device, and the cytosolic fraction was separated from particulate material by centrifugation at 450,000 x g for 30 min. Small molecular weight components were removed by concentration of the cytosolic fraction on Centricon-30 devices (Amicon Corp.), followed by several washes with lysis buffer. This step also served to concentrate the samples to a protein content of 5–10 mg/ml.

**Purification of β-Arrestin and Arrestin**—β-Arrestin and arrestin were purified from the cytosolic preparations of transfected COS7 cells. In addition, arrestin was also purified in larger quantities from frozen bovine retinae. The purification procedures were developed by modifying those used for retinal arrestin by Dorey et al. (1982) and Zigler et al. (1984). All steps were carried out at 4 °C.

Protein from the cytosolic preparations described above was first precipitated with 35% saturated ammonium sulfate (2 h) and centrifuged at 100,000 x g for 30 min. The resultant supernatant was brought to 50% saturated ammonium sulfate, left for another 2 h, and centrifuged as above. The pellet, which contained most of the arrestin or β-arrestin, was resuspended in 250 µl of 100 mM sodium phosphate buffer, pH 7.4, and injected onto a Superose-12 HR 10/30 gel filtration column (Pharmacia LKB Biotechnology Inc.). The column was eluted with the same buffer at a flow rate of 0.25 ml/min. Elution of β-arrestin was monitored by measuring the ability of the fractions to inhibit the β2-receptor-stimulated GTPase activity of G, or by Western blots (see below). Elution of arrestin was determined by Western blots only.

Two peak fractions (equal to 1 ml) were pooled, diluted 5-fold with water and loaded onto a 5-cm HA-Ultrogel (IBF) hydroxylapatite column. Flow rates were 0.35 ml/min. The column was washed with 20 mM sodium phosphate buffer, pH 7.4, and then eluted with a linear 20–300 mM sodium phosphate buffer gradient (pH 7.4). Elution of β-arrestin and arrestin was monitored by Western blots only, since the variations in phosphate concentrations precluded accurate GTPase assays. The peak fractions, which eluted at about 80–100 mM sodium phosphate, were pooled and diluted 5-fold with 10 mM Tris-HCl, pH 7.4.

These diluted fractions were then injected onto a 0.5 × 5-cm Mono-Q 5/5 anion exchange column (Pharmacia LKB Biotechnology Inc.) at a flow rate of 0.5 ml/min. The column was eluted with 10 mM Tris-HCl, pH 7.4, and eluted with a linear 0–500 mM NaCl gradient in the same buffer. Peak fractions were identified by Western blot in GTase assays. Purity of the preparations was checked by silver stains of 12% SDS-polyacrylamide gels.

**Purification of Receptors and G Proteins—β2-Adrenergic receptors were purified from hamster lungs to >90% homogeneity as described by Benovic et al. (1984). G, was purified to >90% homogeneity from human erythrocytes as described by Codina et al. (1984). β2-Adrenergic receptor kinase was purified from bovine brain as described by Benovic et al. (1988); purity was about 70% for screening experiments (i.e. Western blots and autoradiograms) but 90% for quantitative experiments (i.e. those shown in Figs. 7-9). Rhodopsin kinase-phosphorylated rhodopsin was purified from rod outer segments as described by Wilde n and Kuhn (1982) and Phillips et al. (1989). In brief, bovine rod outer segments were phosphorylated by means of their endogenous rhodopsin kinase. This was achieved by incubation at 25 °C in the presence of ATP under bright light. The phosphorylated rhodopsin was subsequently regenerated with 11-cis-retinal (Wilden and Kuhn, 1982) and then solubilized under dim red light with 20 mM CHAPS and purified to >95% over concanavalin A-Sepharose (Pharmacia LKB Biotechnology Inc.) as described by Phillips et al. (1989). Transducin (G) was purified from bovine rod outer segments to >95% homogeneity according to Gier schik et al. (1984).

**Reconstitution Assays—β2-Adrenergic receptors and rhodopsin were reconstituted into phospholipid vesicles as described by Cerione et al. (1984) and Phillips and Cerione (1988). Reconstituted β2-receptors were phosphorylated by purified βARK according to Benovic et al. (1987a). The extent of phosphorylation of the receptors, as well as that of rhodopsin (see above), was monitored by adding small amounts of [γ-³²P]ATP to the phosphorylations and always exceeded 3 mol of phosphate/mol of receptor (or rhodopsin). PhosphorylatedPool of β2-receptors by PKA were done as described by Benovic et al. (1985) and resulted in <1 mol of phosphate/mol of receptor.

To measure receptor function, the respective G proteins were added on ice to the vesicles, using 50 fmol of β2-receptor (or rhodopsin) and 25 fmol of G, (or G) tube containing 50 µl of 10 mM Tris-HCl, pH 7.4, 100 mM NaCl. β-Arrestin or arrestin were also added on ice, and both were allowed to stay on ice for 30 min. The vesicles were then placed in the incubator at 4 °C for 5–30 min. The phosphorylations (adapted from Cerione et al., 1984) were initiated by adding 50 µl of assay mixture (final concentrations in assay were 10 mM Tris-HCl, 25 mM Tris-HCl, 2 mM EDTA, pH 7.4, G, or G, as appropriate, and 0.5 mM [γ-³²P]ATP to the phosphorylations and always exceeded 3 mol of phosphate/mol of receptor (or rhodopsin). Phosphorylated Pool of β2-receptors by PKA were done as described by Benovic et al. (1985) and resulted in <1 mol of phosphate/mol of receptor. To measure receptor function, the respective G proteins were added on ice to the vesicles, using 50 fmol of β2-receptor (or rhodopsin) and 25 fmol of G, (or G) tube containing 50 µl of 10 mM Tris-HCl, pH 7.4, 100 mM NaCl. β-Arrestin or arrestin were also added on ice, and both were allowed to stay on ice for 30 min. The vesicles were then placed in the incubator at 4 °C for 5–30 min. The phosphorylations (adapted from Cerione et al., 1984) were initiated by adding 50 µl of assay mixture (final concentrations in assay were 10 mM Tris-HCl, 25 mM Tris-HCl, 2 mM EDTA, pH 7.4, G, or G, as appropriate, and 0.5 mM [γ-³²P]ATP to the phosphorylations and always exceeded 3 mol of phosphate/mol of receptor (or rhodopsin). Phosphorylated Pool of β2-receptors by PKA were done as described by Benovic et al. (1985) and resulted in <1 mol of phosphate/mol of receptor.
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pH 7.4, 10 mM MgCl₂, 1 mM EDTA, 1 mg/ml bovine serum albumin, 0.2 mM dithiothreitol, 0.2 mM App(NH)p, 0.1 µM [γ-³²P]GTP, ≈0.2 µCi). β-receptors were stimulated with 10 µM (−)-isoproterenol, rhodopsin in the presence of 1 µM alprenolol for the β-receptor/Gᵢ system, and in the absence of rhodopsin, for Gα (since even under very dim red light, sufficient rhodopsin becomes activated to stimulate Gα). Incubations were at 30 °C for 10 min and were terminated by adding 10 µl of 100 mM sodium phosphate, pH 7.4, followed by 500 µl of 1% activated charcoal. The samples were centrifuged at 15,000 x g for 10 min, and the clear supernatant was transferred to tubes containing 1.6 ml of 10% HCl with 1.25% ammonium molybdate. The samples were vortexed, and the phosphate was extracted with 2 ml of isobutanol:toluene (1:1). Radioactivity was determined in the organic phase.

Western Blots—Proteins were transferred from 12% Laemmli SDS-polyacrylamide gels onto nitrocellulose membranes by semidy blotting. The blots were blocked with 5% nonfat dried milk in phosphate-buffered saline. Arrestin and β-arrestin were detected with the monoclonal antibody S6H8 raised against purified retinal arrestin (Faure et al., 1984), which recognizes an epitope common to both proteins (between amino acids 40-50 in arrestin and 36-46 in β-arrestin, i.e. PVDGVVLVDPE (Stiemer et al., 1992)), using peroxidase-coupled goat anti-mouse second antibodies (Bio-Rad) and diaminobenzidine (Kodak).

Results—Quantitative data are presented as means of at least three independent experiments. Concentration response curves were fitted to the Hill equation as described earlier (Lohse et al., 1984). Chromatographic data and photographs of blots and gels are representative of at least three similar experiments.

RESULTS

β-Arrestin and arrestin were expressed in similar quantities in transfected COS7 cells (Fig. 1). Both proteins could be visualized with the monoclonal antibody S6H8 raised against arrestin. Standardization with known amounts of retinal arrestin showed that the expressed proteins amounted to about 0.05-0.1% of the total protein in the cytosolic preparations from COS7 cells (not shown). Whereas arrestin was always seen as a sharp band at about 45 kDa, β-arrestin sometimes appeared as a somewhat broader band at about 50 kDa (Fig. 1).

We had shown earlier that crude cytosolic preparations derived from β-arrestin transfected COS7 cells were capable of inhibiting the function of βARK-phosphorylated β-receptors (Lohse et al., 1990a). To demonstrate this functional effect of β-arrestin unequivocally, however, it was necessary to produce such effects with the pure protein in a reconstituted system. Therefore, we undertook the purification of β-arrestin from transfected COS7 cells. For purposes of comparison, we also purified arrestin from similarly transfected COS7 cells.

To validate the functional integrity of the proteins overexpressed in COS7 cells, we also purified arrestin from bovine rod outer segments, where it represents the most abundant cytosolic protein (about 3% of the total according to Dorey et al. (1982)) and from which it can be recovered in large yields. In the following sections, only the purification of β-arrestin is presented in detail.

β-Arrestin was precipitated from the cytosolic fractions by adding increasing concentrations of ammonium sulfate. Western blots of the pellets, as compared with those of the initial material, showed that very little β-arrestin was precipitated by up to 40% saturated ammonium sulfate (Fig. 2), but up to 50% of the total cytosolic proteins were precipitated under these conditions (not shown). Almost all β-arrestin was precipitated at 50% saturation, and quantitative recovery was obtained in pellets at 60% saturation. Using a 35-50% differential precipitation, most β-arrestin was recovered, together with about a quarter of the total cytosolic protein.

The resulting preparation was then subjected to gel filtration on a Superose-12 column. Since most of the endogenous GTPase activity was resolved from the preparation at this stage, β-arrestin elution could also be monitored by its inhibitory effects on the stimulation of Gα by βARK-phosphorylated β-receptors (Fig. 3). β-Arrestin eluted from this column close to the standard protein ovalbumin, i.e. in agreement with its molecular mass of about 48,000, and was recovered as a sharp peak close to the major protein peak.

The pooled peak fractions from this step were further purified on HA-Ultrogel. Elution was monitored by Western blots only, since the phosphate gradient interfered with the GTPase assay. β-Arrestin eluted as a sharp peak within the major protein peak in the gradient close to 100 mM phosphate (Fig. 4). It was readily identified in silver stains of these fractions and represented an estimated 5-10% of the total protein content (not shown).

Anion exchange chromatography on a Mono-Q column was used as the final step of the purification. β-Arrestin eluted at 200-250 mM NaCl, behind most other proteins (Fig. 5). This peak had high inhibitory activity in GTPase assays and was apparently homogeneous as assessed by silver staining of SDS-polyacrylamide gels (Fig. 6). Fig. 6b shows similar silver stains for preparations of arrestin purified along the same lines from either transfected COS7 cells or rod outer segments.

The yields of either β- or arrestin were about 2 pmol of purified proteins per 15-cm Petri dish of subconfluent transfected COS7 cells. Based on quantitation by Western blots and activity in GTPase assays, the purification factors achieved in the peak fractions of the different purification steps were 3% ± 3% of the total according to Dorey et al. (1982).

Fig. 1. Expression of arrestin and β-arrestin in COS7 cells. COS7 cells were transfected with pBC-β-arrestin or pBC-arrestin and harvested 3 days later. Shown is a Western blot of cytosolic preparations probed with the monoclonal antibody S6H8, which recognizes an epitope common to arrestin and β-arrestin.

Fig. 2. Ammonium sulfate precipitation of β-arrestin from cytosolic preparations of transfected COS7 cells. Different concentrations of ammonium sulfate (given as percent saturation) were added to the cytosolic preparations of COS7 cells transfected with pBC-β-arrestin (see Fig. 1). Pellets were obtained after 2 h by centrifugation, resuspended, and loaded onto an SDS-polyacrylamide gel. The total (unfractionated) preparation was also loaded (far left). Shown is the Western blot probed with the antibody S6H8.
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Fig. 3. Gel filtration of β-arrestin. A 35–50% differential ammonium sulfate precipitate from cytosolic preparations of β-arrestin-expressing COS7 cells (see Fig. 2) was resuspended in 100 mM sodium phosphate buffer, pH 7.4, and separated on a Superose-12 gel filtration column. The fractions were monitored for their ability to inhibit the stimulation of Go,GTPase by reconstituted, βARK-phosphorylated β2-receptors. They were also assayed by Western blotting using the antibody S6H8 (inset). BSA, bovine serum albumin; OVA, ovalbumin; STI, soybean trypsin inhibitor.

Fig. 4. Hydroxylapatite chromatography of β-arrestin. The peak fractions of the gel filtration (Fig. 3) were diluted 5-fold with water and loaded onto a HA-ultrogel column. The column was washed with 20 mM sodium phosphate buffer, pH 7.4, and eluted with a linear 20–300 mM sodium phosphate gradient at pH 7.4. Fractions were assayed by Western blotting using the antibody S6H8 (inset).

Fig. 5. Anion-exchange chromatography of β-arrestin. The peak fractions of the hydroxylapatite chromatography (Fig. 4) were pooled and diluted 5-fold with 10 mM Tris-HCl, pH 7.4. These diluted fractions were then injected onto a 0.5 × 5-cm Mono-Q column. The column was washed with 10 mM Tris-HCl, pH 7.4, and eluted with a linear 0–500 mM NaCl gradient in the same buffer. The fractions were monitored for their ability to inhibit the stimulation of Go,GTPase by reconstituted, βARK-phosphorylated β2-receptors. They were also assayed by Western blotting using the antibody S6H8 (inset).

Fig. 6. Purified preparations of β-arrestin (a) and arrestin (b). The peak fractions of the Mono-Q chromatography (Fig. 5) and of analogous preparations of arrestin from either transfected COS7 cells or bovine retina were electrophoresed on 4/10% SDS-polyacrylamide gels. The gels were silver-stained or blotted onto nitrocellulose membranes that were probed with the antibody S6H8.

The effects of these purified proteins on receptor-G protein interactions were then assessed in the rhodopsin/Gα system. Arrestin was clearly more potent in inhibiting the function of phosphorylated rhodopsin than was β-arrestin (Fig. 7). The difference was at least 100-fold; an accurate determination was not possible, since β-arrestin could not be produced in sufficient amounts to produce significant inhibition of rhodopsin-activated GTPase. To ascertain the functional integrity of the proteins produced in COS7 cells, we also tested arrestin purified from bovine retina. It was found to have approximately equal inhibitory activity, as compared with the protein purified from COS7 cells (Fig. 7). Maximal inhibition was 60–80% of the control activity. Half-maximal inhibition was found at 75 fmol of arrestin/tube in the assay system used, which contained 50 fmol of rhodopsin. Thus, under the present conditions, about a 1:1 molar ratio of arrestin to phosphorylated rhodopsin seemed to result in inhibition of the ability of rhodopsin to activate Gα.

The reverse specificity was found in the β2-receptor/Gα system (Fig. 8). In this assay, β-arrestin was much more potent than arrestin, and caused up to 70% inhibition of β2-receptor-stimulated GTPase activity. Although arrestin was also capable of inhibiting β2-receptor function, it did so only in concentrations more than 100-fold higher than those of β-arrestin. For β-arrestin, half-maximal inhibition occurred at 90 fmol/tube, containing 50 fmol of β2-receptor. Thus, again...
Fig. 7. Inhibition of rhodopsin-stimulated Gt-GTPase by purified arrestin and β-arrestin. Purified phosphorylated and regenerated rhodopsin was reconstituted into phospholipid vesicles together with purified Gt in the presence of varying amounts of the purified preparations of either arrestin or β-arrestin. GTPase activity was stimulated by bright light. Shown is the light-induced activity measured as the difference in GTPase activity with or without rhodopsin. Values are means of three experiments. Control activity (in the absence of arrestin and β-arrestin) was 0.18 ± 0.05 mol of P_i/mol of Gt/min.

Fig. 8. Inhibition of β2-receptor-stimulated Gt-GTPase by purified β-arrestin and arrestin. Purified β2-receptors were reconstituted into phospholipid vesicles and phosphorylated by βARK. The vesicles were washed, and then purified Gt, and varying amounts of the purified preparations of either β-arrestin or arrestin were added. GTPase activity was stimulated by 10 μM (-)-isoproterenol. Shown is the agonist-induced activity measured as the difference in GTPase activity with isoproterenol or 1 μM of the antagonist alpenolol. Values are means of three experiments. Control activity (in the absence of arrestin and β-arrestin) was 0.18 ± 0.05 mol of P_i/mol of Gt/min.

This indicates that β-arrestin specifically recognizes the βARK-mediated phosphorylation pattern of the receptor.

DISCUSSION

The data presented here analyze the mechanism and specificity of inhibition of receptor function by two arresting proteins, the retinal protein arrestin and the widely distributed β-arrestin. Methods to purify β-arrestin have been developed so that studies could be performed with pure proteins. These arresting proteins appear to represent specific additional components of different signaling pathways. They are required to effect an agonist-triggered switching off of receptor-mediated signaling.

The role that these arresting proteins play in this process may be as follows (Wilden et al., 1986; Lohse et al., 1990a). An agonist-induced change in the conformation of the receptor (rhodopsin or the β2-receptor) turns the receptor into a substrate for a specific kinase, which phosphorylates multiple serine and threonine residues at the C terminus of the receptor. This phosphorylation then triggers the binding of a specific arresting protein, which in turn disrupts the coupling between receptor and its G protein. To establish this hypothesis conclusively, it appeared necessary to demonstrate such a mechanism with the purified proteins: receptor, receptor-specific kinase, G protein, and arresting protein.

Such experiments are presented here for the β2-receptor/Gt system and for the rhodopsin/Gt system. These experiments succeeded in reproducing, in a reconstituted system containing only four proteins purified to apparent homogeneity, the well described phenomenon of agonist-induced inhibition of receptor function that has been so extensively studied in intact cells and cell membrane systems. With this work, we can now demonstrate that these four proteins are sufficient to effect this phenomenon at least in a qualitative manner.

In quantitative terms, we have observed up to 60–80% inhibition in the reconstituted models. Using several different approaches, we have estimated that βARK-mediated desen-
sitzation of β2-receptors amounts to more than 50% of receptor function. These experiments involved either mutants of the β2-receptor lacking the presumed phosphorylation sites for βARK (Hausdorff et al., 1989) or the use of inhibitors of βARK in permeabilized cell systems (Lohse et al., 1989, 1990b). Thus, the extent of desensitization that is found in intact cells is similar to that observed in the reconstituted system.

Little is known about the possible mechanisms whereby arrestin and β-arrestin might inhibit the function of their respective receptors. In particular, the amounts of the arresting proteins required to produce inhibition were unknown. In the experiments presented here, the stoichiometries of arrestin to rhodopsin or β-arrestin to β2-receptors causing half-maximal inhibition were 1.5:1 and 1.8:1, respectively. Although a more detailed investigation of the exact stoichiometries will be necessary, these preliminary results suggest that inhibition is effected by binding of one arresting protein to one receptor. Thus, it appears unlikely that the arresting proteins inactivate receptors by an enzymatic catalytic process.

Receptor phosphorylation has been proposed to be important for the inhibitory effects of the arresting proteins both in the rhodopsin and in the β2-receptor systems (Wilden et al., 1986; Benovic et al., 1987a). However, the kinase specificity of this requirement had not been investigated. In particular, it was unknown whether β-arrestin might play a role in PKA-mediated heterologous desensitization of β2-receptors by potentiating the inhibitory effects that PKA-mediated phosphorylation has on receptor function. Our experiments show an important role of βARK-mediated receptor phosphorylation for the inhibitory effects of β2-arrestin. However, in contrast to earlier data obtained with arrestin (Benovic et al., 1987a), we find that receptor phosphorylation is not an absolute requirement. Rather, it increases the potency of β2-arrestin by a factor of 10–30. Thus, βARK-mediated phosphorylation appears to alter an equilibrium in the interactions between receptor, Gα, and β-arrestin.

This triggering function is specific for βARK, since PKA-mediated phosphorylation was without effects on the potency of β-arrestin. Thus, we conclude that β-arrestin plays no role in PKA-mediated heterologous desensitization, but is a specific component of βARK-mediated homologous (i.e. receptor-specific) desensitization. βARK phosphorylates multiple serine and threonine residues in the distal part of the C terminus of the receptor (Dohlman et al., 1987; Hausdorff et al., 1989), whereas PKA-mediated phosphorylations occur close to the membrane both in the third cytoplasmic loop and in the C terminus (Blake et al., 1987; Bouvier et al., 1989; Clark et al., 1989; Hausdorff et al., 1989). β-Arrestin appears to specifically recognize the βARK-mediated phosphorylations. The extent of βARK-mediated phosphorylation that may be required for this triggering is unknown at present. Our experiments were carried out with receptors containing an average of 4.5 mol of phosphate/mol receptor. There are multiple serine and threonine residues in the β2-receptor C terminus, and in vitro, up to eight phosphates can be incorporated into the receptor by βARK. However, in intact cells, the extent of phosphorylation caused by βARK is about equal to that caused by PKA (Hausdorff et al., 1989; Lohse et al., 1990b), i.e. no more than two phosphates/receptor. Since βARK-mediated desensitization amounts to >50% under these conditions (Lohse et al., 1990b), this suggests that phosphorylation at 2 residues of the receptor should be sufficient to allow β-arrestin to act. The small amounts of receptors that can be prepared at present have so far not allowed a determination of these apparently essential phosphorylation sites.

High stoichiometries of rhodopsin phosphorylation have been suggested to reduce per se the coupling to Gα (Shichi et al., 1984). Others have failed to observe such a direct effect of phosphorylation (Wilden et al., 1986). In the case of the β2-receptor, the effects of βARK-mediated phosphorylation alone are only marginal, even at high phosphorylation stoichiometries (Benovic et al. (1987a) and this report).

Both arrestin and β-arrestin show remarkable specificity for their respective receptors: arrestin is about 100-fold more potent than β-arrestin in inhibiting rhodopsin, and β-arrestin is about 100-fold more potent at β2-receptors. It has been suggested (Shinohara et al., 1987; Lohse et al., 1990a) that similarities between arrestin or β-arrestin with their respective G proteins might form the basis for an interaction with the receptors. If such parallels between the binding of receptors to G proteins and to arresting proteins do indeed exist, it is reasonable to expect the discovery of more arresting proteins that might correspond to the complexity of G proteins.

The biological significance of the inhibition of receptor function by these specific mechanisms seems to lie in an adaptation to the intensity of a stimulus (Kühn, 1984; Hausdorff et al., 1990; Dohlman et al., 1991; Lohse, 1992). This adaptation can result in changes of sensitivity of several orders of magnitude, allowing the receptors to function over a wide dynamic range. At the same time, they might serve to protect the signaling systems and the cells from excessive stimulation. Among the many mechanisms that have been shown to regulate the function of β-adrenergic receptors (Hausdorff et al., 1990), the βARK- and β-arrestin-mediated mechanism described here seems to be particularly important. First, quantitatively, it is the most important of the desensitization mechanisms (Hausdorff et al., 1989; Ligget et al., 1989; Lohse et al., 1990b), and second, it is the most rapid of these mechanisms. Recent data indicate that it operates with a half-time of less than 15 s (Roth et al., 1991).

At least in qualitative terms we have reproduced this form of desensitization with the purified proteins. Also, the extent of inhibition seems to be similar in vivo and in the reconstituted system. However, the study of the kinetics of the inhibition will require new experimental designs. Such studies might reveal the contribution of further proteins to the fine regulation of this process. The existence of such additional factors in the retinal system has been postulated from experiments showing that cytosolic extracts from rod cells enhance the kinetics of arrestin-mediated inhibition of rhodopsin (Wagner et al., 1988a, 1988b).

In summary, we have shown using purified proteins that agonist-induced specific inhibition of receptor function can indeed proceed as has been postulated. Agonist-dependent phosphorylation of β2-receptors followed by binding of β-arrestin is sufficient to impair coupling to Gα. Our data suggest that inhibition occurs simply by binding of β-arrestin to the receptors and not by a catalytic process. In inhibiting the function of their respective receptors, rhodopsin and β2-receptors, the two known arresting proteins, arrestin and β-arrestin, show a remarkable receptor specificity in spite of their high degree of structural similarity. Specificity in the β2-receptor system also exists at the kinase level, since βARK-but not PKA-mediated receptor phosphorylation serves as a trigger for the inhibitory effects of β-arrestin. These findings underline the importance of specific and stringent regulation of signaling pathways in biological systems. Finally, the high specificities of these regulatory proteins suggest that addi-
tional members of the receptor kinase and arrestin families are likely to exist and to mediate desensitization of other receptor-G protein systems.

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