Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 10439–10443, November 1993
Biochemistry

Specific enhancement of β-adrenergic receptor kinase activity by defined G-protein β and γ subunits

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Communicated by R. Huisgen, August 9, 1993

ABSTRACT **The β and γ subunits of heterotrimeric guanine nucleotide-binding proteins (G proteins) have recently been shown to play an active role in signal transduction. Among other effects they enable translocation of the β-adrenergic receptor kinase (βARK) from the cytosol to the plasma membrane and thus permit phosphorylation and ultimately desensitization of β-adrenergic receptors and other G-protein-coupled receptors. To investigate the specificity of this effect, we have purified various combinations of recombinant β and γ subunits expressed in Sf9 cells and measured their effects on βARK-catalyzed phosphorylation of β_2-adrenergic receptors and of rhodopsin. The combinations tested were $\beta_1\gamma_2$, $\beta_1\gamma_3$, $\beta_2\gamma_2$, $\beta_2\gamma_3$, and transducin βγ ($\beta_1\gamma_1$). There were clear differences in enhancement of rhodopsin phosphorylation, with an order of efficacy $\beta_2\gamma_2 > \beta_1\gamma_2 \gg \beta_2\gamma_3 \approx \beta_1\gamma_3 \approx \beta_1\gamma_1$. The first two combinations had larger effects than a mixed βγ preparation from bovine brain. In enhancing phosphorylation of β_2-adrenergic receptors, $\beta_1\gamma_2$ was more efficient and potent than all other combinations. These data suggest a twofold specificity of βγ complexes in enhancing βARK-catalyzed receptor phosphorylation: the γ subunits may be important in interacting with βARK, with γ_2 being more potent than γ_3, whereas the β subunits may determine coupling to the receptors, with β_2 being more effective than β_1 for rhodopsin and β_1 being more effective than β_2 for β_2-adrenergic receptors.**

G proteins are transducers that serve to transmit signals from receptors such as rhodopsin or the β-adrenergic receptors to effectors such as phosphodiesterases or adenylyl cyclases (1, 2). They consist of three different subunits, termed α, β, and γ. For each of these subunits several isoforms have been identified and their cDNAs have been sequenced (1, 3). Until recently it was thought that only the α subunits were important for the function as well as the specificity of G proteins. In particular it was thought that they were responsible for coupling to the receptors, for activation (through GTP binding), for transmission of the signal (via activation of the effectors), and for deactivation (by hydrolysis of GTP). In contrast, the hydrophobic βγ subunit complex had been proposed to serve in essence as a membrane anchor for the α subunit (4).

More recently, however, it has been recognized that the β and γ subunits play a more active role (reviewed in ref. 5). For example, direct interactions of βγ complexes with receptors have been shown by biochemical and biophysical means (6–8). The βγ subunit complexes have also been shown to be capable of interacting with effectors. Thus, they can inhibit or stimulate different isoforms of adenylyl cyclase (9–11) and can activate certain subtypes of phospholipase C (12–14). βγ complexes can activate the cytosolic β-adrenergic receptor kinase (βARK) by serving as membrane anchors (15–17). βARK is a kinase that phosphorylates agonist-activated β-adrenergic and related receptors (18) and thereby enhances binding of the inhibitor protein β-arrestin (19, 20) to the receptors, a process that is apparently responsible for receptor-specific or homologous desensitization (21, 22).

There are several distinct isoforms of both the β and the γ subunits, and complete cDNA sequences have been published for four β subunits (23–26) and five γ subunits (27–31). However, there is very little information on the potential specificities of these distinct isoforms in any of the effects mentioned above. Only in the case of receptor/Ca^{2+} channel coupling has the use of antisense oligonucleotides revealed distinct functional roles of β and γ subunits. Thus, the G-protein that couples muscarinic acetylcholine receptors to Ca^{2+} channels appears to be composed of α_{o1}, β_3, and γ_4 subunits, whereas a G protein of composition $\alpha_{o2}\beta_1\gamma_3$ couples somatostatin receptors to the same channels (32, 33).

In the present study we have investigated the potential specificity of G-protein β and γ subunits in activating βARK-mediated phosphorylation of receptors. We have expressed defined combinations of β and γ subunits in Sf9 cells using the baculovirus system, purified them to apparent homogeneity, and investigated their interactions with purified recombinant βARK and β_2-adrenergic receptors, as well as rhodopsin.

MATERIALS AND METHODS

Expression of G-protein βγ Subunit Combinations. For the generation of recombinant baculoviruses, the cDNAs of the G-protein subunits β_1, β_2, γ_2, and γ_3 were cloned into the vector pVL1393 (34). A cDNA of the β_1 subunit containing an *Nco* I site at the 5′ end of the open reading frame (35) was cloned into the *Bam*HI site of pVL1393. The sequence was then reverted to the original by site-directed mutagenesis. The β_2 cDNA (24) was partially digested with *Nar* I and *Sma* I and filled in to obtain blunt ends. The resulting 1127-bp fragment was ligated into the blunt-ended *Bam*HI site of pVL1393. An *Nco* I–*Xba* I fragment (218 bp) containing the γ_2 cDNA (28) was cloned into *Bam*HI/*Xba* I-digested pVL1393 vector. The γ_3 cDNA (29) was cut with *Bsu*36I and *Eco*RI, and the resulting 232-bp fragment was ligated into pVL1393 digested with *Bam*HI and *Eco*RI.

Recombinant baculoviruses were generated by cotransfection of monolayers of Sf9 insect cells (2×10^6 cells) with 25 ng of BaculoGold (Dianova, Hamburg, F.R.G.) viral DNA and 200 ng of vector DNA with the use of DOTAP transfection reagent (Boehringer Mannheim) or the calcium phosphate precipitation technique. After amplification of the mixed virus population, single recombinant clones were isolated by plaque assays, and one of each of these recombinant viruses was chosen for further amplification and subsequent infection.

For large-scale preparation of recombinant βγ subunit complexes, 200-ml suspension cultures of Sf9 cells (2×10^6 cells per ml) were coinfected with the recombinant baculoviruses (multiplicity of infection, 10 and 50 for the viruses expressing the β and the γ subunits, respectively). The cells

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Abbreviation: βARK, β-adrenergic receptor kinase.

were harvested 70 hr after infection, washed twice with ice-cold phosphate-buffered saline, and resuspended in homogenization buffer (10 mM Tris/HCl, pH 8.0/25 mM NaCl/10 mM $MgCl_2$/1 mM EGTA/1 mM dithiothreitol/0.1 mM phenylmethanesulfonyl fluoride with soybean trypsin inhibitor at 10 μg/ml, benzamidine at 30 μg/ml, and leupeptin at 5 μg/ml).

Purification of Recombinant $\beta\gamma$ Subunit Complexes. Sf9 cells were disrupted with an Ultra-Turrax homogenizer (twice for 10 sec) and the broken cells were passed through a 22- and a 27-gauge needle (five times each). The lysate was then centrifuged for 40 min at 115,000 × g. The $\beta\gamma$ complexes were solubilized from the particulate fraction with 1% (wt/vol) sodium cholate in homogenization buffer. After centrifugation for 30 min at 100,000 × g, the $\beta\gamma$ complexes were purified from the supernatant by a two-step chromatographic procedure adapted from Sternweis and Robishaw (36). The detergent extracts were diluted 10-fold in TED (20 mM Tris/HCl, pH 8/1 mM EDTA/1 mM dithiothreitol/0.1 mM phenylmethanesulfonyl fluoride/50 μM AlF_3/5 mM $MgCl_2$/10 mM NaF with benzamidine at 15 μg/ml) and applied to a heptylamine-Sepharose (Pharmacia) column equilibrated in TED/0.15% sodium cholate. Elution was achieved with a linear gradient ranging from 0.15% sodium cholate/250 mM NaCl to 2.5% sodium cholate/25 mM NaCl in TED. Fractions containing the $\beta\gamma$ complexes were pooled, diluted 10-fold, and loaded onto a DEAE-Sephacel (Pharmacia) column equilibrated with TED/0.15% sodium cholate. The $\beta\gamma$ complexes were eluted with a 0–500 mM NaCl gradient in TED/0.15% sodium cholate. Purified $\beta\gamma$ complexes were concentrated to about 15 pmol/μl and were stored at −80°C with 5% (vol/vol) glycerol.

ADP-Ribosylation of α_o. The activity of the recombinant $\beta\gamma$ complexes was determined by measuring their ability to support the ADP-ribosylation of α_o purified from bovine brain. The assay was performed as described (7) with minor modifications. Two pmol of α_o were used as substrate in an assay volume of 50 μl containing 600 ng of pertussis toxin (Sigma) and 0.1–3.0 pmol (2–60 nM) of $\beta\gamma$ complexes.

Phosphorylation of Rhodopsin and β_2-Adrenergic Receptors. Urea-treated rod outer segments and purified β_2-adrenergic receptors reconstituted into phospholipid vesicles were phosphorylated by purified recombinant βARK essentially as described (37). For rhodopsin phosphorylation, reaction mixtures (60 μl) contained 10 pmol (167 nM) of rhodopsin, 0.3 pmol (5 nM) of βARK, 0.3–9 pmol (5–150 nM) of $\beta\gamma$ complexes, 10 mM $MgCl_2$, and 50 μM [γ-^{32}P]ATP (Amersham). The $\beta\gamma$ complexes were diluted in cholate solution such that the final cholate concentration was 0.005%. The incubation was carried out at 30°C for 8 min under bright white light. The reaction was stopped, and the samples were analyzed by electrophoresis, autoradiography, and scintillation counting of the rhodopsin bands (37).

Recombinant human β_2-adrenergic receptors were purified from Sf9 cells (M.H. and M.J.L, unpublished work) and reconstituted into phospholipid vesicles as described (38, 39). Reaction mixtures (45 μl) contained 15 nM β_2-adrenergic receptors, 45 nM βARK, and 13–450 nM $\beta\gamma$ complexes. Incubations were carried out at 30°C for 25 min in the presence of 10 μM (−)-isoproterenol. The samples were analyzed as stated above.

Other Methods. G-protein $\beta\gamma$ complexes and α_o subunit from bovine brain and transducin $\beta\gamma$ complexes from bovine retina were purified as described (36, 40, 41). To reduce the contamination of α_o with $\beta\gamma$ to a minimum, the usual purification process was extended by a further chromatography step on a heptylamine-Sepharose column. Bovine βARK was expressed in Sf9 cells and purified to apparent homogeneity as described (37). SDS/polyacrylamide gel electrophoresis was done as described by Laemmli (42) or, for separation of γ subunits, according to Schägger and von Jagow (43). Protein concentrations were determined from Coomassie blue R250-stained polyacrylamide gels and according to Bradford (44).

RESULTS

To investigate effects of $\beta\gamma$ complexes of defined composition, the proteins were produced in Sf9 cells. Cells were coinfected with recombinant baculoviruses encoding the G-protein subunits β_1 or β_2 and γ_2 or γ_3 to give four different permutations, $\beta_1\gamma_2$, $\beta_1\gamma_3$, $\beta_2\gamma_2$, and $\beta_2\gamma_3$. These four $\beta\gamma$ complexes were purified to >95% homogeneity by chromatography on heptylamine-Sepharose and DEAE-Sephacel columns (Fig. 1). Mock preparations of uninfected cells were prepared in the same manner for control purposes. Transducin $\beta\gamma$ ($\beta_1\gamma_1$) and a bovine brain $\beta\gamma$ preparation containing multiple β as well as γ subunits were prepared by established procedures to serve as controls.

The functional integrity of the recombinant purified $\beta\gamma$ complexes was assessed by measuring their effects on pertussis toxin-mediated ADP-ribosylation of α_o. This assay revealed that all four recombinant combinations had similar activity (Table 1). The recombinant proteins were comparable in their activities to the transducin and brain $\beta\gamma$ preparations, indicating that they were fully active. In agreement with recent results (45) these data suggest that the various $\beta\gamma$ combinations show little selectivity toward α_o.

The phosphorylation of rhodopsin by βARK was enhanced by all $\beta\gamma$ complexes investigated (Fig. 2). However, there were marked differences in potency and efficacy of the various $\beta\gamma$ combinations. Whereas $\beta_2\gamma_3$ and $\beta_1\gamma_3$ caused only an ≈2-fold stimulation compared with controls, $\beta_2\gamma_2$ and $\beta_1\gamma_2$ led to >6-fold increases in rhodopsin phosphorylation (Fig. 3). Thus, $\beta\gamma$ complexes containing γ_2 appeared to be superior to those with γ_3 in supporting βARK-mediated phosphorylation of rhodopsin. The EC_{50} value of $\beta_2\gamma_2$ was much lower than that of $\beta_1\gamma_2$, indicating an increased affinity of the β_2-containing complexes either for the kinase or for rhodopsin. Transducin $\beta\gamma$ ($\beta_1\gamma_1$) had the smallest effects and was also the least potent of the $\beta\gamma$ combinations. A mock preparation from noninfected Sf9 cells had no effects on βARK-catalyzed phosphorylation of rhodopsin, indicating

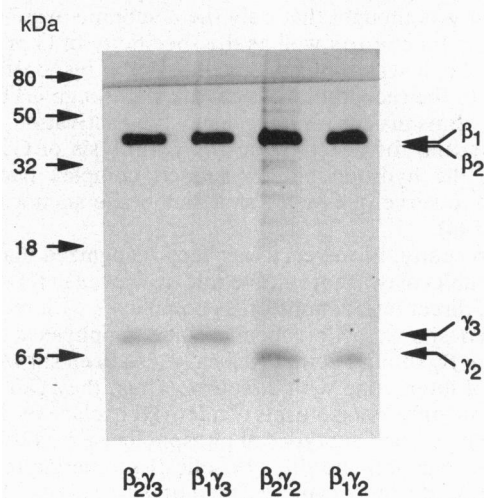

FIG. 1. Purified recombinant G-protein β and γ subunits. Samples (36 pmol per lane) of the $\beta\gamma$ combinations purified from Sf9 cells infected with the corresponding recombinant baculoviruses were analyzed by SDS/polyacrylamide gel electrophoresis according to Schägger and von Jagow (43) followed by staining with Coomassie blue.

Table 1. Enhancement of pertussis-toxin catalyzed ADP ribosylation of α_o by $\beta\gamma$ complexes

$\beta\gamma$ combination	ADP-ribose transferred, mol/mol α_o
None	0.21 ± 0.02
$\beta_1\gamma_2$	0.60 ± 0.10
$\beta_2\gamma_2$	0.67 ± 0.04
$\beta_1\gamma_3$	0.56 ± 0.03
$\beta_2\gamma_3$	0.58 ± 0.06
$\beta\gamma_B$	0.79 ± 0.06
$\beta\gamma_T$	0.66 ± 0.04

α_o (2 pmol, 40 nM) was purified from bovine brain and ADP-ribosylated in the presence of 1 pmol (20 nM) of the various $\beta\gamma$ complexes by 600 ng of pertussis toxin. Incubation time and temperature were 60 min and 30°C. $\beta\gamma_T$ denotes transducin $\beta\gamma$, and $\beta\gamma_B$ denotes a mixed bovine brain $\beta\gamma$ preparation. Data are means ± SEM from three separate experiments.

that the observed effects were indeed due to the recombinant $\beta\gamma$ subunits (Fig. 2).

βARK-mediated phosphorylation of β_2-adrenergic receptors reconstituted into phospholipid vesicles was analyzed in a similar manner in the presence of the various $\beta\gamma$ combinations. In this system, $\beta_1\gamma_2$ was the most potent of the recombinant $\beta\gamma$ combinations (Fig. 4*A*). Whereas $\beta_1\gamma_2$ caused >8-fold stimulation at low concentrations, the other recombinant combinations achieved only 2- to 5-fold stimulation (Fig. 4*B*). The $\beta\gamma$ preparation from bovine brain differed from the recombinant $\beta\gamma$ combinations, in that its maximal effects were similar to those of $\beta_1\gamma_2$ but it had a considerably higher EC_{50} value. The lower potency of this preparation is compatible with the fact that it contains multiple isoforms of both the β and the γ subunit.

DISCUSSION

In the present study we have expressed four different combinations of G-protein β and γ subunits and investigated their effects on βARK-catalyzed receptor phosphorylation. The four purified recombinant combinations—$\beta_1\gamma_2$, $\beta_1\gamma_3$, $\beta_2\gamma_2$, and $\beta_2\gamma_3$—were fully active when tested for their ability to support ADP-ribosylation of α_o by pertussis toxin. In this assay, there were no major differences between the various combinations, suggesting that there is little selectivity among these $\beta\gamma$ combinations with respect to α_o (45). In contrast, there were marked differences between the four recombinant combinations in their ability to enhance βARK-mediated phosphorylation of rhodopsin. The extent of maximal stimulation was much larger for combinations containing the γ_2 subunit (>600%) than those with the γ_3 subunit (≈100%). The $\beta\gamma$ subunit from transducin, which is equivalent to $\beta_1\gamma_1$, had

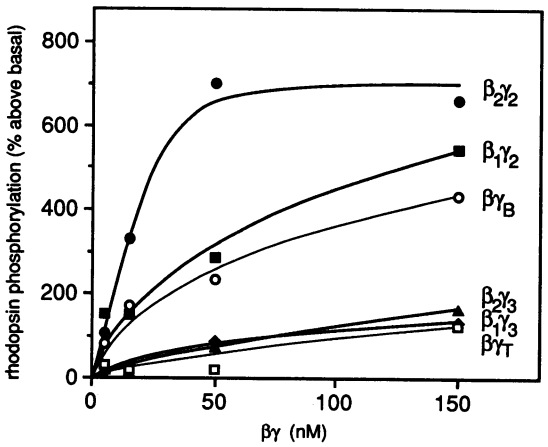

FIG. 3. Concentration dependence of the effects of $\beta\gamma$ complexes on βARK-catalyzed rhodopsin phosphorylation. Rod outer segments were phosphorylated as shown in Fig. 2 in the presence of the indicated concentrations of recombinant $\beta\gamma$ complexes (filled symbols), transducin $\beta\gamma$ ($\beta_1\gamma_1$, shown as $\beta\gamma_T$, □) or a bovine brain $\beta\gamma$ preparation ($\beta\gamma_B$, ○). Non-linear curve fitting to the Hill equation (46) gave the following EC_{50} and E_{max} values: $\beta_1\gamma_2$, 58 nM and 730%; $\beta_2\gamma_2$, 19 nM and 820%; $\beta_1\gamma_3$, 71 nM and 200%; $\beta_2\gamma_3$, 70 nM and 220%; $\beta\gamma_B$, 45 nM and 530%; $\beta\gamma_T$, 120 nM and 190%. Data represent means of six experiments.

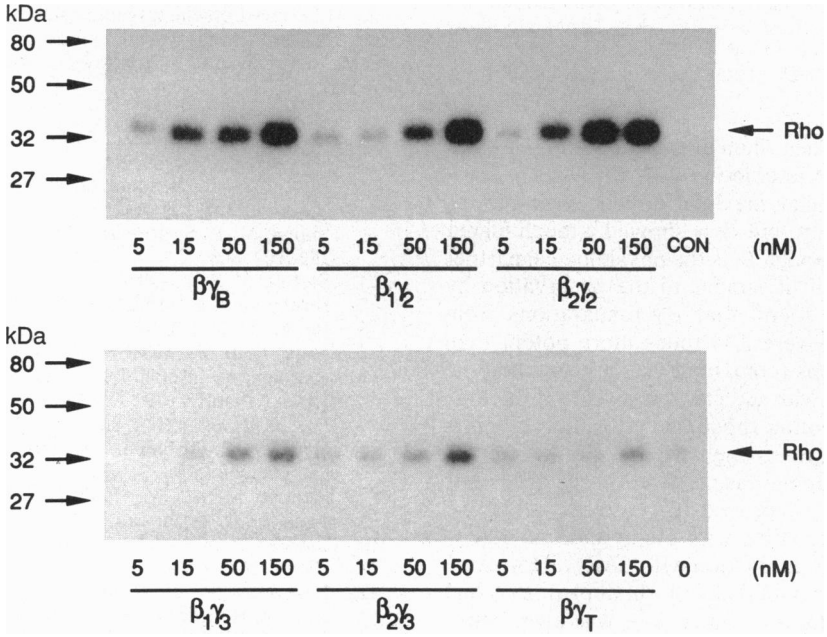

FIG. 2. Enhancement of βARK-catalyzed rhodopsin phosphorylation by $\beta\gamma$ complexes. Rod outer segments (>95% rhodopsin) were phosphorylated by purified βARK in the presence of the indicated concentrations of purified recombinant $\beta\gamma$ complexes. Autoradiogram is shown with the ^{32}P-labeled rhodopsin band (Rho). CON (top panel, right lane) indicates the effects of a mock preparation from uninfected Sf9 cells corresponding to the highest $\beta\gamma$ concentrations. The right lane in the bottom panel contains a control without $\beta\gamma$ complexes. The concentrations of rhodopsin and βARK in the assay were 167 nM (10 pmol per tube) and 5 nM (0.3 pmol per tube), respectively.

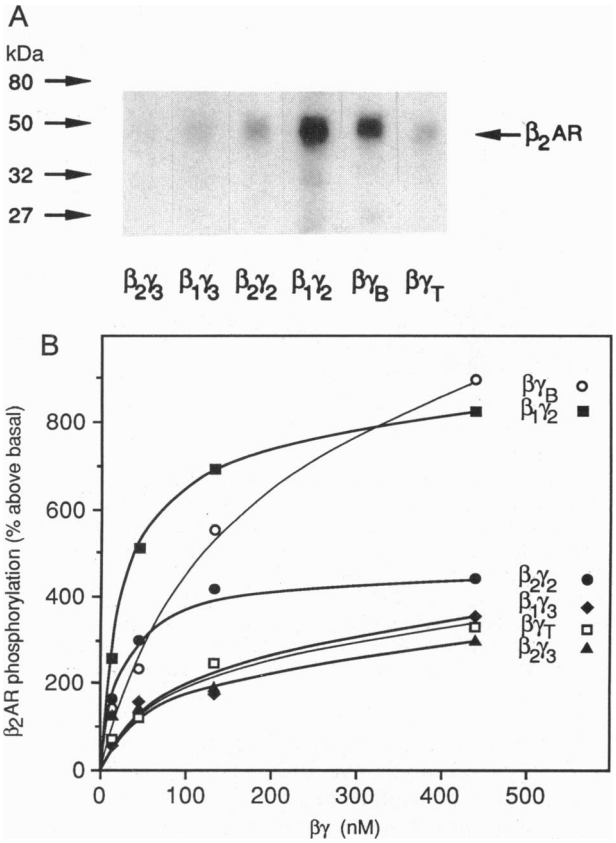

FIG. 4. Enhancement by $\beta\gamma$ complexes of βARK-catalyzed β_2-adrenergic receptor phosphorylation. Purified recombinant β_2-adrenergic receptors were reconstituted into phospholipid vesicles and phosphorylated with 45 nM βARK in the presence of various concentrations of $\beta\gamma$ complexes. (*A*) Autoradiogram of the ^{32}P-labeled receptor (β_2AR) phosphorylated in the presence (130 nM) of the various $\beta\gamma$ combinations. (*B*) Concentration dependence of the effects of the $\beta\gamma$ complexes: recombinant $\beta\gamma$ (filled symbols), transducin $\beta\gamma$ ($\beta\gamma_T$, □), or a bovine brain $\beta\gamma$ preparation ($\beta\gamma_B$, ○). Non-linear curve fitting to the Hill equation (46) gave the following EC_{50} and E_{max} values: $\beta_1\gamma_2$, 30 nM and 870%; $\beta_2\gamma_2$, 25 nM and 500%; $\beta_1\gamma_3$, 125 nM and 430%; $\beta_2\gamma_3$, 40 nM and 280%; $\beta\gamma_B$, 165 nM and 1200%; $\beta\gamma_T$, 80 nM and 380%. Data represent means of three experiments.

also only small effects. These data suggest that the γ subunit may be important for the interaction with βARK.

$\beta_1\gamma_2$ and $\beta_2\gamma_2$ had similar maximal effects in promoting rhodopsin phosphorylation, but $\beta_2\gamma_2$ showed a much higher affinity than $\beta_1\gamma_2$, even though β_1 is the physiological partner for rhodopsin. This result is similar to the observation by Fawzi *et al.* (47), who found that $\beta\gamma$ preparations from placenta (containing β_2) were 3–4 times more potent than transducin $\beta\gamma$ preparations (containing β_1) in promoting the interaction of rhodopsin with α_t. This suggests that the high potency of $\beta_2\gamma_2$ in promoting rhodopsin phosphorylation is due to the (nonphysiological) high-affinity interaction between β_2 and rhodopsin. In the case of β_2-adrenergic receptor phosphorylation, only $\beta_1\gamma_2$ proved to be a very effective combination. This is compatible with the hypothesis that γ_2 is required for an efficient interaction with βARK. However, in contrast to the findings obtained with rhodopsin, $\beta_2\gamma_2$ had much smaller effects than $\beta_1\gamma_2$. Since $\beta_2\gamma_2$ was even better than $\beta_1\gamma_2$ in promoting rhodopsin phosphorylation by βARK, this indicates that the β subunits determine the receptor selectivity in this system. Thus, our results suggest that the β_1 subunit couples more efficiently to β_2-adrenergic receptors than the β_2 subunit.

While these data indicate that $\beta_1\gamma_2$ is the most efficient $\beta\gamma$ combination of those tested here in promoting βARK-catalyzed phosphorylation of β_2-adrenergic receptors, this does not mean that it is the only combination that effects signaling by these receptors. In fact, it is tempting to speculate that receptors may utilize different $\beta\gamma$ subunit combinations in different systems and that, as a consequence, there may be pronounced βARK-mediated desensitization in some systems but not in others. Furthermore, the bovine brain $\beta\gamma$ preparation was comparable in its maximal effects to $\beta_2\gamma_2$ in promoting phosphorylation of β_2-adrenergic receptors. This raises the possibility that there are other $\beta\gamma$ combinations that are similarly efficient both in coupling to the receptors and in permitting translocation of βARK.

The hypotheses derived from the present work complement the data on apparent specificities of the $\beta\gamma$ complexes in receptor/Ca^{2+} channel coupling (32, 33), as well as recent observations by Wu *et al.* (48) on the effects of $\beta\gamma$ complexes on phospholipase C. Using cotransfection assays, those authors observed differences in the ability of various $\beta\gamma$ combinations to activate phospholipase C. They conclude that the β subunit may be responsible for effector interactions, whereas the γ subunit may determine specificity for α subunits. Our data appear to assign an additional function to each of the subunits: receptor interaction to the β subunit and βARK interaction to the γ subunit. The assignment of such different specific functions to the individual subunits may explain why the tightly coupled $\beta\gamma$ complex is composed of two separate subunits.

Receptor phosphorylation and, consequently, desensitization by βARK have already been shown to occur by a complex multistep process. The observation that $\beta\gamma$ complexes of defined composition may serve as membrane anchors for βARK adds another level of specificity to this system and supports the concept that G-protein β and γ subunits may play active and very specific roles in transmembrane signaling.

We thank Drs. Lutz Birnbaumer and Juan Codina for generously providing the β-subunit cDNAs and Drs. Narasimhan Gautam and Melvin Simon for the γ-subunit cDNAs. The support by grants from the Deutsche Forschungsgemeinschaft, Fonds der Chemischen Industrie, and Bundesgesundheitsamt is gratefully acknowledged. S.M. was supported by a fellowship from the Boehringer Ingelheim Fonds.

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