The α2A-Adrenergic Receptor Discriminates between G\textsubscript{i} Heterotrimers of Different βγ Subunit Composition in SF9 Insect Cell Membranes*

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In view of the expanding roles of the βγ subunits of the G proteins in signaling, the possibility was raised that the rich diversity of βγ subunit combinations might contribute to the specificity of signaling at the level of the receptor. To test this possibility, SF9 cell membranes expressing the recombinant α2A-adrenergic receptor were used to assess the contribution of the βγ subunit composition. Reconstituted coupling between the receptor and heterotrimeric G protein was assayed by high affinity, guanine nucleotide-sensitive binding of the α2-adrenergic agonist, [\textsuperscript{3}H]UK-14,304. Supporting this hypothesis, the present study showed clear differences in the abilities of the various βγ dimers, including those containing the β\textsubscript{1} subtype and the newly described γ\textsubscript{1γ}, γ\textsubscript{11} subtypes, to promote interaction of the same α\textsubscript{1} subunit with the α2A-adrenergic receptor.

Consistent with the steadily increasing number of G protein\textsuperscript{1} β and γ subtypes that has been revealed in recent years (1), in vivo studies have indicated a role for this structural diversity in the specificity of signaling. In this regard, antisense studies by Kleuss et al. (2, 3) have demonstrated a specific requirement for the β\textsubscript{1} and γ\textsubscript{1} subunits in the somatostatin receptor signaling pathway in rat pituitary GH\textsubscript{3} cells, with a similarly specific requirement for the β\textsubscript{2} and γ\textsubscript{1} subunits in the muscarinic receptor signaling pathway. Also, a ribozyme study by Wang et al. (4) has shown a specific involvement of the γ\textsubscript{1} subunit in the β\textsubscript{2}-adrenergic receptor signaling pathway in human kidney 293 cells. Taken together, these in vivo studies indicate that the composition of the βγ dimer has important ramifications for the fidelity of signaling that is probably manifested at the level of the receptor.

A growing body of in vitro evidence supports a direct interaction between the receptor and the βγ dimer (5). In particular, direct interaction of transducin βγ with rhodopsin has been shown with a fluorescence energy transfer technique (6). This association was blocked by a synthetic peptide derived from the carboxyl-terminal tail of rhodopsin, suggesting a site of direct contact between βγ and rhodopsin. Moreover, cross-linking studies have confirmed a receptor contact site on the β subunit. A synthetic peptide derived from the carboxyl-terminal portion of the putative third cytoplasmic loop of the α2A-AR could be cross-linked to the carboxyl-terminal region of the β subunit (7).

To date, in vitro studies examining the contribution of a limited number of βγ dimers to the specificity of receptor coupling have not yielded the same high degree of discrimination shown in the in vivo studies cited above (8, 9). The present study extended this analysis to the α2A-adrenergic receptor and to βγ dimers that represent the most extensive degree of structural diversity examined to date. Since baculovirus expression has been shown to be an effective means for producing functional G protein subunits (10–13) as well as G protein-coupled receptors (8, 9, 14, 15), this system was used to measure the level of interaction between the recombinant α2A-adrenergic receptor expressed in SF9 cell membranes and reconstituted in the presence or absence of purified G\textsubscript{i} proteins of varying β or γ subtype composition. Among the two β subtypes or eight γ subtypes tested, 30-fold differences were observed in their relative abilities to support coupling of the same α subunit to the recombinant α2A-adrenergic receptor. These data demonstrate that the specificity of α2A-adrenergic receptor-G protein interactions is affected by the βγ dimer composition.

EXPERIMENTAL PROCEDURES

Expression of α2A-Adrenergic Receptor—A pVL1392 transfer vector containing α2A-adrenergic receptor cDNA was generously provided by Dr. H. Kurose and R. LeKowitz (Duke University, Durham, NC). Recombinant baculovirus encoding the α2A-adrenergic receptor was generated by co-transfection of pBac-PVL1392 with a linearized lethal deletion mutant of Autographa californica as directed by the supplier (BaculoGold, PharMingen Corp.). Expression by recombinant baculovirus was identified by specific binding of the α2-adrenergic radioligand, [\textsuperscript{3}H]yohimbine (described below). A positive recombinant was isolated through four rounds of plaque purification. Receptors were expressed by inoculating SF9 insect cells at an m.o.i. of 1 in IPL-41 medium, 1× lipid concentrate, and 1% heat-inactivated fetal bovine serum (Life Technologies, Inc.) at a density of 2 × 10\textsuperscript{6} cells/ml. After 72 h, cell pellets were lysed by nitrogen cavitation (500 pounds/square inch for 30 min at 4 °C) in 100 ml of ice-cold lysis buffer (25 mM Tris, pH 7.4, 1 mM EDTA, 10 mM MgCl\textsubscript{2}, 100 mM NaCl, 0.02 mg/ml phenylmethylsulfonyl fluoride, 0.03 mg/ml leupeptin, and 1 mM benzamidine) and centrifuged at 4 °C for 10 min at 600 × g. The supernatant was centrifuged at 40,000 × g for 40 min at 4 °C. The pellets were resuspended, washed once in lysis buffer (40,000 × g, 40 min), and resuspended in 10 ml of lysis buffer. Protein concentration was determined by Coomassie assay (Pierce). Particulate fraction protein was snap-frozen with liquid N\textsubscript{2} in aliquots of 300 μg each and stored at −80 °C. Receptor expression was quantitated by saturation binding of [\textsuperscript{3}H]yohimbine, as described under.
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"Experimental Procedures." A single 500-ml expression culture yielded adequate material to carry out all of the reconstitution experiments.

Expression and Purification of G Protein Subunits—Recombinant baculoviruses directing the expression of \( \gamma_2 \), \( \gamma_3 \), \( \gamma_5 \), and \( \gamma_7 \) recombinant baculovirus were described previously (16, 17). Isolation of human \( \beta_3 \) and \( \gamma_2 \) recombinant baculovirus was described previously (18). In these cases, recombinant baculoviruses were obtained by co-transfection of Sf9 insect cells with pVL1393 transfer vectors containing \( \beta_3 \), \( \gamma_2 \), \( \gamma_5 \), or \( \gamma_7 \) and a linearized deletion mutant of \( A. californica \) nuclear polyhedrosis virus as directed by the supplier (BaculGoLd, Pharmingen). Recombinant baculoviruses were identified by immuno-blotting of Sf9 cell lysates for the expression of the appropriate subunit.

Subtype-specific antibodies were encoded previously (20, 21). Recombinant baculovirus encoding \( \alpha_1 \), containing a hexahistidine tag at amino acid position 121 was kindly provided by Dr. T. Kozasa (Southwestern Medical Center, Dallas, TX). One-liter cultures of Sf9 insect cells in IPL-41 medium, 1% heat-inactivated fetal bovine serum, and 1x lipid mix (Life Technologies, Inc.) were inoculated at a density of 2 x 10^5 cells/ml simultaneously with recombinant baculoviruses encoding \( \alpha \), \( \beta \), and \( \gamma \) subunits as follows: \( \alpha_{2A} \) at m.o.i. = 2, \( \beta \), or \( \gamma \) at m.o.i. = 3, and each of the \( \gamma \) subtypes at m.o.i. = 1. Under this condition, those \( \gamma \) subtypes predicted to contain a C-14 geranylgeranyl group are appropriately modified. However, those few \( \gamma \) subtypes predicted to contain a C-15 farnesyl group are variably modified at high levels of protein expression (10).

Therefore, to optimize the addition of a C-14 farnesyl moiety, cultures of Sf9 cells expressing \( \gamma_1 \) and \( \gamma_2 \) subtypes were also infected with recombinant baculovirus encoding both subunits of the mammalian farnesyltransferase at m.o.i. = 0.2. This virus was kindly provided by Dr. Thomas Kast (Glaxo Corp.). Cultures of Sf9 cells infected with the farnesyltransferase virus displayed greater than 15-fold higher activity toward the Ha-Ras fusion protein substrate than cultures not so infected. Moreover, cultures of Sf9 cells infected with the farnesyltransferase virus resulted in the majority of the \( \gamma_1 \) and \( \gamma_2 \) subtypes being modified with the C-15 farnesyl moiety, as shown previously (10). Expression of G protein \( \beta \) and \( \gamma \) subunits in particular cultures was confirmed 72 h later by immunoblotting with subtype-specific antibodies (22).

Recombinant \( \beta \) and \( \gamma \) heterodimers were purified to apparent homogeneity using the procedure described by Rozaska and Gilman (11) for purification of \( \beta_2 \gamma_2 \). Following cholate extraction of particulate fractions, the extract was dialyzed against 4% sodium cholate with 20 mM HEPES, pH 8.0, 100 mM NaCl, 1 mM MgCl\(_2\), 10 mM \( \beta \)-mercaptoethanol, 10 \( \mu \)M GDP, and 0.5% polyoxyethylene 10-lauryl ether. The cholate-soluble extract was loaded onto a 4-ml Ni-NTA resin bed at 300 mM NaCl and 5 mM imidazole. The cholate-soluble protein was diluted to 0.2% sodium cholate with 20 mM HEPES, 0.7% CHAPS, and 0.04%.

Following dialysis, purified \( \beta \gamma \) subunits were further purified by fast protein liquid chromatography on a Mono Q column (Amersham Pharmacia Biotech, HR 5/5) eluted with a linear NaCl gradient from 0 to 400 mM. Peak fractions were collected and dialyzed overnight (3 buffer changes) against 20 mM HEPES, pH 8.0, 1 mM EDTA, 3 mM DTT, 3 mM MgCl\(_2\), and 0.7% CHAPS (Spectra/Por tubing, 6000–8000 molecular weight cut-off, Spectrum Medical Industries, Houston, TX). A mixture of \( \beta \gamma \) subunits purified from bovine brain by a previously described method (23) was also further purified on a Mono Q column by the same procedure. Following dialysis, purified \( \beta \gamma \) subunits were concentrated to approximately 0.1 mg/ml in an Amicon ultrafiltration device (PM10 membrane), and the final protein concentrations were determined by staining with Amido Black. Purified \( \beta \gamma \) subunits were snap-frozen in small aliquots and stored at -80 °C.

The \( \alpha_{2A} \gamma_1 \) subunit was expressed alone (m.o.i. = 3) in a 1-liter culture of Sf9 insect cells for subsequent purification. Protein extraction, loading, and washing of the Ni-NTA column were identical to that described for \( \beta \gamma \) subunits. The \( \alpha_{2A} \gamma_1 \) was eluted from the Ni-NTA column with the following buffer, 20 mM HEPES, pH 8.0, 100 mM NaCl, 10 mM \( \beta \)-mercaptoethanol, 1 mM MgCl\(_2\), 0.5% polyoxyethylene 10-lauryl ether, 10 \( \mu \)M GDP, and 150 mM imidazole, and was subsequently purified further on a Mono Q column (fast protein liquid chromatography) using the same procedure described for \( \beta \gamma \) subunits with the exception that collection tubes contained an aliquot of GDP to yield a final concentration of 10 \( \mu \)M GDP in each of the column fractions. Subsequent handling of the \( \alpha_{2A} \gamma_1 \) was identical to that of the native \( \alpha_{2A} \gamma_1 \) subunit (for review, see Ref. 25). Moreover, this moderate level of recombinant receptor was optimal for the purpose of this study since this level of recombinant receptor expression was readily measurable but required minimal amounts of purified G proteins for reconstitution. Finally, the properties of the recombinant \( \alpha_{2A} \gamma_1 \) subunit were
characteristic of those of the native receptor. The specific $[^3H]$yohimbine binding showed a NaCl sensitivity that is typical of the native receptor (25), i.e. the $B_{max}$ binding plateau was 45–55% lower in the presence of 100 mM NaCl (data not shown). Prazosin and oxymetazoline displaced the specific $[^3H]$yohimbine binding from the recombinant receptor with the same order of potency of the native receptor ($K_D$ values of 4 and 0.022, respectively). Taken together, these data confirmed the suitability of SF9 cells for the expression of recombinant $\alpha_{2A}$-adrenergic receptor that is functionally similar to the native receptor (26).

Reconstitution of $\alpha_{2A}$-Adrenergic Receptor-G Protein Coupling in SF9 Cell Plasma Membranes—Coupling of the $\alpha_{2A}$-adrenergic receptor was examined in the presence and absence of exogenous $G_i$ protein using the agonist $[^3H]$UK-14,304. The direct agonist binding technique is generally considered to be more sensitive than agonist displacement studies for detecting receptor-G protein complexes (25). When SF9 cell membranes expressing the recombinant $\alpha_{2A}$-adrenergic receptor were incubated in the absence of exogenous $G_i$ protein, a low level of specific $[^3H]$UK-14304 binding was detected, accounting for ~15% of the binding that was later observed in the presence of added $G_i$ protein (Fig. 2). By contrast, when SF9 cell membranes expressing the recombinant $\alpha_{2A}$-adrenergic receptor were incubated in the presence of exogenous $G_i$ protein (at a molar ratio of 100:1 $G_i$:receptor), the level of specific $[^3H]$UK-14304 binding was increased by more than 5-fold, representing coupling of the recombinant receptor to the added $G_i$ protein (Fig. 2). Moreover, the increased level of $[^3H]$UK-14304 binding was reversed by the addition of GTPyS, reflecting uncoupling of the recombinant receptor from the added $G_i$ protein. Thus, reconstituted coupling was easily distinguishable from the background coupling in this experimental system, thereby confirming the suitability of this experimental system for measuring the coupling of the recombinant receptor to added $G_i$ proteins of varying $\beta_y$ composition. For optimal resolution between the reconstituted and background coupling, a 4 mM concentration of $[^3H]$UK-14,304 was used in subsequent experiments.

Receptor to G Protein Stoichiometry—The requirements of the $[^3H]$UK-14,304 binding assay for the G protein $\alpha$ and $\beta\gamma$ subunits were examined further. Consistent with previous studies of the $\alpha_1$ adenosine receptor (9), the combined interaction of both the G protein $\alpha$ and $\beta\gamma$ subunits was required in order to detect the high affinity state of the recombinant $\alpha_{2A}$-adrenergic receptor with this binding assay. As shown in Fig. 3A, when the recombinant receptor was reconstituted with the $6his_{1-14}$ subunit alone (at a molar ratio of 50:1 receptor), the level of $[^3H]$UK-14304 binding was low and was indistinguishable from that observed with no added $G_i$ heterotrimer. This result attests to the validity of the $[^3H]$UK-14,304 binding assay to evaluate differences in the ability of $\beta\gamma$ dimers of varying composition to induce the high affinity state of the
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recombinant receptor. Next, the recombinant receptor was reconstituted with a constant amount of 6hisα1 subunit and increasing amounts of βγ dimer. As shown in Fig. 3B, raising the amount of βγ dimer increased the fraction of receptor in the high affinity state as measured by the higher level of [3H]UK-14,304 binding. When the amounts of βγ dimer and 6hisα1 subunit approached a 1:1 ratio, the level of [3H]UK-14,304 binding reached a plateau, accounting for ~60% of the total receptor population as determined by [3H]lyo-histidine binding. A similar, maximal level of coupling was observed previously for the A1 adenosine receptor (9), suggesting that not all of the recombinant receptors are accessible for reconstitution with added G proteins. Under these conditions, any observed differences in the magnitude of [3H]UK-14,304 binding can be assumed to be attributable to selective interactions of βγ dimers of varying composition with the receptor rather than to alterations in G protein α-βγ subunit interactions.

Purification of G Protein βγ Dimers—To produce recombinant βγ dimers of varying composition, the β1 subtype was chosen since it has previously been shown to interact with all of the γ subtypes (17, 27, 28). The recombinant βγ dimers were purified using a procedure originally described by Kozasa and Gilman (11). Sf9 cells expressing the 6hisα1, β1, and one of the following γ1, γ2, γ7, γ4, γ5, γ7, γ10, or γ11 subunits were prepared. Then, cholate-solubilized membrane extracts from these cells were bound to Ni-NTA agarose columns by virtue of the 6-histidine tag on the α1 subunit; the βγ dimers were eluted from the columns by activating the bound heterotrimers with AMF; and the 6hisα1 subunit was subsequently eluted from the columns with high imidazole. Further purification of the recombinant βγ dimers and the 6hisα1 subunit was achieved by applying their enriched fractions from the Ni-NTA columns to Mono Q columns. The purity of the 6hisα1 subunit was assessed by SDS-PAGE and silver staining (29). As shown in Fig. 4A, the purified 6hisα1 preparation contained one major band of the size expected for the α1 subunit taking into account the added amino-terminal tag. The purity of the recombinant βγ dimers was also compared by SDS-PAGE and silver staining (Coomassie was used in the case of β1γ1). As shown in Fig. 4A, each purified βγ preparation was composed of two predominant bands by protein staining as follows: a 36-kDa band representing the β1 subunit, and a 5–8-kDa band representing the α1 subunit. Antibodies specific for each γ subtype. Antibodies specific for the newly described γ1, γ2, γ3, γ5, and γ7 subunits were used for this purpose previously (20). However, antibodies specific for the γ4, γ5, γ7, or γ11 subunits needed to be generated against synthetic peptides based on the unique amino acid sequences of these proteins. As shown in Fig. 4B, the identities of the purified β1γ1, β1γ10, and β1γ11 preparations were confirmed by immunoblotting with these antibodies.

Comparison of βγ Dimers of Varying γ Composition in Terms of Coupling to the α2A-Adrenergic Receptor—G heterotrimers of varying γ composition were tested for their relative abilities to couple with receptor as measured by the level of high affinity [3H]UK-14,304 binding. As shown in Fig. 5, all combinations of the 6hisα1, β1, and various γ subunits were capable of inducing high affinity [3H]UK-14,304 binding, with the level of binding showing dependence on the γ composition. In all cases, the [3H]UK-14,304 binding was completely abolished by addition of GTPγS (data not shown). The β1γ1 dimer supported only a very low level of coupling to the recombinant α2A-adrenergic receptor. By contrast, the β1γ11 dimer produced a high level of coupling to the recombinant α2A-adrenergic receptor. Since the γ1 and γ11 subtypes are closely related, showing 76% identity
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(19), the observation that they promote very different levels of coupling suggests that the relatively small number of amino acid differences between the two subtypes are important for recognition by the receptor. The β_{1γ2α}, β_{1γ3α}, β_{1γ4α}, and β_{1γ7α} dimers also produced high levels of coupling with the recombinant α_{2Aγ}-adrenergic receptor. These four γ subtypes are closely related, showing 66–74% homology at the amino acid level, and therefore, the finding that they produce essentially identical levels of coupling is not unexpected. Finally, the β_{1γ5α} and β_{1γ10α} dimers yielded intermediate levels of coupling with the recombinant α_{2Aγ}-adrenergic receptor. These two subtypes are only distantly related, showing less than 53% homology to each other or to other γ subtypes. Taken together, these results showed measurable differences between βγ dimers of varying γ composition to support coupling of the same α subunit to the recombinant α_{2Aγ}-adrenergic receptor. Statistical analysis revealed the β_{1γ1α} and β_{1γ10α} dimers supported the lowest levels of coupling. Previously, several groups have reported that the β_{1γ1α} dimer was less effective than other βγ dimers in supporting coupling to numerous receptors (8, 9, 30) and that this problem could not be overcome by increasing its concentration. To extend further these observations, we showed that increasing the concentration of the β_{1γ1α} dimer did not raise the level of receptor coupling (Fig. 5B), indicating that the γ_{1} subtype has a lower intrinsic ability to interact with the recombinant α_{2Aγ}-adrenergic receptor. Based on results for both the β_{1γ1α} and β_{1γ10α} dimers, a similar result could be predicted for the moderately effective β_{1γ10α} dimer.

The lower activities of the β_{1γ1α}, β_{1γ5α}, and β_{1γ10α} dimers could be due to differences in affinities for the α subunit of the G protein rather than for the α_{2Aγ}-adrenergic receptor itself. To evaluate this possibility, the affinities of representative βγ dimers for the α_{4α} subunit were measured by the pertussis toxin-dependent ADP-ribosylation assay. Under the reconstitution conditions used in this study, which employed relatively high concentrations of βγ dimers, there were no real differences in the affinity of these γ subtypes for the α_{4α} subunit (Fig. 6). Thus, the observed differences between βγ dimers of varying γ composition reflect their intrinsic abilities to interact with the receptor, suggesting structural diversity among γ subtypes plays a role in agonist-stimulated receptor-G protein complex formation.

Purification of G Protein βγ Dimers—A variety of approaches has been used to examine the ability of the 6 β and 11 γ subtypes to form βγ dimers (17, 19, 27, 28). Overall, these approaches have provided consistent results showing that all the known γ subtypes are able to interact with the βα subtype and, to a lesser extent, the β_{3γ} subtype. However, these approaches have yielded conflicting results regarding the abilities of the known γ subtypes to interact with the β_{4α} subtype. In this regard, the lack of a βγ dimer containing the β_{4α} subtype to serve as a positive control in the various assays has been a particular hindrance. To this end, the method of Kosasa and Gilman (11) was used to obtain a βγ dimer containing the β_{3γ} subtype. SF9 cells were infected with recombinant viruses encoding the α_{4α}, β_{3γ}, and γ subunits either simultaneously or separately, and the β_{3γ} γ subunits were then purified by the procedure described above. As shown in Fig. 7A, the co-expression of the α_{4α}, β_{3γ}, and γ subunits resulted in the appearance of β_{3γ} and γ subunits in the AMF activation fractions as detected by immunoblotting. This result was consistent with the release of
β3γ5 dimer from the 6his-i1 subunit in response to AMF activation. As shown in Fig. 7B, the identity of the AMF-released β subunit as the β3 subunit was confirmed by immunoblotting with a β3 subtype-specific antibody (B-34). Taken together, these results supported the conclusion that the β3 and γ5 subunits interact to form a functional βγ dimer.

An example of the purity of the β3γ4, β3γ5, and β3γ11 subunits that can be obtained by this procedure is shown by silver staining. As shown in Fig. 8A, each purified β3γ subunit was composed of two predominant bands by silver staining as follows: a 37-kDa band representing the β3 subunit and a 5–8-kDa band representing one of the γ subtypes. Confirmation that the β3 and γ5 subunits interact to form a functional βγ dimer is also shown using a previously developed tryptic digestion procedure (19, 27). This method is based on the finding that β monomers are cleaved at numerous sites by trypsin. By contrast, functional βγ dimers are cleaved at a single site, resulting in the appearance of a 26-kDa fragment of the β subunit that is resistant to further digestion by trypsin. Whereas the appearance of this stable 26-kDa fragment is a reliable marker for the formation of βγ dimers containing the β1 and β2 subtypes, it is not clear whether formation of βγ dimers containing the β3 subtype yields the appearance of a similar protected fragment. To date, such a protected fragment has not been detected for the β3 subtype, but these results are difficult to interpret due to the lack of availability of a positive control at that time (19). As shown in Fig. 8B, purified preparations of both the β3γ4 and β3γ3 subunits produced a 26-kDa protected fragment when digested under identical conditions with trypsin, as detected in each case by immunoblotting with a commercial β-antibody (DuPont SW/1, carboxyl terminus). Since equal amounts of β3γ4 and β3γ3 subunits were loaded, the differences in intensities of the β3 and β2 bands presumably reflect differences in affinities of the antibody used for immunoblotting. Taken together, these results confirmed that the β3 and γ5 subunits are able to interact to form a functional βγ dimer. Moreover, these results extended the utility of the trypsin digestion method as a reliable marker of βγ dimer formation to the β3 subtype.

Comparison of βγ Dimers of Varying β Composition in Terms of Coupling to the α2A-Adrenergic Receptor—As shown in Fig. 9, all combinations of the 6his-i1 subunit with the various βγ dimers were capable of inducing high affinity [3H]UK-14,304 binding. Again, in all cases, the [3H]UK-14,304 binding was completely abolished by addition of GTPγS (data not shown). The β3γ5 and β3γ11 dimers showed similar abilities to reconstitute coupling with the recombinant α2A-adrenergic receptor. Likewise, the β1γ11 and β3γ11 dimers had essentially identical activities. On the other hand, the β3γ5 dimer showed a substantially higher level of coupling with the recombinant α2A-adrenergic receptor than the β1γ11 dimer. Increasing the concentration of the β1γ5 dimer did not raise the level of coupling (Fig. 5B), indicating the β1 subtype, when in association with the γ5 subtype, has a lower intrinsic ability to interact with the recombinant α2A-adrenergic receptor. Taken together, these differences support the conclusion that the receptor recognition of the G protein is dependent on the particular combination of β and γ subtypes.

DISCUSSION

The present study examined the potential of the α2A-adrenergic receptor to couple to G proteins differing in their βγ subunit composition only. The selectivity of coupling was directly assessed by a high affinity agonist binding assay. Importantly, this assay was found to require the addition of the βγ subunits in order to detect the interaction of the α subunit with the receptor. From previous studies, this requirement for the βγ subunits appears to reflect not only a general role of the βγ subunits to stabilize the α subunit (31) but also a specific role of the βγ subunits to interact directly with the receptor (6, 7).
FIG. 9. Comparison of $\beta_1$ and $\beta_2$ in reconstitution of $\alpha_{2A}$-AR coupling. $G_i$ heterotrimer composed with the $\beta\gamma$ dimers indicated was reconstituted into Sf9 plasma membranes expressing $\alpha_{2A}$-adrenergic receptor at a molar ratio of 1:125:100 for receptor,$^{66}\alpha_i;\beta\gamma$. Binding of 4 nM [H]UK-14,304 was assayed in the absence or presence of 100 $\mu$M GTPgS. High affinity agonist binding was calculated as the difference between total binding and binding in the presence of GTPgS. Background (i.e. high affinity agonist binding without added G) was subtracted from each value to show only the reconstituted coupling. $\beta_1\gamma_5$ supported significantly less coupling than $\gamma_5\gamma_5$ ($p \leq 0.05$, Student’s $t$ test).

In view of these roles and the rich diversity of $\beta\gamma$ subunit combinations, the possibility was suggested that the nature of the $\beta\gamma$ subunits might contribute to the selectivity of the receptor interaction. Supporting such a possibility, the present study showed clear differences in the abilities of the various $\beta\gamma$ dimers, including those containing the $\beta_5$ subtype and the newly described $\gamma_4$, $\gamma_10$, and $\gamma_11$ subtypes, to promote interaction of the same $\alpha_i$ subunit with the $\alpha_{2A}$-adrenergic receptor.

Influence of $\beta\gamma$ Composition on Receptor Coupling—Several lines of evidence support the validity of using Sf9 insect cell membranes expressing the recombinant $\alpha_{2A}$-adrenergic receptor as a suitable system for examining the specificity of coupling to purified, recombinant $G$ proteins. First, the recombinant $\alpha_{2A}$-adrenergic receptor displayed the binding affinities and pharmacologic properties characteristic of the native receptor. Second, the recombinant $\alpha_{2A}$-adrenergic receptor showed a mostly uncoupled phenotype in the absence of added $G$ proteins and a largely coupled phenotype in the presence of added $G$ proteins of defined composition and stoichiometry. Using high affinity agonist binding as a quantitative measure of the coupled phenotype, this system was first used to examine the influence of the $\gamma$ component on receptor coupling. $G_i$ proteins were produced from $^{66}\alpha_i;\beta_1$, and varying $\gamma$ subtypes. Among the eight $\beta\gamma$ dimers tested, 30-fold differences were observed in their abilities to support coupling of the $^{66}\alpha_i;\gamma_1$ subunit to the $\alpha_{2A}$-adrenergic receptor, with the $\beta_1\gamma_2$, $\beta_1\gamma_3$, $\beta_1\gamma_5$, $\beta_1\gamma_7$, and $\beta_1\gamma_{11}$ dimers displaying the most efficacy, the $\beta_1\gamma_5$ and $\beta_1\gamma_{11}$ dimers showing intermediate efficacies, and the $\beta_1\gamma_1$ dimer exhibiting the least efficacy. With the exception of the $\gamma_1$ subtype, the observed differences segregated with the structural diversity of the $\gamma$ component along subclass lines. As defined previously, the human $\gamma$ subunit family has been divided into three subclasses, with each subclass showing less than 50% amino acid homology to other subclasses (1). On this basis, subclass I contains the $\gamma_1$, $\gamma_6$, and $\gamma_11$ subtypes, which are modified by the less common C-15 farnesyl group; subclass II includes the $\gamma_2$, $\gamma_4$, $\gamma_4$, $\gamma_7$, and $\gamma_{12}$ subtypes, which are modified by the more common C-20 geranylglycerol group; and subclass III contains the $\gamma_6$ and $\gamma_{10}$ subtypes, which again receive the more common C-20 geranylglycerol group. The present study represents the most extensive functional analysis of the $\gamma$ subunit family to date.

Next, this system was used to examine the influence of the $\beta_1$ versus the $\beta_3$ subtype on receptor coupling. In vitro studies have revealed little or no functional differences due to the $\beta$ subunit (16, 17). Since only the closely related $\beta_1$ and $\beta_3$ subtypes were examined, however, the present study extended this analysis to the more divergent $\beta_5$ subtype. For this purpose, the method of Kozasa and Gilman (11) was used to produce and then purify functional $\beta\gamma$ dimers containing the $\beta_5$ subtype. In addition to providing a source of material of defined composition, this approach also revealed new information on the selectivity of $\beta\gamma$ interaction by confirming the ability of the $\beta_3$ subtype to interact with the $\gamma_4$, $\gamma_5$, and $\gamma_11$ subtypes. Whereas interaction between the $\beta_5$ and $\gamma_4$ subtypes had been predicted (2, 3), the ability of the $\beta_3$ subtype to interact with the $\gamma_1$ subtype was unexpected in view of the high homology between the $\gamma_1$ and $\gamma_4$ subtypes and the reported failure of the $\gamma_1$ subtype to interact with the $\beta_3$ subtype (28). When the various $\beta\gamma$ dimers were tested for receptor coupling, only the $\beta_3\gamma_4$ and $\beta_3\gamma_5$ dimers showed substantive differences in their abilities to support coupling of the $^{66}\alpha_i;\gamma_1$ subunit to the $\alpha_{2A}$-adrenergic receptor. No such differences were observed with the $\beta_5\gamma_4$ and $\beta_5\gamma_5$ dimers nor the $\beta_5\gamma_{11}$ and $\beta_5\gamma_{11}$ dimers. These results suggested that it is the particular combination of $\beta$ and $\gamma$ subtypes that ultimately determines receptor recognition. Interestingly, the $\beta_5\gamma_5$ dimer was shown previously to interact preferentially with a G protein-coupled receptor kinase, GRK3, indicating a role of the $\beta$ subtype in selective receptor desensitization (32).

Taken together, these data show that $G_i$ proteins containing different $\beta\gamma$ dimers produce different levels of coupling to the $\alpha_{2A}$-adrenergic receptor. This result could arise because the composition of the $\beta\gamma$ subunits alters the formation or stability of the $G$ protein, the affinity of the $\beta\gamma$ dimer for the receptor, or some combination therefrom. Our data (17) and those from other laboratories (9, 33) suggest that formation of the $G$ protein is the least likely reason since the affinity of the $\alpha_i$ subunit for the various $\beta\gamma$ dimers is similar. Instead, our data are most consistent with the composition of the $\beta$ and, particularly, the $\gamma$ component affecting the affinity of the $G$ protein for the $\alpha_{2A}$-adrenergic receptor. Studies of the A1-adenosine receptor support a similar conclusion (33).

Sites of Interaction of $\gamma$ Component with Receptor—The observed differences in the abilities of various $\beta\gamma$ dimers to support coupling to the $\alpha_{2A}$-adrenergic receptor reside primarily in the $\gamma$ component. Although cross-linking studies have yet to detect receptor-$\gamma$ contact sites (7), several studies point to the importance of the carboxyl-terminal amino acid region and the type of prenyl group on the $\gamma$ subunit in determining the interaction of the $\beta\gamma$ dimer with receptor (34, 35). These latter studies may explain the lower reconstitutive activity of the $\beta_5\gamma_4$ dimer in the present study. In this regard, the $\gamma_4$ subtype sequence is the most divergent of those determined to date, and its lipid modification is a C-15 farnesyl group rather than the C-20 geranylglycerol group found on most other $\gamma$ subtypes (1). With regard to the latter, a recent study comparing $\beta\gamma$ dimers with the two types of prenyl groups showed that the wild type, geranylglyceranylated $\gamma_4$ subunit and the mutant, geranylglyceranylated $\gamma_4$ subunit had similar abilities to interact with the A1 adenosine receptor (34). By contrast, the wild type, farnesylated $\gamma_4$ subunit and the mutant, farnesylated $\gamma_4$ subunit were much less effective. Whereas these data indicate that type of prenyl group is critical, the primary structure of the $\gamma$ subunit is of equal or greater importance. Underscoring this point, a synthetic peptide derived from the carboxyl-terminal sequence of the $\gamma_4$ subtype was able to stabilize the light-activated state of rhodopsin receptor to a much greater degree when the peptide was farnesylated than not (35). However, the effect was greatly attenuated when the amino acid sequence of the peptide changed by only two amino acid substitutions (F64T and
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L67S) even though the farnesylated state was preserved. Thus, both the primary structure and the prenylation state of the γ1 subunit are likely to contribute to its poor affinity for the α2A-adenrenergic receptor in the present study and its contrastingly high affinity for the rhodopsin receptor in previous studies (35). When compared with the β1γ1 dimer, the higher reconstitutive activity of the β1γ111 dimer was unexpected since the newly described γ11 subtype is farnesylated and has a carboxy-terminal tail nearly identical to γ1 subtype. This result suggests the importance of regions other than the carboxy-terminal tail of the γ11 subtype in promoting its strong interaction with the α2A-adrenergic receptor. By focusing on the few amino acid differences between the γ1 and γ1 subtypes and making the appropriate mutations, it should be possible to pinpoint other regions of γ protein structure that are selectively recognized by the α2A-adrenergic receptor. Given the wide tissue distribution of the γ11 subtype (19) in contrast to the restricted expression of the γ1 subtype, it is perhaps not so surprising that a receptor other than rhodopsin would exist which prefers the farnesylated γ11 subtype in tissues other than the retina.

Finally, the high reconstitutive activities of the β1γ1, β1γ9, β1γ11, or β1γ12 dimers compared with the intermediate activities of the β1γ1 and β1γ10 dimers underscore the importance of primary structure in another way. Since all of the aforementioned γ subtypes are modified by the C-20 geranylgeranyl group (19), the observed differences between the two groups must relate to the differences in protein structure. In agreement with this, the γ1, γ10, γ4, and γ11 subtypes share a high degree of amino acid homology (66–74%), and predictably, βγ dimers containing these γ subtypes produce comparably strong levels of coupling of the thisγ1 subunit to the α2A-adrenergic receptor. By contrast, the γ1 and γ11 subtypes share only 35–50% identity with the aforementioned group of γ subunits (19), and accordingly, βγ dimers containing these γ subtypes yielded significantly lower levels of coupling in comparison with the mean value calculated from the grouping of βγ dimers consisting of the γ2, γ3, γ4, and γ12 subtypes (ρ = 0.025 or 0.05, respectively; Student’s t test). Taken together, these data indicate that the primary structure, including regions other than the carboxy-terminal tail, of the γ subunit is a most important factor in determining the selectivity of interaction with the α2A-adrenergic receptor. In this instance, the type of prenyl group is a less critical factor.

Sites of Interaction of β Component with Receptor—Although the β subunits are highly conserved in their predicted amino acid sequences, the observed difference between the β1γ1 and β1γ9 dimers indicates that selective receptor recognition does occur on the basis of the β subtype. Cross-linking studies have revealed a site of contact between the 7th or possibly 6th WD-40 repeat of the β subunit and a peptide derived from the third intracellular loop of the α2A-adrenergic receptor (7). The crystal structure of βγ indicates that residue 303, which lies at the center of this segment, resides on an exposed surface of the βγ dimer (36). Thus, this site has the potential to participate in the preferential coupling of the α2A-adrenergic receptor to Gβ1 heterotrimers containing the β1γ9 dimer over those containing the β1γ11 dimer. Other possibilities are suggested: 1) regions other than the carboxy-terminal tail of the β subunit may interact with receptor; 2) a concerted interaction between the β and γ subunits within the G protein binding pocket of the receptor; or 3) some combination therefrom.

Influence of βγ Composition on Coupling to Other G Protein-coupled Receptors—Various βγ dimers show a different order of potency depending on the type of receptor (8, 9, 37). It is speculated that protein-protein interactions between the βγ subunits and receptors, as well as hydrophobic interactions due to the prenylation state of the γ subunit, will be important elements in modeling selective recognition between G protein and receptors. Distinct, yet to be revealed, structural features within the G protein binding regions of receptor subtypes must also be taken into account in such a model. For example, a previous study showed that the 5HT1A receptor interacts similarly with G proteins containing β1γ1, β1γ9, or β1γ12 dimers (8), whereas the present study revealed that the α2A-adrenergic receptor prefers G proteins containing the β1γ1 or β1γ12 dimer over that containing the β1γ11 dimer. Thus, the G protein binding pockets of the α2A-adrenergic receptor and the 5HT1A may possess subtle structural differences that result in either more or less receptor to Gαβγ contact depending on the identity of the γ subunit. Active-state receptors may possess discrete elements contacting α, β, and γ subunits within the same G protein that complement one another to some degree in order to activate the heterotrimer. The experimental approach used here is quite amenable to manipulating both the receptor and the G protein subunit composition in order to bring together selected elements of signaling proteins.

Summary—The data in the present study demonstrate the specificity of α2A-adrenergic receptor-G protein interactions is affected by the βγ dimer composition, with protein-protein interactions forming the basis for the observed differences. These in vitro results complement a growing body of in vivo results demonstrating the βγ subunit composition is an important determinant of the specificity of signaling pathways. Strikingly, antisense suppression of the β1γ9 or β1γ12 subtypes disrupts coupling between inhibition of a calcium channel and the somatostatin or muscarinic receptors, respectively, in GH3 pituitary cells (2, 3). Similarly, riboyme suppression of the γ1 subtype attenuates coupling between stimulation of adenylcyclase and the β-adrenergic receptor in HEK293 cells (4). Although these results could arise from an ordered arrangement of signaling proteins in the cell membrane, the in vitro results presented here implicate receptor-βγ “recognition” as an additional mechanism for determining the specificity of signaling. It is expected that a combination of in vitro and in vivo approaches will provide some of the answers needed for construction of a mechanistic model of specificity in G-protein-mediated signaling pathways.

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