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Role of G-protein availability in differential signaling by alpha 2-adrenoceptors

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Abstract

The impact of G-protein expression on the coupling specificity of the human α_{2B}-adrenergic receptor (α_{2B}-AR) was studied in Sf9 cells. The α_{2B}-AR was shown to activate both coexpressed G_s- and G_i-proteins in a [^{35}S]GTPγS binding assay. Noradrenaline and the synthetic agonist UK14,304 were equally potent and efficacious in stimulating G_i activation. At the effector level (adenylyl cyclase), both ligands stimulated cAMP production. In the presence of forskolin, the effects of the agonists were more complex. Noradrenaline stimulated cAMP production, while UK14,304 showed a biphasic concentration–response curve with inhibition of stimulated cAMP production at low agonist concentrations and further stimulation at high agonist concentrations. G_s coexpression caused a monophasic stimulatory response with both ligands. Coexpression with G_i resulted in a biphasic concentration–response curve for noradrenaline and a monophasic inhibition with UK14,304. Experiments with a panel of agonists demonstrated that the more efficacious an agonist is in stimulating cAMP production, the weaker is its ability to couple to inhibition of cAMP accumulation via exogenous G_i. To be able to explain the mechanistic consequences of dual G-protein coupling described above, we developed a mathematical model based on the hypothesis that an agonist induces different conformations of the receptor having different affinity for different G-proteins. The model reproduced the profiles seen in the concentration–response curves with G_s and G_i coexpression. The model predicts that the affinity of the receptor conformation for G-proteins as well as the availability of G-proteins will determine the ultimate response of the receptor.

Keywords: α 2 receptors; G-proteins; cAMP; GTPγS; Sf9 cells; Model

1. Introduction

The α_2-adrenergic receptors (α_2-ARs), represented by three genetically distinct subtypes α_{2A}, α_{2B}, and α_{2C}, control the function of different organs via central and peripheral mechanisms. The effects can be either inhibitory or stimulatory depending on the organ in question [1]. This is likely to be due to the ability of these receptors to activate multiple signal transduction pathways [2]. The classical cellular response observed upon activation of endogenously expressed α_2-ARs is an inhibition of stimulated cAMP production [3] or inhibition of voltage-gated Ca^{2+} channels in neuronal cells [4,5]. Both of these responses are mediated by $G_{i/o}$-type pertussis toxin-sensitive G-proteins. However, ectopic expression of α_2-ARs in different cell lines has revealed a pertussis toxin-insensitive increase rather than a decrease in cAMP production in response to α_2-AR activation [6–9]. In many cell lines, this can be seen as a biphasic concentration–response curve with inhibition at low and stimulation at high agonist concentrations [7,10,11]. In some systems, in particular with the α_{2B} subtype, an exclusively stimulatory concentration–response curve is obtained [7–9,11], suggesting a productive receptor–G_s interaction. A large number of G-protein-coupled receptors have been shown to interact with multiple G-proteins [12]. The promiscuity can occur not only within one class of G-proteins, such as G_i and G_o, but also with G-proteins from different classes. Using immunoprecipitation, it has been shown that the α_{2A}-AR interacts with both G_i- and G_s-proteins [10]. Promiscuous G-protein coupling has often been ascribed to high expression levels of receptors, but this does not always hold true [9]. It is of course possible that promiscuous G-protein coupling seen in heterologous expression systems could be an artefact due to altered processing of the receptors. The molecular basis for receptor–G-protein coupling

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Abbreviations: α_2-AR, α_2-adrenergic receptor; AC, adenylyl cyclase; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); IBMX, 3-isobutyl-1-methylxanthine; UK14,304, 5-bromo-*N*-(4,5-dihydro-1H-imidazol-2-yl)-6-quinoxalinamine.

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promiscuity is, however, not well understood and a more likely explanation is that overexpression reveals secondary coupling pathways also utilized by endogenous receptors.

Data from analysis with chimeric or mutated α_2-ARs receptors suggests that the G_i/G_s coupling requires largely separate receptor domains for interaction with the two G-proteins [13–15]. In addition, certain agonists can selectively direct the coupling of the α_{2A}-AR to either inhibition [16] or stimulation [17], indicating agonist-directed signal trafficking. The above-mentioned issues strongly suggest that the two responses depend upon different conformational changes in the receptor protein altering the avidity for different G-proteins. A factor that also influences the adenylyl cyclase activity is the receptor expression level [10], suggesting that the stoichiometry between receptor and G-protein may influence coupling.

To gain insight into these issues, we chose to study the regulation of cAMP production in Sf9 cells by the human α_{2B}-AR expressed either alone or coexpressed together with G-proteins. Insect Sf9 cells have been used successfully earlier as a cellular reconstitution system for determining receptor–G-protein interactions [18–20]. The human α_{2B}-AR produces a monophasic stimulatory cAMP response when expressed in these cells [8]. Our aim was to find out how altered levels of G-proteins affect the receptor-mediated cAMP response and in this context to get insights into partial agonism in the light of G-protein accessibility.

2. Materials and methods

2.1. Reagents and cell culture

[^{3}H]Adenine, [^{14}C]cAMP, and [^{3}H]RX821002 were purchased from Amersham Corp. [^{35}S]GTPγS was from New England Nuclear. Noradrenaline, 3-isobutyl-1-methylxanthine (IBMX), and forskolin were from Sigma. 5-bromo-*N*-(4,5-dihydro-1*H*-imidazol-2-yl)-6-quinoxalinamine (UK14,304) was from RBI. Other chemicals used were of analytical grade quality.

Sf9 cells were grown in glass spinner bottles at 26° in Grace's insect medium supplemented with lactalbumin hydrolysate and yeastolate (Life Technologies). Additional supplements were 8% (v/v) fetal bovine serum (Life Technologies), 100 U/mL of penicillin (Sigma), and 80 U/mL of streptomycin (Sigma). The cultures were maintained at a density of 1–3 $\times$ 10^6 cells/mL.

2.2. Recombinant baculovirus and expression

The generation of recombinant baculoviruses for the human α_{2B}- and α_{2A}-ARs has been described in detail previously [8]. Baculovirus transfer vectors harbouring the genes for $G\alpha_s$, $G\alpha_{i1}$, and $\beta_1\gamma_2$, all of bovine origin, were gifts from Dr. T. Haga (University of Tokyo, Tokyo, Japan). The cDNA for $G\alpha_s$ [21] in pVL1392, $G\alpha_{i1}$ [22] in pVL1392, and $\beta_1\gamma_2$ [23] in pVL1393 were cotransfected with linearized baculovirus DNA (Pharmingen) and the resultant virus stocks were subjected to one round of plaque purification before generation of high-titer virus stocks.

For expression of the α_2-AR in this study, Sf9 cells were plated on plastic tissue culture dishes (diameter 94 mm) and allowed to attach for at least half an hour. Thereafter, the culture medium was removed and 5 mL of a high-titer virus stock was added. After 30–60 min, the virus stock was removed and the culture medium re-added. The infection was then allowed to proceed for the indicated time. In the case of coexpression with G-proteins, high-titer stocks of the α subunit virus and $\beta\gamma$ subunits virus (ratio 1.5:1) were added together for 30 min whereafter receptor virus was added for another 30 min. Using this procedure with wild-type polyhedrin-producing virus and a virus expressing green fluorescent protein, we could observe coexpression of polyhedrin crystals and green fluorescent protein in over 90% of the cells.

2.3. Membrane preparation and [^{3}H]RX821002 binding

Infected cells were washed and harvested in a buffer containing 1 mM Na_2HPO_4, 10.5 mM KH_2PO_4, 140 mM NaCl, and 40 mM KCl, pH 6.2. Homogenization was conducted on ice with an Ultra-Turrax homogenizator in a buffer of pH 7.4 containing 20 mM HEPES, 1 mM $MgCl_2$, 100 mM NaCl, 1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride. Unbroken cells were sedimented by low-speed centrifugation and the supernatant was centrifuged for 30 min at 30,000 $\times$ g at +4°. The pellet was washed once in the same buffer and finally resuspended in the same buffer containing 1 mM dithiothreitol at a concentration of 1–2 mg protein/mL. For receptor-stimulated [^{35}S]GTPγS binding to G_s-proteins, the membranes were washed two additional times in the same buffer. Protein amounts were determined with the Bradford dye reagents (Bio-Rad). Membranes were stored at −80° until use.

Saturation binding experiments were performed on membrane preparations using [^{3}H]RX821002 as radioligand. Membrane preparations were diluted to a final concentration of 200 μg protein/mL in $NaPO_4$ buffer, pH 7.4. Membranes (10 μg protein) were incubated with 0.1–100 nM [^{3}H]RX821002 in a total volume of 150 μL for 40 min at 22° with agitation. Non-specific binding was determined with 10 μM phentolamine. The reactions were terminated by rapid filtration through prewashed GF/B filters with five subsequent washes with cold buffer containing 180 mM NaCl, 25 mM $MgCl_2$, and 20 mM HEPES, pH 7.4, in a microplate harvester (Packard Instrument). The radioactivity was determined in a microplate scintillation counter (Packard Instrument).

2.4. Immunoblotting

SDS/polyacrylamide electrophoresis was performed according to [24]. Membrane proteins were solubilized and subjected to electrophoresis in a gel containing 10% (w/v)

acrylamide. Gel-separated proteins were transferred onto Hybond-P membranes (Amersham) and allowed to react with rabbit anti-Gα_s IgG or rabbit anti-Gα_i IgG (Calbiochem) according to the manufacturers' instructions. Immunoreactive bands were visualized with donkey anti-rabbit Ig F(ab′)^2 fragment coupled to horseradish peroxidase (Amersham), and an ECL Western blotting system (Amersham) was used to stain the bands before exposure to Hyperfilm x-ray films (Amersham).

2.5. [^{35}S]GTPγS binding

Membranes were diluted to a final concentration of 50 μg protein/mL in binding buffer containing 100 mM NaCl, 3 mM $MgCl_2$, 1 mM EDTA, 20 mM HEPES, and 1 mM dithiothreitol, pH 7.4. Diluted membranes (2.5 μg protein/well) were preincubated with 1 μM GDP for 10 min before addition of 0.2 nM [^{35}S]GTPγS and agonist or vehicle for determination of the basal binding. The binding mixture in a total volume of 150 μL was incubated for 60 min at 22° with agitation. The reactions were terminated by rapid filtration through prewashed GF/B filters (Packard Instrument) with five subsequent washes with cold buffer containing 180 mM NaCl, 25 mM $MgCl_2$, and 20 mM HEPES, pH 7.4, in a microplate harvester (Packard Instrument). The radioactivity was determined in a microplate scintillation counter (Packard Instrument).

2.6. Measurement of cellular cAMP production

The cells were incubated with 5 μCi/mL of [^{3}H]adenine in culture medium for 2–3 hr before they were washed and harvested in 2-(*N*-morpholino) ethanesulfonic acid (MES)-buffered medium (MBM) composed of 130 mM NaCl, 5.4 mM KCl, 1 mM EGTA, 10 mM glucose, 1.2 mM $MgCl_2$, 4.2 mM $NaHCO_3$, 7.3 mM NaH_2PO_4, 63 mM sucrose, and 20 mM MES, pH adjusted to 6.3. The pelleted cells were resuspended in MBM containing 0.5 mM IBMX. The cells were preincubated with 0.5 mM IBMX for 10 min at 25° after which stimulants were added. After another 10-min incubation with the drugs, the reactions were stopped by rapid centrifugation, removal of supernatants, and addition of 0.33 M perchloric acid containing about 1000 cpm [^{14}C]cAMP. Cyclic AMP was isolated by sequential Dowex/alumina ion-exchange chromatography [25] and radioactivity was determined in a liquid scintillation counter. The conversion of [^{3}H]ATP to [^{3}H]cAMP was calculated as a percentage of total recovered [^{3}H]ATP and normalized to the recovery of [^{14}C]cAMP. In cases where data is given as percent of forskolin, the change in cAMP level is related to the forskolin response (= 100%).

2.7. Data analysis and computer modeling

Student's non-paired two-tailed *t*-test was used to calculate statistical significance and non-linear curve fitting of the concentration–response data was performed using SigmaPlot for Windows 4.00 (Jandel Scientific).

The agonist activation of the receptor was modeled according to the

$$[R]_{act} = \frac{[agonist][R]_{act\text{-max}}}{[agonist] + \mathrm{EC}_{50\text{-R}}}$$

($[R]_{act}$ = concentration of activated receptors; $[R]_{act\text{-}max}$ = maximum concentration of receptors that can be activated; $\mathrm{EC}_{50\text{-R}}$ = the concentration of agonist producing the half-maximal receptor activation). The efficacy could be regulated by changing the $[R]_{act\text{-}max}$ in an analogous fashion as in [26] and $\mathrm{EC}_{50\text{-R}}$. This activated receptor acts as an activator for two G-proteins, G_i and G_s, according to

$$[G_{i\text{-}act}] = \frac{[R_{act}][G_{i\text{-max}}]}{[R_{act}] + \mathrm{EC}_{50\text{-G}_i}\left(1 + \frac{[G_{s\text{-max}}]}{G_{s\text{-}I_{50}}}\right)}$$

$$[G_{s\text{-}act}] = \frac{[R_{act}][G_{s\text{-max}}]}{[R_{act}] + \mathrm{EC}_{50\text{-G}_s}\left(1 + \frac{[G_{i\text{-max}}]}{G_{i\text{-}\mathrm{I}_{50}}}\right)}$$

($[G_{i\text{-act}}]$ and $[G_{s\text{-act}}]$ = concentrations of activated G proteins; $[G_{i\text{-}max}]$ and $[G_{s\text{-}max}]$ = maximum concentrations of G-proteins that can be activated; $\mathrm{EC}_{50\text{-G}_i}$ and $\mathrm{EC}_{50\text{-G}_s}$ = the concentrations of activated receptors producing the half-maximal G-protein activation). $1 + [G_{s\text{-}max}]/G_{s\text{-}I_{50}}$ and $1 + [G_{i\text{-}max}]/G_{i\text{-}I_{50}}$ are the factors by which the G-proteins can be made to competitively inhibit the activation of each other ($G_{s\text{-}I_{50}}$ and $G_{i\text{-}I_{50}}$ are the inhibitory constants of G_s and G_i, respectively, on activation of each other). The overall adenylyl cyclase (AC) response to the G_i and G_s activation is calculated from

$$\text{response}_{AC} = \frac{\frac{[S]}{S_{0.5}}\,\text{response}_{AC\text{-max}}}{\frac{[S]}{S_{0.5}} + \frac{1 + \frac{[G_i]}{G_{i0.5}} + \frac{[G_s]}{G_{s0.5}} + \frac{[G_i]}{G_{i0.5}}\frac{[G_s]}{G_{s0.5}}}{1 + \alpha_i\frac{[G_i]}{G_{i0.5}} + \alpha_s\frac{[G_s]}{G_{s0.5}} + \alpha_i\alpha_s\frac{[G_i]}{G_{i0.5}}\frac{[G_s]}{G_{s0.5}}}}$$

($[S]$ = [substrate]; $S_{0.5}$ = EC_{50}(substrate) on AC; $G_{i0.5}$ = $\mathrm{EC}_{50}(G_i)$ on AC; $G_{s0.5}$ = $\mathrm{EC}_{50}(G_s)$ on AC; α_i = maximum effect(G_i); α_s = maximum effect(G_s)). This model is based on an enzyme kinetic equation in [27]. In this modification, we are using G_i and G_s as allosteric modifiers, and we have also simplified the equation by fixing the cooperativity factor for substrate binding to 1. Terms α_i and α_s determine whether the modifier is a positive modifier ($\alpha > 1$) or a negative modifier ($\alpha < 1$). The values of α_i and α_s were thus fixed always to be <1 and >1, respectively, in the simulations. Also, the interaction of the G-proteins with the AC is made independent of each other. The following arbitrary constants were used:

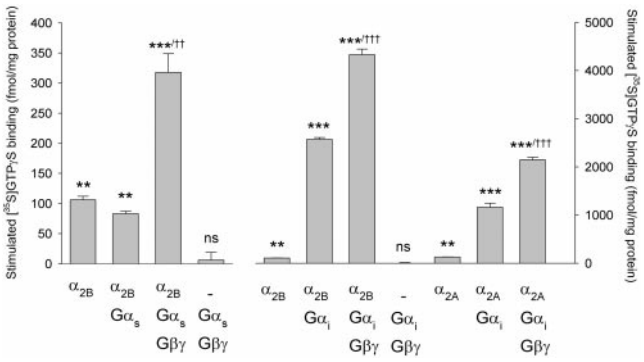

Fig. 1. Functional characterization of expressed G proteins. Sf9 cell membranes coexpressing receptors and different G-proteins were assayed for receptor-catalyzed [^{35}S]GTPγS binding. The experiments were performed with membranes isolated from cells infected for 44–48 hr. The binding was stimulated for 60 min with 100 μM noradrenaline. Data are given as stimulated [^{35}S]GTPγS binding in fmol/mg protein and represent the means ± SEM of three experiments. Observe different ordinates on right and left panel. Basal binding was determined with vehicle addition. Statistically significant changes are indicated with ** and ***; $P < 0.01$ and $P < 0.001$, respectively, compared to basal, †† and †††; $P < 0.01$ and $P < 0.001$, respectively, compared to binding with receptor alone, ns; not significant ($P > 0.05$) compared to basal binding. The B_{max} values (mean ± SEM, N = 3) for receptors, determined with [^{3}H]RX821002, were in pmol/mg protein: 63.2 ± 2.0 (α_{2B}), 55.7 ± 0.8 ($G_s\alpha + \alpha_{2B}$), 50.1 ± 2.9 ($G_s\alpha\beta\gamma + \alpha_{2B}$), 54.1 ± 3.1 ($G_i\alpha + \alpha_{2B}$), 49.8 ± 3.4 ($G_i\alpha\beta\gamma + \alpha_{2B}$), 9.8 ± 0.3 ($\alpha_{2A}$), 7.6 ± 0.2 ($G_i\alpha + \alpha_{2A}$), 6.2 ± 0.3 ($G_i\alpha\beta\gamma + \alpha_{2A}$).

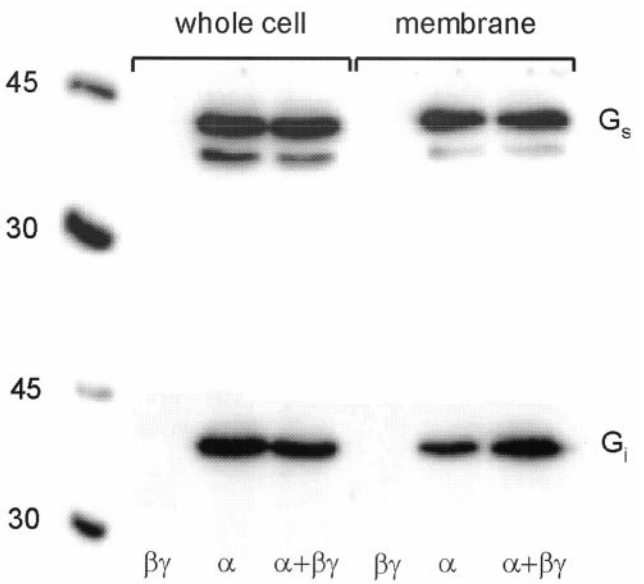

Fig. 2. Western blot analysis of expressed G-proteins. Representative Western blot analysis of G-protein α_s and α_i subunits. Sf9 cells were infected with virus for $\beta\gamma$ subunits (controls for antibody specificity), the respective α subunit, or α and $\beta\gamma$ subunits together for 40 hr. Aliquots of whole cell homogenates were taken before preparation of isolated membranes and run on the gels as controls for total expression levels. The amounts of protein were 10 μg per lane of whole cell homogenates and 5 μg per lane of isolated membranes. The molecular mass markers in kDa are as indicated on the left. Densitometric scanning of the blots, with corrections for protein amounts, gave the following values (arbitrary units): 18.11 (α_s homogenate), 17.47 (α_s + $\beta\gamma$ homogenate), 25.92 (α_s membrane), 26.42 (α_s + $\beta\gamma$ membrane), 18.81 (α_i homogenate), 12.79 (α_i + $\beta\gamma$ homogenate), 16.18 (α_i membrane), 37.66 (α_i + $\beta\gamma$ membrane).

$\text{response}_{\text{AC-max}} = 1000$; $[S] = 10$; $S_{0.5} = 100$; $G_{s0.5} = 10$; $G_{i0.5} = 10$; $\alpha_s = 10$; $\alpha_i = 0.3$. The used variables were $G_{s\text{-}max}$, $G_{i\text{-}max}$, $EC_{50\text{-}G_s}$, $EC_{50\text{-}G_i}$, $G_{s\text{-}I_{50}}$ or $G_{i\text{-}I_{50}}$.

3. Results

3.1. Functional expression of G proteins

Stimulation of Sf9 cells expressing the human α_{2B}-AR with noradrenaline results in a significant elevation of the cellular cAMP content compared to the basal level [8]. This stimulation presumably results from receptor activation of endogenous G_s proteins. To characterize the G-protein coupling, we coexpressed the receptor with bovine $G\alpha_s$ alone or in combination with $\beta\gamma$ subunits and measured the noradrenaline-stimulated increase in [^{35}S]GTPγS binding (Fig. 1; left panel). In membranes without exogenous G-proteins, there was a 1.5-fold stimulation of [^{35}S]GTPγS binding with noradrenaline compared to basal binding. The receptor-stimulated [^{35}S]GTPγS binding without exogenous G-proteins was not affected by the infection time between 26 and 48 hr (data not shown). Expression of the $G\alpha_s$ subunit did not significantly alter this binding. Expression of G_s (α_s and $\beta\gamma$) resulted in a 3-fold increase in noradrenaline-stimulated [^{35}S]GTPγS binding as compared to receptor alone. This increase was rather small, although statistically significant, compared to the binding obtained with G_i coexpression. For comparison, the agonist-stimulated binding obtained with a primarily G_s-coupled receptor (adenosine A_{2A} receptor) under similar conditions was 538 ± 17 fmol/mg protein with G_s coexpression compared to 55 ± 10 fmol/mg protein in cells expressing receptor alone (not shown). Noradrenaline had no effect on cells expressing the G-protein alone, ruling out potential endogenous receptors.

Coexpression of G_i and α_{2B}-AR resulted in an increase in receptor-stimulated [^{35}S]GTPγS binding that amounted to a 40-fold increase compared with receptor alone at 48-hr postinfection (Fig. 1; right panel). Coexpression of α_i and $\beta\gamma$ subunits greatly enhanced the receptor-stimulated [^{35}S]GTPγS binding as compared to α_i subunit alone. Data from experiments with the α_{2A}-AR are included for comparison as this subtype, in contrast to α_{2B}-AR, is coupled to inhibition of cAMP production in these cells [8]. Representative Western blot analysis (Fig. 2) shows the heterologous expression of α_s and α_i in Sf9 cells. The immunoblots also demonstrated that coexpression with $\beta\gamma$ increases the amount of heterologous α_i subunits in the membrane fraction from approximately 20% of total cellular α_i subunits in the absence of $\beta\gamma$ to approximately 75% in the presence of $\beta\gamma$ based on densitometric scanning (the membrane fraction contains roughly 25% of total cellular protein). Such an effect of $\beta\gamma$ could not be demonstrated with the α_s subunit despite the similar effect in the [^{35}S]GTPγS binding assay.

Expression of G_s increased both the basal and forskolin-

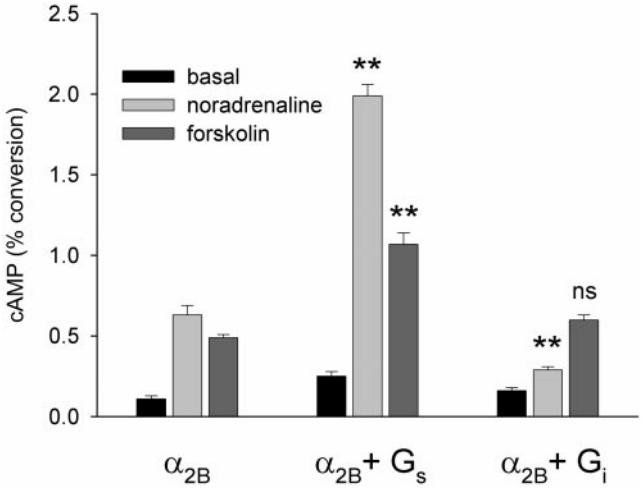

Fig. 3. Effects of G-protein expression on receptor- and forskolin-stimulated cAMP production. Sf9 cells coexpressing the α_{2B}-AR and G_s- or G_i-proteins (α and $\beta\gamma$) were assayed for stimulated cAMP production. The infection time was 26–28 hr. The data are given as percent conversion of ATP to cAMP and represent the means ± SEM of three experiments. Noradrenaline (100 μM) was used to stimulate the receptor and forskolin (10 μM) was used to stimulate AC directly. Basal level denotes unstimulated cells. Statistically significant changes are indicated with **; $P < 0.01$ and ns; not significant ($P > 0.05$) compared to control (α_{2B}-AR).

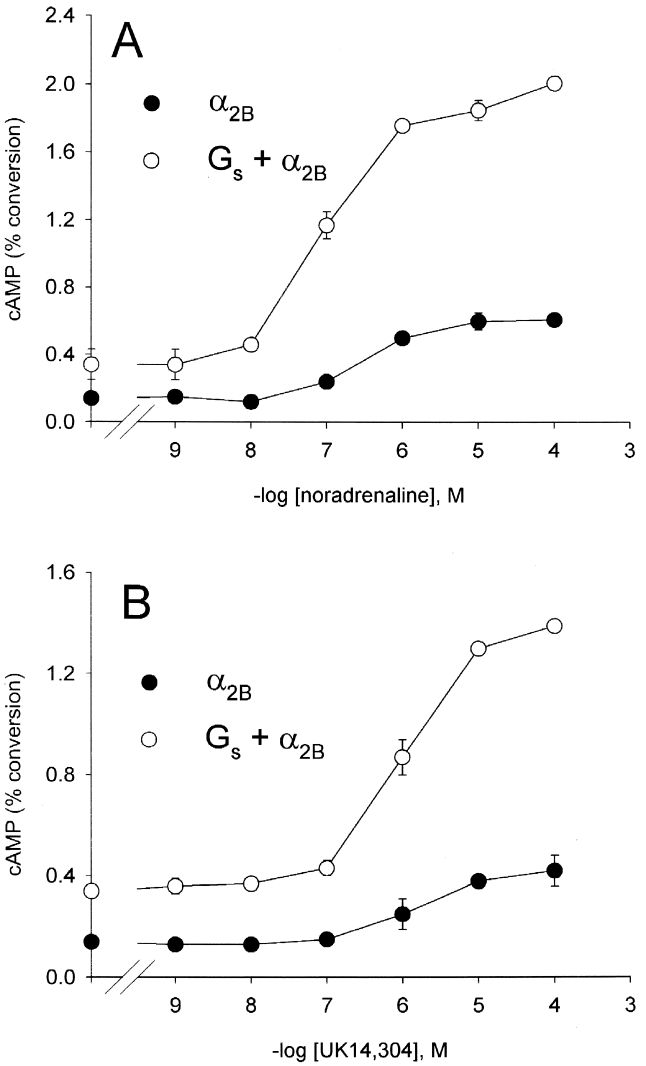

Fig. 4. Concentration–response relationships for α_{2B}-AR-mediated regulation of cAMP production. Sf9 cells expressing the α_{2B}-AR alone or together with G_s-proteins were assayed for concentration-dependent stimulation of cAMP production by noradrenaline (A) and UK14,304 (B). The infection time was 26–28 hr. The data are given as percent conversion of ATP to cAMP and represent the means ± SEM of three experiments.

stimulated level of cAMP (Fig. 3). This increase was independent of receptor coexpression, indicating a certain unstimulated basal activity of the G-protein itself. The noradrenaline-induced cAMP accumulation was considerably enhanced by expression of G_s-proteins (Fig. 3). With G_i expression, the response to noradrenaline was attenuated, while the forskolin-stimulated response remained largely unaffected (Fig. 3). This demonstrates that both G-proteins interact with the receptor and the effector enzyme. The total amount of receptors in membranes from G-protein coexpression at 26 hr postinfection (24.2–25.0 pmol/mg protein, N = 6) was not significantly different from control cells expressing receptor alone (24.6 ± 1.3 pmol/mg protein, N = 3) as determined by radioligand binding.

3.2. Effect of G_s expression on agonist potency

Increased availability of G_s-proteins should in the case of interaction with the receptor affect the concentration-dependence of stimulation. This has been shown for G_s and β_2-AR [28]. Concentration–response relationships for noradrenaline and UK14,304 in cells coexpressing G_s and the α_{2B}-AR are shown in Fig. 4. With both ligands, there was a clear increase in the maximal cAMP accumulation. With noradrenaline, the E_{max} was enhanced 3.4-fold and the EC_{50} was reduced ($P < 0.001$) when compared to receptor expressed alone (pEC_{50} 7.03 ± 0.02 with G_s and pEC_{50} 6.50 ± 0.01 without G_s). With UK14,304 the E_{max} was increased 3.6-fold with no significant ($P > 0.05$) change in the potency (pEC_{50} 5.99 ± 0.03 with G_s and pEC_{50} 5.83 ± 0.11 without G_s). UK14,304 behaved as a partial agonist for the stimulation both in the absence and presence of exogenous G_s proteins.

3.3. Effect of G_s and G_i on cAMP production in the presence of forskolin

A bell-shaped concentration–response relationship, with inhibition of forskolin-stimulated cAMP production at low agonist concentrations and stimulation at higher concentrations, has been observed with both the α_{2A}-AR and the α_{2B}-AR using different expression systems [7,10,11,14]. As shown in Fig. 5A, no significant inhibitory component can be observed with the expressed α_{2B}-AR as also reported earlier [8]. When the receptor was coexpressed with G_s

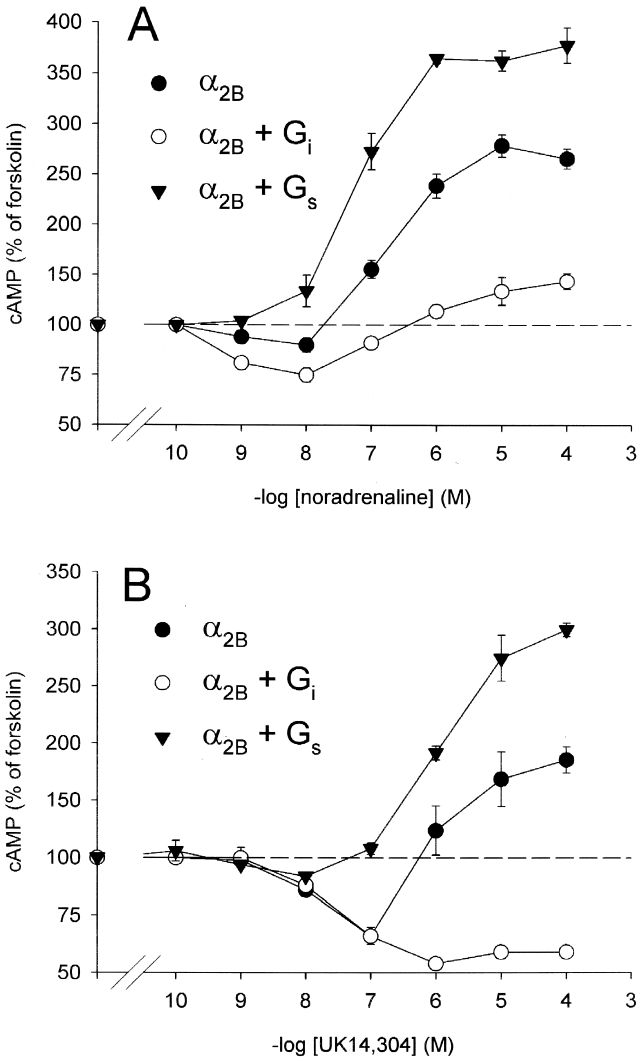

Fig. 5. Concentration–response relationships of α_{2B}-AR in the presence of forskolin. The α_{2B}-AR was expressed alone or together with G_i- or G_s-proteins and the concentration-dependent changes in forskolin-stimulated cAMP production were determined. The infection time was 26–28 hr. The concentration of forskolin was 10 μM. The data are given as percent of forskolin (forskolin alone taken as 100%, represented by the dashed line) and represent the means ± SEM of three experiments.

proteins, there was an enhancement of the stimulatory response seen as an increase in the maximal response and potency of the agonist (Fig. 5A). When the receptor was coexpressed with G_i, a biphasic concentration–response curve was seen with inhibition of cAMP accumulation at low agonist concentrations followed by an increased cAMP accumulation at higher concentrations (Fig. 5A). The increase exceeded the stimulation by forskolin alone but was considerably reduced as compared to conditions without G-protein coexpression.

When UK14,304 was used to stimulate α_{2B}-ARs, a biphasic concentration–response curve was obtained with inhibition of cAMP accumulation (maximal inhibition about 40%) at low ligand concentrations followed by an increase in cAMP production at higher concentrations (Fig. 5B).

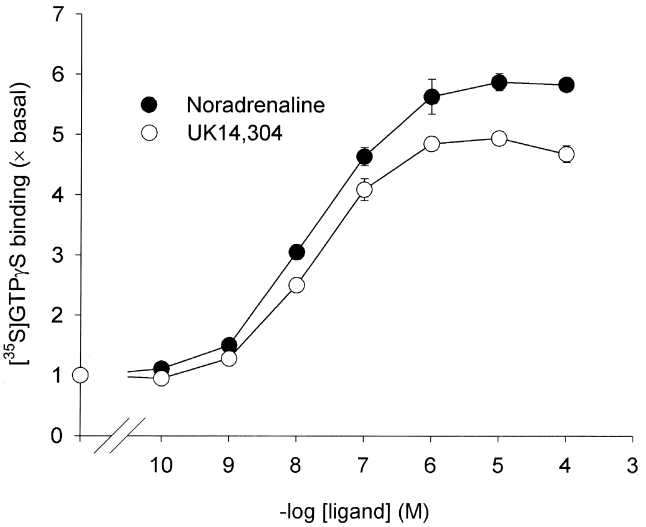

Fig. 6. Receptor-catalyzed [^{35}S]GTPγS binding to G_i-proteins. Sf9 cell membranes with coexpressed α_{2B}-AR and G_i-proteins (43–46 hr post-infection) were assayed for concentration-dependent stimulation of [^{35}S]GTPγS binding. Membranes (2.5 μg) were stimulated with different concentrations of noradrenaline or UK14,304 in the presence of 1 μM GDP and 0.2 nM [^{35}S]GTPγS for 60 min. The data are given as fold increase compared to basal binding and represent the means ± SEM of three experiments.

This response was converted to a pure stimulation of cAMP production if the receptor was coexpressed with G_s and to an inhibition with G_i coexpression over the whole concentration range of the ligand. Although the responses were different at the effector level, UK14,304 was about equally effective as noradrenaline in stimulating [^{35}S]GTPγS binding to coexpressed G_i proteins (Fig. 6).

Table 1 summarizes the effects of a panel of agonists on the coupling of α_{2B}-AR to endogenous G-proteins and coexpressed G_i-proteins. There seems to be an inverse rela-

Table 1
Agonist-dependent signaling through α_{2B}-AR to stimulation and inhibition of cAMP production

Agonist	cAMP response	
	α_{2B}	α_{2B} + G_i
Adrenaline	+159 ± 13	+37 ± 6
Noradrenaline	+148 ± 5	+22 ± 4
Oxymetazoline	+150 ± 9	+13 ± 2
d-medetomidine	+142 ± 14	+14 ± 5
α-methyl-NA	+130 ± 11	+7 ± 4
Guanabenz	+95 ± 7	−5 ± 6
UK14,304	+83 ± 3	−8 ± 7
BHT-933	+60 ± 7	−21 ± 3
Clonidine	+54 ± 8	−20 ± 5

Sf9 cells were infected with α_{2B}-AR alone or together with G_i for 25 hr and agonist-mediated changes in forskolin-stimulated cAMP production were determined. All agonists were used at 100 μM. α-Methyl-NA stands for α-methyl-noradrenaline. The data are given as percent increase (+) or percent decrease (−) and represent the means ± SEM from three experiments.

tionship between stimulatory and inhibitory coupling. Those ligands which couple more weakly to stimulation are more effective for inhibition of cAMP accumulation. At longer infection times (equaling increased levels of G-proteins as well as receptors), an inhibition is also revealed with the ligands that are full agonists for stimulation (data not shown).

3.4. Effect of G_s on the inhibitory α_{2A}-AR

In previous studies on the α_{2A}-AR expressed in Sf9 cells, an inhibition of forskolin-stimulated cAMP accumulation has been reported contrary to the stimulation seen with the α_{2B}-AR [8]. A concentration–response curve for the effect of noradrenaline on the forskolin-stimulated cAMP accumulation is shown in Fig. 7. Noradrenaline causes an inhibitory response at the whole concentration range tested. There is, however, a tendency toward a stimulatory phase in the concentration–response curve at 100 μM noradrenaline. Coexpression with G_s reduces the inhibition, causing a biphasic concentration–response curve with stimulation at high agonist concentrations.

3.5. Modeling the adenylyl cyclase response with G-protein ratios

In order to illustrate the interaction of the two G-proteins with α_2-ARs, a model was constructed where the G-proteins interact with the receptor and compete for the same binding site on the receptor. This does not necessarily mean that the G-proteins would compete for the same binding site, but that the interaction with one G-protein excludes interaction with the other. The AC response was modeled using a modification of the enzyme kinetic model in [27]. The three different concentration–response patterns, pure inhibition, biphasic, and pure stimulation, seen with noradrenaline and UK14,304 on the α_{2B}-AR could be reproduced using this model if it was assumed that the noradrenaline-induced conformation has equal affinities for G_s and G_i (Fig. 8A) and that the UK14,304 induced conformation has 10 times higher affinity for G_i than for G_s (Fig. 8B). For α_{2A}-AR, the affinity for G_i was assumed to be 300 times higher than for G_s (Fig. 8C). Thus, with equal affinity a biphasic concentration–response curve is obtained only upon increasing the amount of G_i. With lower affinity for G_s than for G_i, a biphasic concentration–response curve is obtained. Increased amounts of the respective G-protein can convert this to either pure stimulation or almost pure inhibition. With a further reduction in the affinity for G_s, a tendence of stimulation is obtained with increased levels of G_s but the stimulatory concentration–response curve is shifted to the right and does not show saturation within this concentration range. It should be noted that affinity in this context does not in physiological situations necessarily mean the binding affinity of the G-protein for the receptor, as other factors such as adaptor proteins could also promote the interaction.

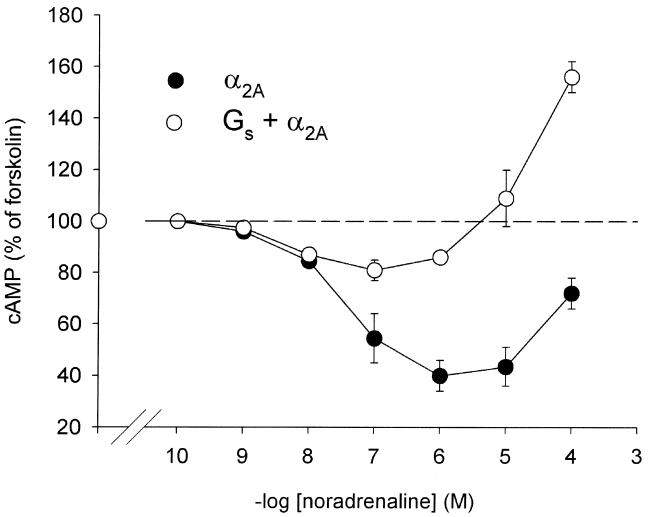

Fig. 7. Effect of G_s coexpression on cAMP production with the α_{2A}-AR subtype. The α_{2A}-AR was expressed alone or together with G_s-proteins and the concentration-dependent changes in forskolin-stimulated cAMP production were determined. The infection time was 44–48 hr. The concentration of forskolin was 10 μM. The data are given as percent of forskolin (forskolin alone taken as 100%, represented by the dashed line) and represent the means ± SEM of three experiments.

4. Discussion

We and others have previously shown that the α_{2B}-AR shows preferential coupling to an increase in cAMP production in certain cell types when compared to the other subtypes, α_{2A}-AR and α_{2C}-AR, which seem to be primarily coupled to inhibition of stimulated cAMP production [7,8, 11]. It has been shown, however, that all three subtypes can couple to stimulation if uncoupled from G_i by pertussis toxin treatment [10,29]. The results of the present study demonstrate that the availability of G-proteins significantly affects the outcome of these dually coupled receptors at the cellular level.

In an earlier study, we found that when transiently expressed in Sf9 insect cells the human α_{2B}-AR stimulated whereas the α_{2A}-AR inhibited cAMP production [8]. As the endogenous insect G-proteins may not provide optimal coupling to mammalian receptors, we chose in this study to investigate the interaction with mammalian G-proteins. Coexpression of G_s- or G_i-proteins with the α_{2B}-AR resulted in an enhanced receptor-stimulated GTPγS binding, reflecting a direct receptor–G-protein interaction. Comparison with the coupling of the inhibitory α_{2A}-AR to G_i indicated that the expressed α_{2B}-AR subtype also functionally couples efficiently to G_i. Although we do not have a measure for how efficiently the chosen G$\beta\gamma$ of $\beta_1\gamma_2$ subunit composition interacts with Gα_s and Gα_i, several groups have successfully coexpressed G$\beta_1\gamma_2$ and Gα_i or Gα_s for the study of G-protein–receptor interactions [19,20,30–32]. In this study, the enhanced receptor-stimulated GTPγS binding to both Gα_s and Gα_i with coexpressed G$\beta\gamma$ indicates that

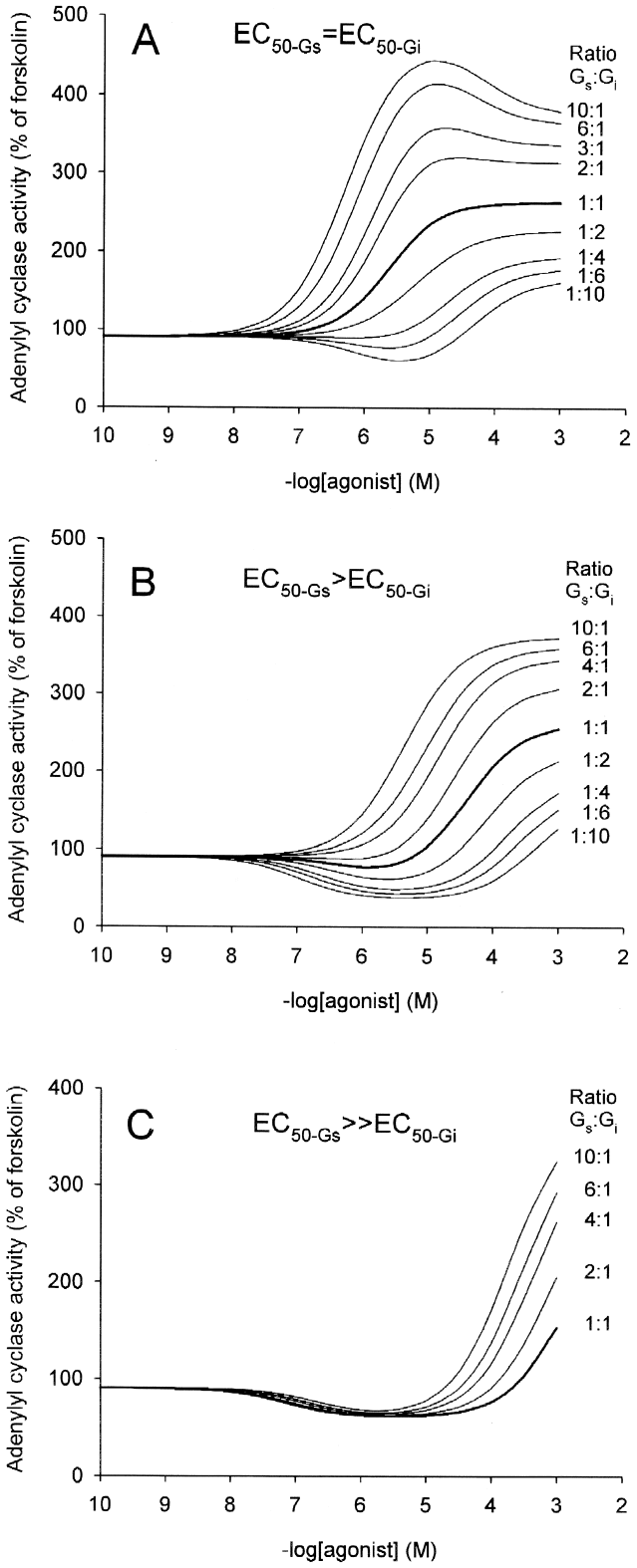

Fig. 8. Prediction of cAMP responses in relation to G-protein levels. The mathematical model, described in the Methods section, was used to predict the activity of adenylyl cyclase through receptor activation of inhibitory and stimulatory G-proteins. In (A), the receptor affinities for G_i and G_s ($EC_{50\text{-}G_s}$, $EC_{50\text{-}G_i}$) and their inhibitory constants for mutual interaction with the receptor ($G_{s\text{-}I_{50}}$ or $G_{i\text{-}I_{50}}$) are the same (0.1). The respective G-protein levels ($G_{s\text{-}max}$, $G_{i\text{-}max}$) are varied and the G_s:G_i ratios are shown next to the graphs. With these parameters, the model reproduces the curve pattern obtained with noradrenaline stimulation of the α_{2B}-AR. In (B), the receptor affinity for G_s is reduced 10-fold ($EC_{50\text{-}G_s}$ and $G_{s\text{-}I_{50}}$ = 1). With these parameters, the model reproduces the approximate curve patterns obtained with UK14,304 stimulation of the α_{2B}-AR. In (C), the receptor affinity for G_s is 300-fold less than that of G_i ($EC_{50\text{-}G_s}$ and $G_{s\text{-}I_{50}}$ = 30) and $G_{s\text{-}max}$ is varied. With these parameters, the model reproduces the approximate curve patterns obtained with noradrenaline-stimulation of the α_{2A}-AR.

functional heterotrimers of both G-protein subfamilies are formed and interact with the receptor.

Results from previous studies on α_2-ARs indicate that inhibitory and stimulatory coupling to cAMP production show different structural and/or conformational requirements for the receptor. Stimulatory coupling is usually seen at higher agonist concentrations than inhibitory coupling causing biphasic concentration–response curves [10]. This could indicate that the different conformations induced or stabilized require a different occupance of the ligand–receptor complex. Mutagenesis studies indicate that different intracellular domains of the receptor are involved in inhibitory and stimulatory coupling [13,14,33]. This indicates that the receptors adopt different conformations that differ in their preference for interaction with different G-proteins. In some cells, all α_2-AR subtypes couple only to inhibition of the cAMP accumulation response [7,34], suggesting that the cellular environment can direct the signaling. One variable in this context could be the expression level of G-proteins or accessory proteins. As shown in this study, increased levels of G_s-proteins cause shifts in the concentration–response curves for noradrenaline via the α_{2B}-AR. The inhibitory phase revealed with G_i coexpression as well as the enhanced noradrenaline-stimulated GTPγS binding demonstrate that the α_{2B}-AR also couples efficiently to G_i-proteins if their availability is increased, although it appeared to couple only to a stimulation of cAMP accumulation without G-protein coexpression. With the α_{2A}-AR on the other hand, a stimulatory phase was revealed with G_s coexpression resulting in a biphasic concentration–response curve. G-protein availability thus seems to be an important factor for the ability of α_2-ARs to direct cellular signaling via cAMP.

Some agonists show preference for coupling to inhibition over stimulation with the α_2-ARs [14,16,29,35]. In this study, UK14,304 elicited a biphasic concentration–response curve in the absence of exogenous G-proteins. The phenomenon where certain agonists can selectively activate specific signal pathways has been termed agonist-specific coupling [36] or agonist trafficking (of receptor signals) [37]. If ligand-induced, different active receptor conformations will preferentially activate specific G-proteins, a distinct activation of different signaling pathways by different agonists will be observed. A possible explanation for the difference between noradrenaline and UK14,304 is that the receptor conformation induced by UK14,304 interacts more weakly

with G_s- compared to G_i-proteins. This is further supported by the observation that exogenous G_i was unable to fully override the stimulatory response with noradrenaline, while UK14,304 elicited a monophasic inhibitory response. Both ligands activated G_i to the same extent in the GTPγS binding assay. This indicates that the difference between these two ligands is not due to an overall lower efficacy of UK14,304 to activate the receptor but that they differ in their ability to activate G_s-proteins. Increased levels of G_s-proteins converted the α_{2B}-AR to a stimulatory receptor also with UK14,304. Extending this to the α_{2A}-AR suggests that the noradrenaline-activated α_{2A}-AR receptor is considerably more restricted with respect to its interaction with G_s compared to the α_{2B}-AR. The effect of an altered G_s-protein level on α_{2A}-AR signaling has been described earlier [38]. The potency of UK14,304 to promote G_i activation was found to be 1000-fold higher than that for G_s activation. This supports our observation of α_{2A}-AR being much less promiscuous than the α_{2B}-AR subtype.

Using a panel of agonists, we could demonstrate that the agonist properties of UK14,304 are not unique, but that all agonists exhibiting partial agonism for the stimulatory response are more efficacious for inhibition via exogenous G_i-proteins. The ultimate response of the receptor would thus depend on both the affinity of receptors for different G-proteins as well as the receptor–G-protein stoichiometry. An effect of G-protein coexpression on agonist efficacy has also been demonstrated using NIH-3T3 cells transfected with the α_{2A}-AR [39].

The mathematical model presented herein can be used to assess the impact of altered G-protein levels for receptor coupling. There are several interesting predictions that can be made using the model presented. One is that in truly promiscuous receptors the relatively small changes in G-protein availability can significantly direct trafficking of signals in this case from a stimulatory response to an inhibitory and vice versa. Even if G_s can override the effect of G_i at the level of cAMP production, G_i would still have an ability to activate other pathways such as ion channels. In more G-protein stringent receptors, trafficking of signals will be obtained only under extreme conditions. However, as shown here, promiscuous responses can be obtained even if the affinity of the secondary site (G_s site) is 300-fold lower than that of the primary site (G_i site).

In dealing with recombinant expression of receptors, one usually reaches expression levels that may be beyond the physiological situation. This does not mean that coupling to several signal pathways is a phenomenon only seen with overexpressed receptors. Coupling of α_2-ARs to stimulation of cAMP production has also been observed with endogenous receptors [40,41] though the mechanism has not been fully elucidated. Two reports have shown that the receptor expression level does not alter the G-protein coupling profile of α_2-ARs, but does have an impact on the regulation of cAMP production [9,35]. It is more likely that overexpression of receptors reveals coupling mechanisms which may function as a physiological pathway in certain cells or under certain conditions. In this study, we have shown that the G protein level as well has a major impact on receptor regulation of effector enzymes. The readout we have used, i.e. cAMP production, may actually be blunted as the G-proteins studied converge on AC giving opposite effects. In another setting where two pathways diverge to regulate different effector systems, the impact of G-protein levels may be even more pronounced. There are a number of studies demonstrating differential regulation of G_s expression in the course of normal development, differentiation, and acclimatization [reviewed in 42]. Given the high promiscuity of the α_{2B}-AR for coupling to G_i and G_s, this receptor subtype should be the focus of future studies on the physiological relevance of dual G-protein coupling.

Acknowledgments

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