Analysis of the relative interactions between the $\alpha 2C10$ adrenoceptor and the guanine-nucleotide-binding proteins $G_{\alpha 1}$ and $G_{\alpha 2}$ following co-expression of these polypeptides in Rat 1 fibroblasts

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Rat 1 fibroblasts which had been transfected to express the human $\alpha 2C10$ adrenoceptor (clone 1C) were further co-transfected with a plasmid containing the hygromycin-B-resistance gene and a plasmid containing a cDNA encoding the α -subunit of the rat pertussis-toxin-sensitive G-protein $G_{\alpha 1}$. In clone 3 the receptor was expressed at some 2.2 pmol/mg of membrane protein, and $G_{\alpha 1}$ at approx. 100 pmol/mg of membrane protein. The interaction of these two polypeptides and that between the receptor and $G_{\alpha 2}$ (endogenously expressed at some 50 pmol/mg of membrane protein) were studied. Agonist activation of $G_{\alpha 1}$ was observed in membranes of the $\alpha 2C10$ -adrenoceptor⁺- $G_{\alpha 1}$ ⁺ cells (clone 3), but not in $\alpha 2C10$ -adrenoceptor⁺- $G_{\alpha 2}$ ⁺ cells (clone 1C), whereas similar agonist-

dependent activation of $G_{\alpha 2}$ was observed in both cell types. $\alpha 2C10$ -adrenoceptor activation of $G_{\alpha 1}$ and $G_{\alpha 2}$ in clone-3 membranes was produced with similar agonist-dose-effect curves. These observations indicate that the receptor interacts with equivalent affinity with each of these G-proteins. Agonist-dependent cholera-toxin-catalysed [³²P]ADP-ribosylation of $G_{\alpha 1}$ was terminated when the $\alpha 2$ -adrenoceptor antagonist yohimbine was added subsequent to agonist-induced initiation of the reaction and release of GDP, demonstrating the conformational requirement for this reaction to be the ternary complex of agonist-occupied receptor and guanine-nucleotide-denuded G-protein.

INTRODUCTION

The family of $\alpha 2$ adrenoceptors represents the prototypic example of receptors which mediate inhibition of adenylate cyclase via agonist-mediated stimulation of one or more members of the family of pertussis-toxin-sensitive G-proteins [1]. This function is transduced, at least in both platelets [2] and NG108-15 neuroblastoma $\times$ glioma hybrid cells [3], by $G_{\alpha 2}$. However, $\alpha 2$ adrenoceptors are also able to regulate the activity of a number of other effectors. In a variety of circumstances $\alpha 2$ -adrenoceptor agonists are able to inhibit Ca^{2+} currents [4], and, at least in situations in which $\alpha 2$ adrenoceptors have been expressed heterologously, reports of the regulation of K^{+} -channel flux [5], of stimulation of each of adenylate cyclase [6], of phospholipases of each of the A2 [7], C [8] and D [9] classes and of the mitogen-activated protein (MAP) kinase cascade [10], have been recorded. As fast inhibition of I_{Ca} by $\alpha 2$ adrenoceptors is probably the result of a direct action of activated G_{α} subunits on N-type Ca^{2+} channels both in neuroblastoma $\times$ glioma hybrid cells [11] and in rat sympathetic neurons [12], this pathway may represent the mechanism by which presynaptic α -adrenoceptors inhibit neurotransmitter release, and may reflect a more important function in the central nervous system than agonist-mediated inhibition of adenylate cyclase.

The potential interactions of $\alpha 2$ adrenoceptors with G_{α} have been examined in a number of systems, with somewhat varying results. For example, in reconstitution assays Kurose et al. [13], while noting high-affinity interactions of the $\alpha 2A$ receptor with $G_{\alpha 3}$ and $G_{\alpha 2}$, were unable to record a stable interaction of this

receptor with $G_{\alpha 1}$, whereas using a co-immunoprecipitation approach Okuma and Reisine [14] found a strong physical interaction between this receptor and $G_{\alpha 1}$. Furthermore, Coupry et al. [15] have provided evidence for a selective coupling between the $\alpha 2C$ receptor and $G_{\alpha 2}$.

To study more fully potential interactions between $\alpha 2$ adrenoceptors and G_{α} , and the relative affinity of this interaction in comparison with that between the receptor and $G_{\alpha 2}$, in this study we have isolated clones of Rat 1 fibroblasts dually transfected to express both the $\alpha 2C10$ (pharmacologically defined as the $\alpha 2A$) adrenoceptor and the $G_{\alpha 1}$ splice variant. We demonstrate effective and functional coupling between these two proteins in membranes of these cells, and also that functional interactions between the receptor and the G-protein ($G_{\alpha 2}$) which allows $\alpha 2$ -adrenoceptor-mediated inhibition of adenylate cyclase in these cells [16] is not disrupted by co-expression of $G_{\alpha 1}$. These results further indicate that a single $\alpha 2$ -adrenoceptor subtype population can concurrently activate both of these G-proteins, a hypothesis originally suggested by the ability of an $\alpha 2$ -adrenoceptor agonist to mediate both inhibition of adenylate cyclase and inhibition of Ca^{2+} currents in NG108-15 cells [4].

MATERIALS AND METHODS

Materials

All reagents for tissue culture were from GIBCO/BRL. [³H]Yohimbine was purchased from Amersham International. Hygromycin B and cholera toxin were from Sigma.

Cells

The generation of clone-1C cells (Rat 1 cells expressing the $\alpha 2C10$ adrenoceptor) has previously been described in detail [17]. Briefly this involved transfection of Rat 1 fibroblasts, using calcium phosphate precipitation, with genomic DNA encoding the human $\alpha 2C10$ adrenoceptor [18] subcloned into the mammalian expression vector pDOL. Further clones, including clone 3, which is analysed in this study, were generated from clone-1C cells by co-transfection in a 10:1 ratio of a cDNA encoding rat $G_o1\alpha$ in the mammalian expression vector pcEXV-3 [19] and the plasmid pBABE hygromycin-B-resistance marker, by using DOTAP (Boehringer Mannheim) according to the manufacturers' instructions. After transfection the cells were maintained in medium containing hygromycin B (200 $\mu\text{g}/\text{ml}$) for 7 days, at which point individual colonies were selected and subsequently expanded. Cells were grown in Dulbecco's modification of Eagle's medium supplemented with 5% newborn-calf serum, penicillin (100 units/ml) and streptomycin (100 $\mu\text{g}/\text{ml}$) in 5% CO_2 at 37 °C. Membranes from the cells were prepared by homogenization with a Teflon/glass homogenizer and differential centrifugation as described for a variety of cells [20]. Membranes from a number of independent clones isolated from such transfections were analysed for the expression of both the $\alpha 2C10$ adrenoceptor and $G_o1\alpha$ (results not shown).

Immunological studies

The generation of antisera SG1 [21], ON1 and OC1 [22] has been described previously.

Immunoblotting studies

After SDS/PAGE, proteins were transferred to nitrocellulose (Costar) and blocked for 2 h in 5% gelatin in PBS, pH 7.5. Primary antisera were added in 1% gelatin in PBS containing 0.2% Nonidet P40 (NP40) and incubated for at least 2 h. The primary antiserum was then removed and the blot washed extensively with PBS containing 0.2% NP40. Secondary antiserum (donkey anti-rabbit IgG coupled to horseradish peroxidase) (Scottish Antibody Production Unit, Wishaw, Scotland, U.K.) was added (1:200 dilution in 1% gelatin in PBS containing 0.2% NP40) and incubated with the nitrocellulose for 2 h. The antiserum was then removed and, after extensive washing of the blot with PBS containing 0.2% NP40, and finally with PBS alone, the blot was developed by using *o*-dianisidine hydrochloride (Sigma) as the substrate for horseradish peroxidase as previously described [23]. Relative quantification of immunoblots was performed with a Bio-Rad model GS-670 imaging densitometer. Quantification of membrane levels of $G_o1\alpha$ in clone 3 cells was performed by quantitative immunoblotting, employing standard curves constructed with various amounts of purified Sf9-cell-expressed $G_o1\alpha$ following infection with a recombinant baculovirus (kindly given by Dr. M. Freissmuth, Department of Pharmacology, University of Vienna, Austria). This recombinant protein was also used to determine levels of $G_o\alpha$ in a preparation of rat brain cortex, and this preparation was then used routinely in subsequent standardizations. A similar strategy was used to quantify levels of $G_{i2}\alpha$ in these cells.

Immunoprecipitation studies

Membranes were suspended in 200 μl of 1% (w/v) SDS containing protease inhibitors (0.5 mM phenylmethanesulphonyl fluoride and 0.15 μM aprotinin) and boiled for 4 min. Subse-

quently 800 μl of ice-cold buffer 1 [1.25% (w/v) Triton X-100, 190 mM NaCl, 6 mM EDTA, 50 mM Tris/HCl, pH 7.5] plus protease inhibitors was added. After 1 h at 4 °C, non-solubilized material was removed by centrifugation (14000 *g*, 10 min at 4 °C). Immunoprecipitation was achieved by addition of between 5 and 10 μl of the relevant antiserum and incubation for 12 h at 4 °C. Subsequently, 50 μl of Pansorbin (25 μl of packed gel) (Calbiochem) was added and the incubation continued for a further 3 h with occasional mixing. Immuno-complexes were collected by centrifugation (14000 *g*, 3 min, 4 °C). These pellets were washed with 3 $\times$ 1 ml of a buffer consisting of a 4:1 mixture of buffer 1 and 1% (w/v) SDS, and once subsequently with 50 mM Tris/HCl, pH 6.8. The final pellets were resuspended in Laemmli sample buffer and replaced in a boiling-water bath for 5 minutes. Samples were then centrifuged (14000 *g*, 3 min) and the supernatant then resolved by SDS/PAGE [10% (w/v acrylamide) at 60 V. Gels were stained with Coomassie Blue, destained, dried and then autoradiographed using Kodak X-O-Mat X-ray film and intensifying screens, or were exposed to a phosphor storage plate and developed with a FUJIX Bio-imaging analyser.

Cholera-toxin-catalysed [^{32}P]ADP-ribosylation

[^{32}P]ADP-ribosylation of membranes of cells of clones 1C and 3 was performed in the absence of added guanine nucleotide basically as in [17]. However, in the immunoprecipitation assays sodium phosphate, pH 7.0, replaced potassium phosphate, pH 7.0, as difficulties were encountered with the immunoprecipitation protocols in the presence of high concentrations of K^+ ions, due to the relative insolubility of the potassium salt of SDS. Further additions to the assays were as detailed in the text. Dried gels were autoradiographed for up to 14 days by using Kodak X-O-Mat X-ray film or were exposed overnight on a Canberra Packard Instant Imager model 2024.

Binding experiments

These were performed at 30 °C for 30 min in buffer B (10 mM Tris/HCl, 50 mM sucrose, 20 mM MgCl_2 , pH 7.5). In saturation experiments using [^3H]yohimbine, the concentration of ligand was varied between 0.5 and 20 nM. Non-specific binding was defined in all cases by parallel assays containing 100 μM idoxoxan. Non-specific binding increased with ^3H -ligand concentration in a linear manner. Binding experiments were terminated by rapid filtration through Whatman GF/C filters, followed by washing the filter with 3 $\times$ 5 ml of ice-cold buffer B.

Curve fitting and data analysis

These were performed by using the Kaleidograph curve-fitting package driven by an Apple Macintosh computer.

RESULTS

Cells of Rat 1 fibroblasts which had been transfected to express the human $\alpha 2C10$ adrenoceptor (clone 1C) [17] were further transfected with a 1:10 ratio of the plasmids pBABE hygromycin-B-resistance gene, and pcEXV-3, into which a cDNA encoding the rat $G_o1\alpha$ splice variant had been ligated [19]. Clones which displayed resistance to hygromycin B (200 $\mu\text{g}/\text{ml}$) were selected, expanded and tested for maintained expression of the $\alpha 2C10$ adrenoceptor and novel expression of $G_o1\alpha$. A number of clones co-expressing these proteins were recorded (results not shown), and clone 3 was selected for detailed study. This clone expressed 2.23 ± 0.23 pmol

Figure 1 Analysis of levels of the $\alpha 2C10$ adrenoceptor and $G_o1\alpha$ in membranes of clone-3 cells

(a) Quantification of levels of the $\alpha 2C10$ adrenoceptor in clone-3 membranes. Membranes of clone-3 cells were analysed for the presence of an $\alpha 2$ adrenoceptor in saturation assays using different concentrations of $[^3H]$ yohimbine as described in the Materials and methods section. Data from a representative experiment are presented as a Scatchard plot for visual examination, but all quantitative data were calculated by using a non-linear least-squares curve-fitting program as described in the Materials and methods section. In the example displayed, $B_{max} = 2.2$ pmol/mg of membrane protein and the K_d for $[^3H]$ yohimbine was 3.6 nM. (b) and (c) Quantification of $G_o1\alpha$ levels. Amounts of $G_o1\alpha$ in a stock membrane preparation prepared from rat cortex were assessed by quantitative immunoblotting of these membranes against different amounts of $G_o1\alpha$ purified from Sf9 cells infected with a recombinant baculovirus expressing this polypeptide and calculated to be 3.44 ng/ μ g of membrane protein (85 pmol/mg of membrane protein) (results not shown). Immunoblotting various amounts of this membrane preparation (b) [lanes 1–6: 1 = 0, 2 = 5 μ g (17.2 ng of $G_o1\alpha$), 3 = 7.5 μ g (25.8 ng), 4 = 10 μ g (34.4 ng), 5 = 12.5 μ g (43.6 ng), 6 = 15 μ g (51.6 ng)] of this membrane preparation in parallel with various amounts of membrane from clone-3 cells (lanes 7–10: 7 = 5 μ g, 8 = 7.5 μ g, 9 = 10 μ g, 10 = 12.5 μ g) and construction of standard curves from such immunoblots (c) allowed estimation of levels of $G_o1\alpha$ in clone-3 membranes. Analysis of such immunoblots demonstrated that clone 3 expresses 94.3 ± 13.2 pmol of $G_o1\alpha$ /mg of membrane protein (mean $\pm$ S.E.M., $n = 3$).

Figure 2 Cholera-toxin-catalysed $[^{32}P]$ ADP-ribosylation of G-proteins in membranes of clones of Rat 1 fibroblasts: effect of agonist

Membranes (60 μ g) of (a) Rat 1 fibroblasts, (b) clone 1C or (c) clone 3 were incubated with $[^{32}P]$ NAD $^+$ and thiol-activated cholera toxin in the absence of guanine nucleotides and in the absence (1) or presence (2) of UK 14304 (10 μ M) for 2 h as described in the Materials and methods section. Samples were precipitated, resolved by SDS/PAGE (10% acrylamide) and subsequently exposed to a phosphor storage plate and developed.

(per mg of membrane protein) of the $\alpha 2C10$ adrenoceptor (Figure 1a), which bound the $\alpha 2$ -adrenoceptor antagonist $[^3H]$ yohimbine with high affinity (K_d 2.8 ± 0.5 nM) and 94.3 ± 13.2 pmol of $G_o1\alpha$ /mg of membrane protein (Figures 1b and 1c) (means $\pm$ S.E.M., $n = 3$ in each case).

To assess the possible physical interactions of the $\alpha 2C10$ adrenoceptor with pertussis-toxin-sensitive G-proteins in clone-3 cholera-toxin-catalysed $[^{32}P]$ ADP-ribosylation was performed on membranes of clone 3 cells, clone 1C cells and parental untransfected Rat 1 cells in the absence of guanine nucleotides and in the presence and absence of the selective $\alpha 2$ -adrenoceptor agonist UK14304 (10 μ M). Resolution of membrane proteins from each cell type by SDS/PAGE (10% acrylamide) and subsequent autoradiography after such treatment led to the incorporation of radioactivity into the 45 and 42 kDa splice variants of $G_s\alpha$ ($G_s\alpha 45$ and $G_s\alpha 42$) in both the absence and presence of UK14304 in all three membrane preparations (Figure 2). It is unclear why less labelling of the $G_s\alpha$ isoforms was obtained in the parental Rat 1 fibroblast membranes in these experiments, as immunoblotting studies with a $G_s\alpha$ -specific antiserum demonstrated similar amounts of these polypeptides in each cell line (results not shown). In both clone-1C and clone-3 membranes, but not in membranes of Rat 1 cells, there was also incorporation of radioactivity into an apparently single band of some 40 kDa primarily when the experiments were performed in the presence of the receptor agonist (Figure 2), as noted previously for clone 1C [17]. Incorporation of radioactivity into this 40 kDa polypeptide was greater in membranes of clone 3 than in clone 1C. Such data define the interaction of the agonist-occupied $\alpha 2C10$ adrenoceptor with a 'G $_i$ -type' G-protein(s) [17]; however, as the subtypes of $G_i\alpha$ and $G_o1\alpha$ essentially co-migrate in such gels, this does not allow discrimination between interactions of the receptor with $G_o1\alpha$ and with other G_i -like G-proteins expressed endogenously. To explore in detail the molecular identity of the pertussis-toxin-sensitive G-protein(s) activated by the $\alpha 2C10$ adrenoceptor in these clones, we performed cholera-toxin-catalysed $[^{32}P]$ ADP-ribosylation of membranes of both clone 1C and clone 3 in the absence and presence of UK14304, and subsequently immunoprecipitated these samples with antisera which identify $G_i2\alpha$ (antiserum SG) or $G_o1\alpha$ (antiserum ON). SDS/PAGE and autoradiography of these immunoprecipitates demonstrated UK14304-stimulated incorporation of radioactivity into $G_i\alpha$ in both clone 1C and clone 3 and also incorporation of radioactivity

Figure 3 Immunoprecipitation and quantitative analysis of agonist-induced cholera-toxin-catalysed [32 P]ADP-ribosylation of G₂α and G₀1α in membranes from clones of Rat 1 fibroblasts

Cholera-toxin-catalysed [32 P]ADP-ribosylation was performed in the absence of guanine nucleotides and in the absence or presence of UK14304 (10 μ M) on membranes (60 μ g) of clone-1C and clone-3 cells as described in the Materials and methods section. Subsequently samples were immunoprecipitated with either the anti-G₂α antiserum SG or the anti-G₀1α antiserum ON. Immunoprecipitates were resolved by SDS/PAGE (10% acrylamide), dried, and either autoradiographed (a) or radioactivity was detected over a 17 h period by electron capture and quantified by using a Canberra Packard Instant Imager model 2024 (b) as described in the Materials and methods section. Data are displayed from a representative experiment.

into G₀1α in clone 3, but not in clone 1C (Figures 3a and 3b). A small degree of cholera-toxin-catalysed incorporation of radioactivity into G₀1α in membranes of clone-3 cells was also recorded in the absence of UK14304 (Figure 3). Immunoprecipitation of such experiments with a G_α-specific antiserum (CS) confirmed that the 45 and 42 kDa polypeptides radiolabelled by cholera-toxin treatment in both the absence and presence of UK14304 were indeed isoforms of G_α (results not shown).

To assess whether it was necessary to maintain a ternary complex of agonist, receptor and G-protein to allow pertussis-toxin-sensitive G-proteins to act as substrates for cholera-toxin-catalysed [32 P]ADP-ribosylation, or whether once agonist-induced GDP release had been achieved agonist would no longer be required, we incubated membranes of clone-3 cells with UK14304 (100 nM), cholera toxin and [32 P]NAD⁺ for 10 min and then added the α 2-antagonist yohimbine (10 μ M) to half of the assays to displace UK14304 from the ligand-binding site. Immunoprecipitations of G₀1α were subsequently performed at various times. Addition of yohimbine to the assays prevented further cholera-toxin-catalysed [32 P]ADP-ribosylation of this G-protein (Figure 4), demonstrating that the true substrate for the reaction is the guanine-nucleotide-denuded G-protein α subunit in a ternary complex with receptor and agonist.

Figure 4 UK14304-stimulated cholera-toxin-catalysed [32 P]ADP-ribosylation of G₀1α in membranes of clone-3 cells: effect of antagonist

Cholera-toxin-catalysed [32 P]ADP-ribosylation was performed in the absence of guanine nucleotides on membranes (60 μ g) of clone-3 cells as described in the Materials and methods section. UK14304 (100 nM) was added to all samples at $t = 0$. At $t = 10$ min the α 2 antagonist yohimbine (10 μ M) (□) or vehicle (●) was added, and the incorporation of [32 P]ADP-ribose into G₀1α subsequently determined by specific immunoprecipitation, SDS/PAGE and exposure of the gel to a phosphor storage plate. The data displayed are taken from one experiment which was representative of five experiments performed on separate membrane preparations.

Figure 5 Dose-effect curves for UK14304-stimulated cholera-toxin-catalysed [32 P]ADP-ribosylation of G₂α and G₀1α in membranes of clone-3 cells

Cholera-toxin-catalysed [32 P]ADP-ribosylation was performed in the absence of guanine nucleotides and in the presence of various concentrations of UK14304 (CON, control), as described in the Materials and methods section, on membranes (60 μ g) of clone-3 cells. These reaction mixtures were subsequently immunoprecipitated as described in the legend to Figure 3, with either antiserum SG (G₂α; ■) or ON (G₀1α; ○). Data are displayed as radioactivity incorporated into each G-protein in response to UK14304, as a percentage of the incorporation of [32 P]ADP-ribose in the presence of 10 μ M UK14304, and represent mean values $\pm$ S.E.M. from four independent experiments performed on different preparations of membranes of clone-3 cells. Lines are computer-generated best-fit analyses using the Kaleidograph curve-fitting package.

Analysis of dose-effect curves for the stimulation of cholera-toxin-catalysed [32 P]ADP-ribosylation of G₀1α by UK14304 in membranes of clone-3 cells indicated an EC₅₀ for UK14304 of 34 ± 7 nM (mean $\pm$ S.E.M., $n = 4$) (Figure 5), and the measured EC₅₀ for UK14304 stimulation of incorporation of radiolabel into G₂α (22 ± 6 nM; mean $\pm$ S.E.M., $n = 4$) was similar (Figure 5).

Table 1 Effect of sustained exposure of clone-3 cells to UK14034 on membrane-associated levels of G-protein α -subunits

Clone-3 cells were exposed to UK14304 ($10 \mu\text{M}$) for 16 h. Membranes were prepared, and the relative levels of each of a combination of $G_q\alpha/G_{11}\alpha$, of $G_{i2}\alpha$ and of $G_{o1}\alpha$ were measured by immunoblotting with selective antisera as described in the Materials and methods section. Data are presented as means $\pm$ S.E.M. ($n = 3$ from individual membrane preparations).

G-protein α subunit	Levels in membranes of UK14304-treated cells (% of that in untreated cells)
G_q/G_{11}	95 ± 5
G_{o1}	29 ± 4
G_{i2}	35 ± 19

Figure 6 Dose-effect curves for UK14304-induced down-regulation of $G_{i1}\alpha$ and $G_{i2}\alpha$ in clone-3 cells

Clone-3 cells in tissue culture were treated with various concentrations of UK14304 (CON, control) for 16 h; the cells were then harvested and membranes prepared. Quantitative analysis of the relative levels of both $G_{i2}\alpha$ (■) and $G_{i1}\alpha$ (○) was performed on individual immunoblots derived from four separate membrane preparations. Results are presented as means $\pm$ S.E.M., with the lines representing computer-analysed best fit as described in Figure 5.

In many circumstances, sustained exposure of cells expressing a G-protein-coupled receptor to agonist at that receptor results in a selective down-regulation of the α subunit of the G-protein(s) activated by the receptor [24]. After exposure of clone-3 cells to UK14304, a marked down-regulation of both $G_{o1}\alpha$ and $G_{i2}\alpha$ was observed (Table 1), whereas membrane-associated levels of each of a combination of $G_q\alpha + G_{11}\alpha$ (Table 1) and $G_s\alpha$ (results not shown) were unaltered by such treatment. Dose-effect curves for UK14304-mediated down-regulation of both $G_{o1}\alpha$ ($176 \pm 67 \text{ nM}$; mean $\pm$ S.E.M., $n = 4$) and $G_{i2}\alpha$ ($58 \pm 40 \text{ nM}$; mean $\pm$ S.E.M., $n = 4$) (Figure 6) were similar to each other, although occurring at somewhat higher concentrations than those recorded for agonist-induced cholera-toxin-catalysed [^{32}P]ADP-ribosylation.

DISCUSSION

$\alpha 2$ adrenoceptors are classically defined in terms of second-messenger regulation as inhibitors of adenylate cyclase. For both the $\alpha 2A$ (molecularly defined as the $\alpha 2C10$) adrenoceptor of human platelets [2] and the $\alpha 2B$ adrenoceptor of neuroblastoma $\times$ glioma hybrid cells [3], this effector function has been

demonstrated to result from a selective activation of the G-protein G_{i2} . However, it is clear that, in a number of other systems, expression of an $\alpha 2$ adrenoceptor can result in the activation of multiple G-proteins and effector cascades. For example, in both Rat 1 fibroblasts and CHO cells transfected to express the $\alpha 2C10$ adrenoceptor, agonist activation of both G_{i2} and G_{i3} has been recorded [17,25], whereas after transfection of this receptor into LLC-PK1-O cells a more complex pattern has been reported, with the receptor appearing to be able to interact with each of G_{i1} , G_{i2} , G_{i3} and G_o [14].

The question of potential interactions of $\alpha 2$ adrenoceptors with G_o is of particular interest, as $\alpha 2$ adrenoceptors in the central nervous system are known to be able to inhibit flux through N-type Ca^{2+} channels, and receptor regulation of these channels has been established to be mediated via the individual splice-variant subtypes of G_o [26]. Furthermore, regulation of such ionic conductance may be a more significant function in the central nervous system than inhibition of adenylate cyclase. Although the potential interaction of $\alpha 2$ adrenoceptors with multiple G-proteins has been noted above, there is little information on the relative selectivity of interactions of the receptor with various G-proteins in a membrane or whole cell. We have therefore examined the interactions of the $\alpha 2C10$ adrenoceptor with $G_{o1}\alpha$ and compared this with the interaction of the receptor with $G_{i2}\alpha$ after construction of a Rat 1 fibroblast cell line expressing each of these polypeptides.

To assess the relative interactions of the $\alpha 2C10$ adrenoceptor with $G_{o1}\alpha$ and $G_{i2}\alpha$, we performed cholera-toxin-catalysed [^{32}P]ADP-ribosylation on membranes of both clone 1C and clone 3 in the presence and absence of the $\alpha 2$ -adrenoceptor-selective agonist UK14304. Selective immunoprecipitations demonstrated effective activation of $G_{i2}\alpha$ by the receptor in both clones, and of $G_{o1}\alpha$ only in clone 3 (Figure 3). Although it may seem from the data of Figure 3 that the $\alpha 2C10$ adrenoceptor activated more G_{o1} than G_{i2} in membranes of clone 3, as there is greater amount of radiolabel in the $G_{o1}\alpha$ immunoprecipitates than in the $G_{i2}\alpha$ immunoprecipitates, such conclusions must be drawn with caution, as this will be dependent to a large extent on the immunoprecipitation efficiencies of each antiserum, something which is difficult to assess accurately and which may vary from experiment to experiment.

We [17,27] and others [28–31] have previously made use of the ability of agonist to promote cholera-toxin-catalysed [^{32}P]ADP-ribosylation to define pertussis-toxin-sensitive G-proteins which interact with receptors. This reaction occurs effectively in the absence of GTP/GDP and not in their presence. It has thus been concluded that cholera toxin is only able to cause ADP-ribosylation of these G-proteins when the nucleotide-binding pocket is empty. However, as agonist should rapidly lead to the release of GDP, then it might be expected that the maintained presence of agonist would not be required for this reaction to continue. To assess if this was true, or whether the target for cholera-toxin-catalysed ADP-ribosylation is actually a ternary complex of agonist, receptor and pertussis-toxin-sensitive G-protein, we incubated membranes initially in the presence of agonist, but then subsequently displaced the agonist by addition of a receptor-saturating concentration of the antagonist yohimbine. Such treatment prevented further cholera-toxin-catalysed [^{32}P]ADP-ribosylation of $G_{o1}\alpha$ (Figure 4), demonstrating clearly that it is insufficient for agonist simply to cause release of bound GDP, but that the receptor must remain agonist-occupied and presumably physically associated with the guanine-nucleotide-free G-protein α subunit.

To demonstrate further the interaction of the receptor with both G_{i2} and G_{o1} , we took advantage of the growing literature

which indicates that maintained exposure of a receptor to agonist can result in down-regulation of the G-protein(s) which are activated by that receptor without alteration in levels of G-proteins which are not activated by the receptor (see [24] for review). We noted that maintained exposure of clone-3 cells to UK14304 resulted in a substantial and essentially equal degree of down-regulation of membrane-associated levels of $G_{i2\alpha}$ and $G_{o1\alpha}$, but that there was no parallel down-regulation of the phosphoinositidase C-linked G-proteins $G_q\alpha/G_{11\alpha}$. Whereas it has been noted after transfection that α_2 adrenoceptors can in some instances cause the stimulation of a phosphoinositidase C via a pertussis-toxin-insensitive mechanism [8], and thus presumably via activation of G_q and/or G_{11} , we have previously noted in clone 1C that UK14304 is unable to stimulate the production of inositol phosphates [9]. It is thus not surprising that there was no down-regulation of G_q/G_{11} upon agonist occupation of the α_2C10 adrenoceptor.

Because α_2 adrenoceptors have been demonstrated to have the potential to interact weakly with G_s to stimulate adenylate cyclase [6], we wished to examine whether there was a substantial difference in the efficiency of the α_2C10 adrenoceptor in clone-3 cells to activate $G_{i2\alpha}$ and $G_{o1\alpha}$. No large difference in dose-effect curves for the covalent modification of the two G-proteins was recorded, indicating that the receptor is able to activate both G-proteins at similar degrees of receptor occupancy and that activation of $G_{o1\alpha}$ did not occur only at high receptor occupancy.

A recent study has reported on the relative affinity of interaction of the α_2C10 adrenoceptor with various G-proteins following transient co-expression in HEK293 cells [32]. That study, although demonstrating that the α_2 adrenoceptor interacted more effectively with G_i than with G_s or G_o , was unable to assess, as reported herein, either the levels of expression of the various polypeptides or the relative ability of the receptor to activate two G-proteins when they were co-expressed. It is likely that the absolute stoichiometry of activation of two different G-proteins by a single receptor will be dependent on the relative levels of expression of the G-proteins. In clone-3 cells G_{o1} (approx. 100 pmol/mg of membrane protein) (Figure 1) is expressed at somewhat higher levels than $G_{i2\alpha}$ (approx. 50 pmol/mg of membrane protein) [16]; however, these levels are not dissimilar to the measured levels of ' G_o ' and ' G_i ' in mammalian adult brain [33], although it is obviously not yet possible to estimate levels of these G-proteins in individual neurons. This suggests that in the absence of physical constraints α_2 adrenoceptors are likely to be able to activate both G_{i2} and G_{o1} in individual neurones and that the integration of signal information following activation of an α_2 adrenoceptor will depend both on the absolute levels of expression of each G-protein and what mole fraction of the G-protein must be activated to alter significantly the activity of its effector.

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