β3-Adrenoceptor agonist-induced down-regulation of $G_s\alpha$ and functional desensitization in a Chinese hamster ovary cell line expressing a β3-adrenoceptor refractory to down-regulation

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Chinese hamster ovary (CHO) cells transfected to express human β2- or β3-adrenoceptors (β2-CHO and β3-CHO cells) were exposed to the β-adrenoceptor agonist isoprenaline at various concentrations and for differing times. Sustained exposure of the β2-CHO but not β3-CHO cells to isoprenaline resulted in a time- and concentration-dependent down-regulation of the receptor as measured by a reduction in specific binding of [^{125}I]cyanopindolol. Such maintained exposure of cells expressing either receptor to the agonist produced a marked down-regulation of immunologically detectable levels of the α subunit of the stimulatory guanine-nucleotide-binding protein G_s. This effect was specific for $G_s$because levels of both $G_i2\alpha$ and $G_q\alpha/G_{11}\alpha$ were unaltered by isoprenaline treatment of both β2-CHO and β3-CHO cells. The effect of isoprenaline on $G_s\alpha$ down-regulation was some 30-fold more potent in the β2-CHO than in the β3-CHO cells. Time courses of isoprenaline-induced down-regulation of $G_s\alpha$ were not different, however, in the two cell lines. Isoprenaline treatment of the β3-CHO cells produced a desensitization of agonist-mediated regulation of adenylyl cyclase, manifested by a 4-fold reduction in the potency and a 30% reduction in maximal effect of the agonist, whereas desensitization of the β2-CHO cells was considerably greater (25-fold reduction in potency and 70% reduction in maximal effect). These results demonstrate that agonist-induced down-regulation of the G-protein which interacts with a receptor can be produced by both β2- and β3-adrenoceptors. Despite apparent concurrence of down-regulation of receptors and G-proteins in other systems [e.g. Adie, Mullaney, McKenzie and Milligan (1992) Biochem. J. **285**, 529–536], agonist-induced receptor down-regulation does not appear to be a prerequisite for down-regulation of the G-protein. Furthermore, the results suggest that agonist-induced down-regulation of a G-protein may be sufficient, in the absence of receptor regulation, to induce some agonist desensitization of effector function.

INTRODUCTION

It is now well established that prolonged exposure of a cell or tissue to an agonist for a G-protein-coupled receptor can lead to the down-regulation of expressed levels of the receptor and that this process plays its part in the regulation of cellular sensitivity to the sustained presence of the agonist [1]. More recently, it has been shown that, often, another consequence of prolonged agonist exposure is the selective down-regulation of the levels of the G-protein(s) to which the receptor is normally coupled [2].

Agonist-induced G-protein down-regulation appears to be produced primarily by an acceleration of proteolytic degradation of the G-protein [3,4], and in many instances receptor and G-protein down-regulation appear to be equally sensitive to agonist and concurrent [5,6]. These observations have led to the suggestion that receptor and G-protein down-regulation may be coupled [2]. However, it has been shown that the direct activation of the primary effector (e.g. adenylyl cyclase by forskolin) fails to mimic the down-regulation of G-protein observed after prolonged exposure to an agonist [2,7].

It has been noted that the cloned β3-adrenoceptor [8], at least when expressed in Chinese hamster ovary (CHO) cells, is resistant to agonist-induced down-regulation [9], whereas the β2-adrenoceptor has been well established to undergo down-regulation [1]. We have therefore used CHO cells expressing human β2- and β3-adrenoceptors (hereafter named β2-CHO and β3-CHO) to determine whether agonist-induced G-protein down-regulation can be produced in the absence of down-regulation of the associated receptor. Herein we present evidence to show that, in the case of the cloned β3-adrenoceptor expressed in CHO cells, prolonged agonist exposure leads to a down-regulation of $G_s\alpha$ in the absence of receptor down-regulation. In this system, receptor down-regulation and G-protein down-regulation are not coupled. The system therefore allows an assessment of the effect of G-protein down-regulation, in the absence of receptor-density changes, on the sensitivity of the functional response to agonist. We demonstrate that agonist-mediated β3-adrenoceptor-induced down-regulation of $G_s\alpha$ is accompanied by a desensitization of response to this receptor as measured by the potency and maximal effect of the agonist isoprenaline.

METHODS

Constructs and transfection

The human β2-CHO cell line, obtained under licence from A. D. Strosberg (Université Paris VII, Institut Cochin de Génétique Moléculaire, Paris, France), has been described elsewhere [10]. The human β3-adrenoceptor gene was obtained under licence from Dr. S. B. Liggett (Duke University Medical Center, Durham, NC 27710, U.S.A.). It was subcloned into the in-house-constructed dihydrofolate reductase (dhfr) amplifiable expression vector CNOD, before transfection into CHO dhfr$^-$ cells using standard $Ca_3(PO_4)_2$-precipitation methodology. Transformants were grown in selection medium and cloned in microtitre plates.

Abbreviations used: cAMP, cyclic AMP; CHO, Chinese hamster ovary.

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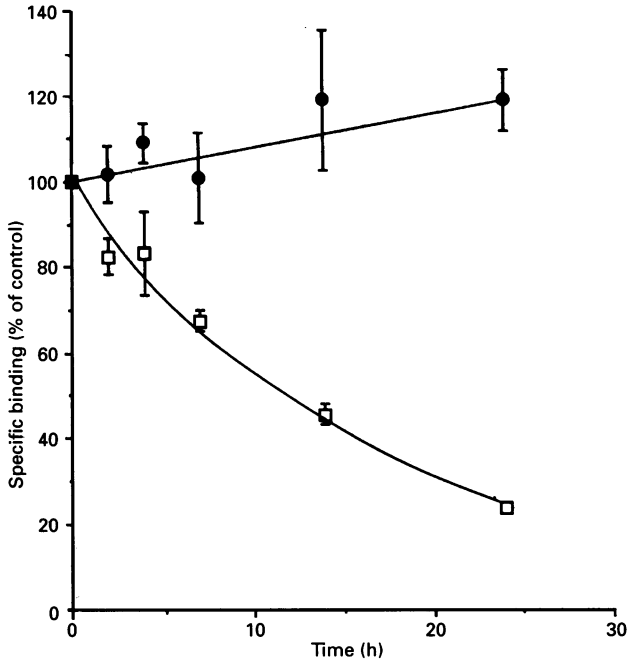

Figure 1 Time course of β-adrenoceptor down-regulation in β2- and β3-CHO cells exposed to isoprenaline

β2- and β3-CHO cells were exposed to isoprenaline (100 μM) for varying times, and their membranes were subsequently prepared. Levels of either the β2- (□) or β3- (●) adrenoceptor (mean ± S.E.M., $n = 3$, of membrane preparations derived from individual cell treatments) were then measured using the specific binding of [^{125}I]CYP as described in the Methods section.

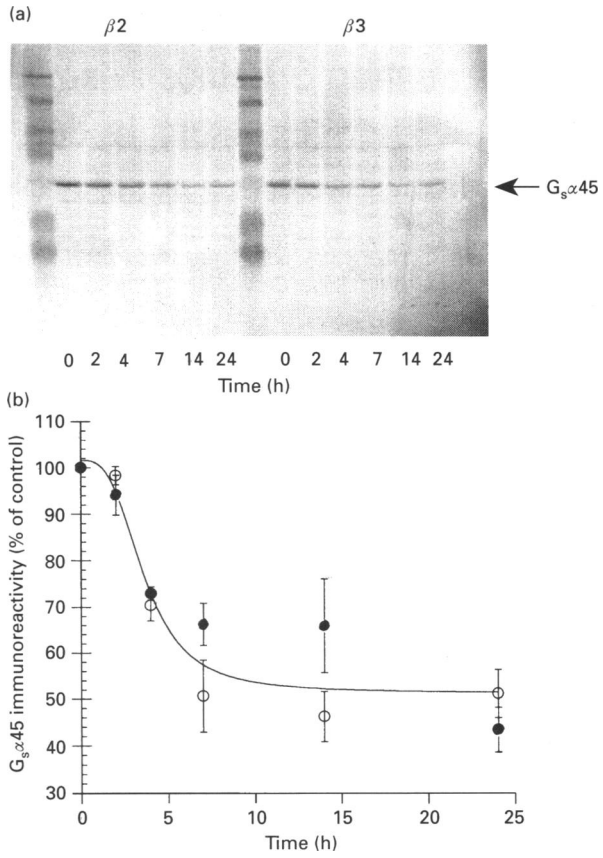

Figure 2 Time course of $G_s\alpha45$ down-regulation in β2- and β3-CHO cells exposed to isoprenaline

Membranes prepared from β2- and β3-CHO cells which had been exposed to isoprenaline (100 μM) for varying times as in Figure 1 were immunoblotted with antiserum CS as primary reagent to detect the presence of $G_s\alpha$. (**a**) A typical immunoblot. (**b**) Quantitative analysis of immunoblot data (○, β2-CHO; ●, β3-CHO), in which data are presented as means ± S.E.M. ($n = 3$) of membrane preparations derived from individual cell treatments. Curve fitting of this data was performed using the Kaleidagraph curve-fitting package as described in the Methods section.

Of 50 clonal cell lines obtained, five expressed significant levels of the β3-adrenoceptor. The highest expression level was 390 ± 83 fmol/mg of membrane protein. This level of expression was stable over a minimum of 30 population doublings. This clone was used to generate, by amplification in increasing concentrations of methotrexate, a clone with further increased expression. Cloning was carried out at each stage, resulting in the clone used in the present study which expressed the β3-adrenoceptor, stably over a minimum of 30 population doublings, at a density of 3000 ± 400 fmol/mg of membrane protein.

Cell culture

Cells were grown to 95% confluence in α-MEM growth medium (Life Technologies) containing 10% (v/v) dialysed foetal calf serum (plus 100 nM methotrexate for the β3-CHO only) in 175 cm^2 flasks at 37 °C in the presence of 5% CO_2, and exposed to 100 μM isoprenaline (unless stated otherwise) for the times indicated. Cells were washed with 4 × 10 ml of ice-cold PBS, gently scraped into PBS using Falcon cell scrapers, then snap-frozen on dry ice.

Ligand binding

All binding assays other than $B_{max.}$ determinations were carried out as follows. Samples of frozen cells were diluted with 7–14 vol. of assay buffer (50 mM Tris, 12.5 mM $MgCl_2$, 2 mM EDTA, pH 7.4, at 37 °C), repeatedly (8–10 times) aspirated and ejected from a pipette tip in order to lyse the cells, and then incubated at 37 °C for 60 min in deep well microtitre plates pretreated the previous day with Sigmacote (Sigma). Incubations with β2-CHO cell membranes contained [^{125}I]-cyanopindolol ([^{125}I]CYP; Amersham International) (specific radioactivity 2000 Ci/mmol) at a concentration of 0.5 nM. β3-CHO membranes were incubated with 5 nM [^{125}I]-CYP (specific radioactivity 300 Ci/mmol). These concentrations of [^{125}I]-CYP were calculated to occupy > 50% of β-adrenoceptors present and were used as single point determinations of the effect of agonist treatment on receptor density. Non-specific binding was determined in the presence of 0.1 mM (−)-propranolol. Bound radioligand was separated from free by rapid filtration through GF/C filters (Whatman Paper Ltd., Maidstone, Kent, U.K.). Binding was normalized to protein (Bradford assay), and expressed as a percentage of the binding observed in cells not exposed to agonist.

$B_{max.}$ determinations were carried out using membranes from cells which had not been exposed to agonist, and which were prepared according to the protocol described below for the adenylyl cyclase assay. Membranes were incubated in the same way as above, except that LP3 tubes pretreated with Sigmacote were used, and membranes were incubated with a range of concentrations of radiolabel: 0.1–12 nM [^{125}I]CYP (specific radioactivity 300 Ci/mmol) for β3-CHO cells, and 0.01–2 nM

[^{125}I]CYP (specific radioactivity 2000 Ci/mmol) for β2-CHO cells.

Membrane preparation for immunological analysis of G-proteins

Frozen cell pellets were suspended in 5 ml of 10 mM Tris/HCl, 0.1 mM EDTA, pH 7.5 (buffer A) and rupture of the cells was achieved with 25 strokes of a hand-held Teflon-on-glass homogenizer. The resulting homogenate was centrifuged at 500 *g* for 10 min in a Beckman L5-50B centrifuge with a Ti 50 rotor to remove unbroken cells and nuclei. The supernatant fraction from this treatment was then centifuged at 48000 *g* for 10 min and the pellet from this treatment was washed and resuspended in 10 ml of buffer A. Following a second centrifugation at 48000 *g* for 10 min, the membrane pellet was resuspended in buffer A to a final protein concentration of 1–3 mg/ml and stored at −80 °C until required.

Production of antisera and immunoblotting

Antiserum CS was produced by a New Zealand White rabbit following immunization with a glutaraldehyde conjugate of keyhole limpet haemocyanin (Calbiochem) and a synthetic peptide, RMHLRQYELL, which corresponds to the C-terminal decapeptide of all forms of the α subunit of G_s. The specificity of this antiserum for $G_s\alpha$ has previously been demonstrated [11]. Immunoblotting with this antiserum was performed as previously described [12]. Antiserum SG was produced in a similar fashion against the C-terminal decapeptide of the α subunit of rod transducin (KENLKDCGLF) [13]. This antiserum identifies both $G_i1\alpha$ and $G_i2\alpha$ as well as transducin α. However, using electrophoretic techniques which we have described previously [12], of these G-proteins we observed only the expression of $G_i2\alpha$ by CHO cells. Antiserum CQ was generated against a synthetic peptide (QLNLKEYNLV) which represents the C-terminal decapeptide which is conserved between $G_q\alpha$ and $G_{11}\alpha$ [14]. This antiserum cannot distinguish between these two polypeptides, as it has been shown directly to identify both polypeptides equally [15]. Molecular-mass determinations were based on pre-stained molecular-mass markers (Bethesda Research Laboratories). SDS/PAGE [10% (w/v) acrylamide] was carried out overnight at 60 V.

Quantification of immunoblots

Following SDS/PAGE, proteins were transferred to nitrocellulose (Schleicher and Schuell) and blocked for 2 h in 5% (w/v) gelatin in PBS, pH 7.5. Primary antisera were added in 1% gelatin in PBS containing 0.2% (v/v) Nonidet P-40 (NP40) and incubated for at least 2 h. The primary antiserum was then removed and the blot washed for 2 × 15 min with PBS containing 0.2% NP40. Secondary antiserum [donkey anti-(rabbit IgG) coupled to horseradish peroxidase (Scottish Antibody Production Unit, Wishaw, 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 following 2 × 15 min washes of the blot with PBS containing 0.2% NP40, and then finally with a single wash with PBS alone, the blot was developed using *o*-dianisidine hydrochloride (Sigma) as the substrate for horseradish peroxidase as previously described [12]. The developed immunoblots were scanned with a Shimadzu CS-9000 dual-wavelength flying-spot laser densitometer on reflectance mode at 450 nm. Background was subtracted by scanning of equivalent sized areas of nitrocellulose which did not contain immunoreactive protein. The results were analysed on a Shimadzu FDU-3 central processing unit, enabling quantification of the immunoblots. Preliminary experiments were performed to assess the range of linearity of the assay for each antiserum. Amounts of membranes used to assess the effects of isoprenaline treatment on levels of the various G-proteins were, in all cases, within the observed linear region.

Adenylyl cyclase activity

The activity of adenylyl cyclase was measured using the method described by Salomon et al. [16]. Cells were grown and washed as described above, except that cells were finally scraped into ice-cold lysis buffer (10 mM Tris/HCl, 2 mM EDTA, 5 μg/ml leupeptin, 5 μg/ml benzamidine and 10 μg/ml soybean trypsin inhibitor, pH 7.4, at 4 °C). Lysed cells were washed twice by centrifugation at 40000 *g* followed by resuspension in lysis buffer. The final resuspension was dispensed into aliquots, snap-frozen on dry ice and stored in liquid nitrogen until assayed. Membrane protein (10–40 μg) was incubated in the presence of 27 mM Tris, 1.8 mM EDTA, 2.5 mM $MgCl_2$, 0.2 mM ATP, 1 μM GTP, 1 mg/ml creatine phosphokinase, 22 mM phosphocreatine, 0.1 mg/ml BSA, 0.1 mM L-ascorbate, 10 mM theophylline and 20–40 μCi/ml [α-^{32}P]ATP in a final volume of 100 μl. Incubations were started by the addition of membrane, and, after 20 min, were terminated by the addition of 1 ml of ice-cold stop solution [0.25% (w/v) SDS, 5 mM ATP, 175 μM [2,8-^{3}H]cyclic AMP (cAMP) (0.2 kBq/ml), 10 mM Tris, 2 mM EDTA). The cAMP was then isolated by sequential chromatography on Dowex cation-exchange resin and alumina. The determinations were in triplicate.

Data analysis

All curve fitting and data analysis was performed using the Kaleidagraph (version 2.1) curve-fitting package driven by an Apple Macintosh computer.

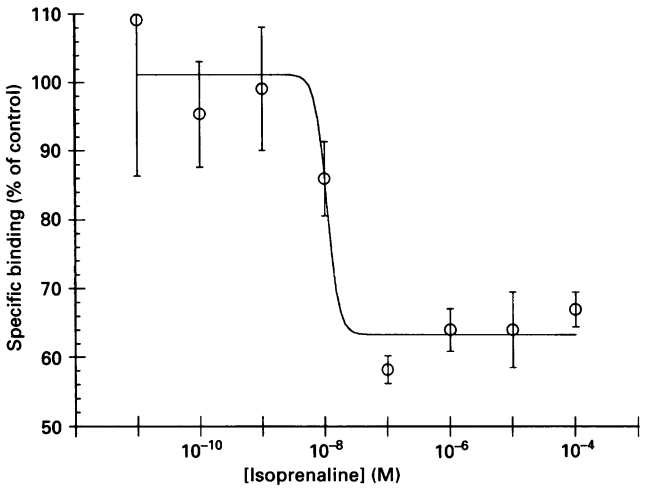

Figure 3 β-Adrenoceptor down-regulation in β2-CHO cells exposed to varying concentrations of isoprenaline

β2-CHO cells were exposed to isoprenaline (0–100 μM) for 7 h. Membranes were prepared and levels of the receptor were determined as in Figure 1 and in the Methods section. Half-maximal down-regulation of the β2-adrenoceptor was produced by some 10 nM isoprenaline. Analysis of this data was performed as described in the legend to Figure 2 and data are presented as means ± S.E.M. ($n = 3$).

RESULTS

CHO cells transfected to express either the β2- (2300 ± 120 fmol/mg of membrane protein) or the β3- (3000 ± 400 fmol/mg of membrane protein) adrenoceptor were treated for varying times with the β-adrenoceptor agonist isoprenaline (100 μM). A steady reduction in levels of the β2-adrenoceptor population in membranes derived from the β2-CHO cells was noted, as assessed by the specific binding of [^{125}I]CYP, which reached some 80% following exposure to the agonist for 24 h (Figure 1). In contrast, no reduction in levels of the β3-adrenoceptor was recorded in membranes derived from the β3-CHO cells (Figure 1).

Parallel estimation of the effect of isoprenaline on membrane levels of the α subunit of the stimulatory guanine-nucleotide-binding protein (G_s) was measured immunologically with an anti-peptide antiserum (CS) which is directed against the C-terminal decapeptide common to all splice variant isoforms of this G-protein [11]. These studies demonstrated that CHO cell membranes expressed primarily a 45 kDa form of G_s, which previously we have demonstrated to be equivalent to $G_s\alpha$ long [5]. This protein was down-regulated substantially during the time course of isoprenaline treatment in β2-CHO cells (Figure 2a). Quantification of immunoblots demonstrated that membrane levels of $G_s\alpha$45 were reduced maximally by some 50% in β2-CHO cells and that the maximal effect was achieved within 7–10 h with half-maximal down-regulation achieved in some 3.7 ± 0.7 h (mean ± S.E.M., $n = 3$) (Figure 2b). When equivalent analyses were performed on membranes of isoprenaline-treated β3-CHO cells, a similar time course of reduction of $G_s\alpha$45 levels was observed (Figures 2a and 2b). This effect, in both β2-CHO and β3-CHO, was specific for $G_s\alpha$. Equivalent immunoblots aimed at detecting membrane levels of both a combination of the phosphoinositidase-C-linked G-proteins $G_q\alpha$ and $G_{11}\alpha$ and of the inhibitory G-protein of the adenylyl cyclase cascade, G_i2, demonstrated that there was no difference in these polypeptides between untreated and isoprenaline-treated β2-CHO or β3-CHO cells (results not shown). Treatment of parental untransfected CHO cells with isoprenaline (100 μM, 24 h) had no effect on membrane levels of $G_s\alpha$ (results not shown).

Treatment of the β2-CHO cells with varying concentrations of isoprenaline for 7 h demonstrated that half-maximal reduction in levels of the β2-adrenoceptor was produced by some 10 nM agonist (Figure 3) [at this time point, maximal reduction, produced by concentrations of isoprenaline of > 0.1 μM, was some 40% (see Figure 1)]. As anticipated from the time course (Figure 1), no effect of isoprenaline on β3-adrenoceptor levels was noted in parallel experiments on the β3-CHO cells when tested at concentrations of up to 10 mM (results not shown). Measurement of membrane levels of $G_s\alpha$45 in the β2-CHO cells following a 7 h treatment with varying concentrations of isoprenaline demonstrated that half-maximal down-regulation of

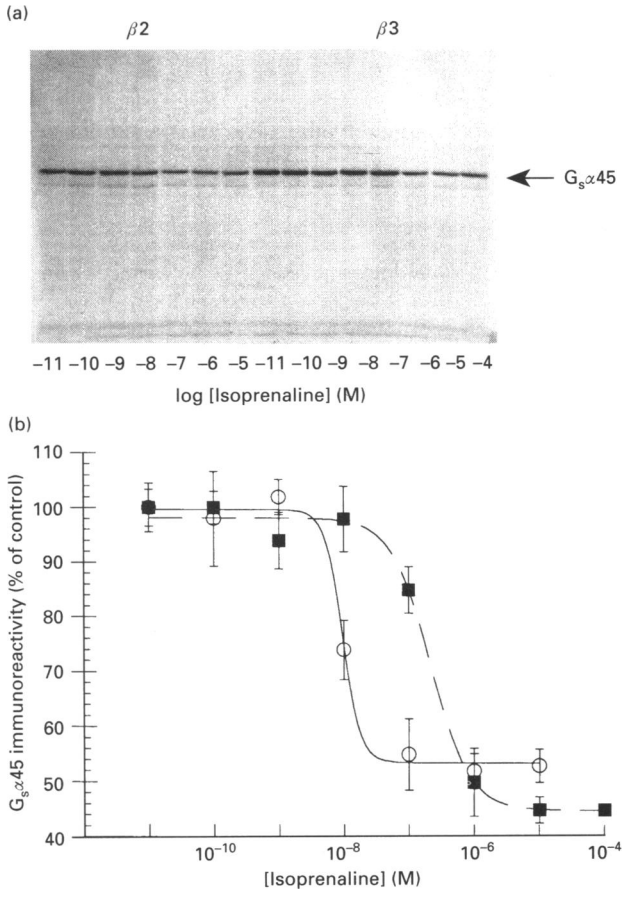

Figure 4 $G_s\alpha$ down-regulation in β2- and β3-CHO cells exposed to varying concentrations of isoprenaline

β2- and β3-CHO cells were exposed to isoprenaline (0–100 μM) for 7 h. Membranes were prepared and immunoblotted as in Figure 2 to detect the presence of $G_s\alpha$45. This polypeptide was down-regulated by exposure to isoprenaline in both the β2- and β3-CHO cells to a similar maximal extent (50–60%) in individual experiments. However, the EC_{50} for isoprenaline-induced $G_s\alpha$45 down-regulation in β3-CHO cells was considerably greater (214 ± 43 nM; ■) than in the β2-CHO cells (~ 9 ± 4 nM; ○) (**b**).

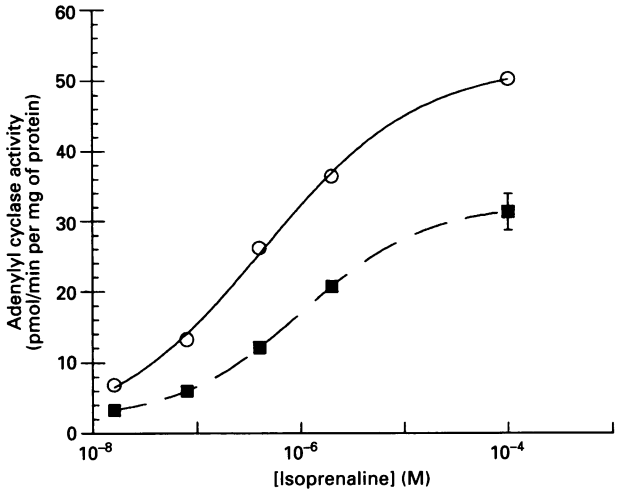

Figure 5 Desensitization of β3-adrenoceptor-mediated stimulation of adenylyl cyclase activity following treatment with isoprenaline

β3-CHO cells were treated with (■) or without (○) isoprenaline (100 μM) for 24 h. The sensitivity of membranes prepared from these cells for isoprenaline-mediated stimulation of adenylyl cyclase activity (mean ± S.E.M., $n = 1$) was then assessed as described in the Methods section. Two further experiments produced similar data. In the experiment displayed, the basal adenylyl cyclase activity was 4.3 ± 0.3 pmol/min per mg of membrane protein in the membranes of control cells, and the estimated EC_{50} for isoprenaline was 220 nM, whereas basal adenylyl cyclase activity was 2.7 ± 0.1 pmol/min per mg of membrane protein and the estimated EC_{50} for isoprenaline was 925 nM in membranes from isoprenaline-treated cells. Maximal response to isoprenaline in this experiment was reduced by 37% by pretreatment with isoprenaline.

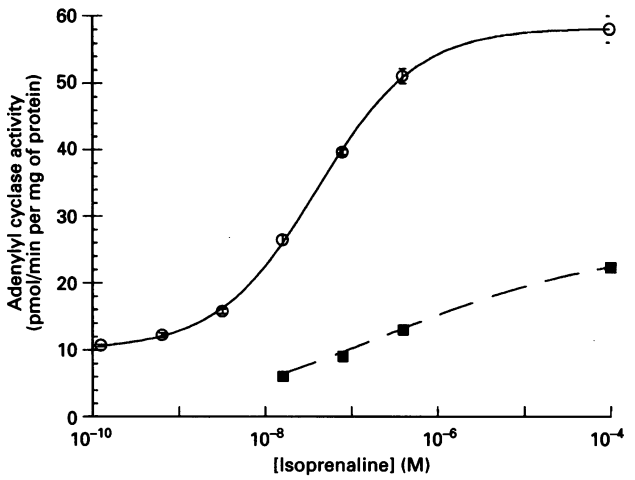

Figure 6 Desensitization of β2-adrenoceptor-mediated stimulation of adenylyl cyclase activity following treatment with isoprenaline

β2-CHO cells were treated with (■) or without (○) isoprenaline (100 μM) for 24 h. The sensitivity of membranes prepared from these cells to isoprenaline-mediated stimulation of adenylyl cyclase activity (mean ± S.E.M., $n = 1$) was then assessed as described in the Methods section. Two further experiments produced similar data. In the experiment displayed, the basal adenylyl activity was 9.6 ± 0.4 pmol/min per mg of membrane protein and the estimated EC_{50} for isoprenaline was 38 nM in the membranes of control cells, whereas basal adenylyl cyclase activity was 4.8 ± 0.2 pmol/min per mg of membrane protein and the estimated EC_{50} for isoprenaline was 560 nM in membranes from isoprenaline-treated cells. Maximal response to isoprenaline in this experiment was reduced by 71% by pretreatment with isoprenaline.

this polypeptide was produced by treatment with 9.6 ± 4.0 nM ($n = 3$) ligand (Figure 4). When equivalent experiments were performed with the β3-CHO cells, although a similar maximal down-regulation of $G_s\alpha$ was observed with concentrations of isoprenaline > 1 μM (Figures 4a and 4b), half-maximal down-regulation of this polypeptide required pretreatment of the β3-adrenoceptor with a substantially higher concentration of isoprenaline (214 ± 43 nM, $n = 3$) (Figure 4b). This observation is consistent with the lower potency of isoprenaline at the β3-adrenoceptor compared with the β2-adrenoceptor (compare Figures 5 and 6).

The sensitivity of the adenylyl cyclase response to isoprenaline was compared in membranes prepared from both cell lines with and without a 24 h exposure to 100 μM isoprenaline before cell harvest (Figures 5 and 6). In both cell lines, desensitization of the functional response to agonist was clearly observed. However, the fold shift in EC_{50} caused by agonist preincubation of the β2-CHO cell line (25 ± 5.0-fold, $n = 3$) was considerably greater than the fold shift observed with the β3-CHO cell line (4.4 ± 1.5-fold, $n = 3$). Moreover, the decrease in maximal response to isoprenaline was larger for the β2-CHO cell line (70 ± 6.0%, $n = 3$) than for the β3-CHO cell line (30 ± 7.2%, $n = 3$).

DISCUSSION

The β3-adrenoceptor plays a major role in mediating the effects of catecholamines upon lipolysis and thermogenesis in white and brown adipose tissue [17]. The potential importance of this receptor in influencing the overall energy balance of the organism has prompted a number of studies of its regulation. Using cloned human receptors, it has been demonstrated that the β3-adrenoceptor, in contrast to the β2-adrenoceptor, exhibits little or no rapid agonist-induced desensitization [8,18]. Similarly, a lack of rapid β3-adrenoceptor desensitization has been demonstrated in non-recombinant cells which express native β3-adrenoceptor, such as isolated rat adipocytes [19].

The loss of cellular levels of a receptor which occurs over longer periods of exposure to agonist is commonly refered to as down-regulation. In the present study, we have shown that the β2-adrenoceptor, when expressed in CHO cells, is down-regulated by > 75% over a 24 h exposure to the β-adrenoceptor agonist isoprenaline. This agrees well with the published reports that β2-adrenoceptor levels in CHO cells are reduced to < 20% of their original levels over the same time period [20]. The molecular basis of this pronounced down-regulation is thought to involve, at least partially, the presence of two tyrosine residues (Tyr-350 and Tyr-354) located in the intracellular C-terminal tail of the β2-adrenoceptor, as replacement of these with alanines by site-directed mutagenesis dramatically reduces the ability of the β2-adrenoceptor to undergo agonist-induced down-regulation [21].

We have now shown that the β3-adrenoceptor, at least when expressed in CHO cells, does not undergo any substantial down-regulation, even when exposed to near-saturating concentrations of agonist over 24 h. This is in agreement with a previous study [18]. Despite this, isoprenaline treatment does result in a substantial down-regulation of $G_s\alpha$ levels. Clearly, therefore, in this system, down-regulation of the G-protein which is activated by the receptor is not coupled to down-regulation of the receptor.

A series of studies has shown that, in a number of other receptor systems, agonist treatment leads to a concurrent down-regulation of both receptor and the G-protein which is activated by that receptor [2]. This has led to suggestions that it may be the coupled receptor·G-protein complex which is the target for down-regulation [22–24]. The observation in the present study, that receptor down-regulation is not a pre-requisite for G-protein down-regulation, allows the consequences of specific G-protein down-regulation to be investigated independently of receptor down-regulation.

The general mechanism(s) by which G-proteins down-regulate in response to agonist occupation of a receptor is poorly understood. In CHO cells transfected with the human muscarinic M1 acetylcholine (HM1) receptor, prolonged exposure to the agonist carbachol leads to enhanced degradation of the phosphoinositidase-C-linked G-proteins $G_q\alpha$ and $G_{11}\alpha$ [3]. We have not been able to assess whether a similar enhanced degradation of G_s results from isoprenaline treatment of the β2- or β3-adrenoceptors in this study because the antibodies which were available to us are not sufficiently effective in immunoprecipitation to allow appropriate experiments to be performed. Concern based on this limitation has also been expressed by others [4] and a potential strategy taking advantage of an epitope-tagged variant of $G_s\alpha$ has been developed [4]. This may, in time, be appropriate to our studies.

In previous studies on agonist-induced down-regulation of $G_s\alpha$, one system which has been employed is the IP prostanoid receptor expressed by the neuroblastoma × glioma hybrid cell line NG108-15 [5,7]. In these cells, even after prolonged exposure to an agonist, the remaining cell-surface receptors remain apparently tightly associated with G_s, as measured by high-affinity binding of a ^{3}H-labelled agonist and by the fact that this is markedly reduced by addition of a poorly hydrolysed analogue of GTP [5]. The β2-adrenoceptor is the prototypic example of a receptor which undergoes rapid uncoupling from G_s as an early component of its desensitization mechanism [1]. However, the

current study demonstrates that prolonged agonist activation of the β2-adrenoceptor, at least when expressed in CHO cells, can still produce a substantial down-regulation of this G-protein. This confirms an earlier observation following expression of the human β2-adrenoceptor in NG108-15 cells [25] and demonstrates that such an 'uncoupling' does not prevent agonist-induced down-regulation of G_s.

The discovery that G-proteins may be down-regulated has led to the proposal that both receptor and G-protein reductions may contribute to the desensitization of the functional response observed after long-term agonist exposure [2,24]. However, because until now these processes had been thought to be coupled, their relative contributions to functional desensitization has been impossible to determine. The system described in the present study allowed assessment of the functional significance of a reduction in G-protein levels in the absence of a loss of receptor, especially as the β3-adrenoceptor is also reported to be largely resistant to short-term agonist-induced desensitization [9].

The results show that, in the β3-CHO cells, sensitivity of adenylyl cyclase activity to isoprenaline is reduced some 4-fold after a 24 h incubation with this agonist and that the maximal response to the agonist is also reduced. This suggests that the agonist-induced down-regulation of G-protein levels alone is capable of reducing the sensitivity of the functional response to an agonist. However, the effect is clearly not as great as that observed when both receptor and G-protein are down-regulated, as in the case of the β2-CHO cell line. The relative contribution of receptors and G-protein down-regulation to functional desensitization in a system is likely to be highly dependent on cell or tissue type and on the relative levels of expression of the receptor and G-protein. Whether short-term agonist treatment of cells expressing the β3-adrenoceptor, which is likely to occur without significant alteration in cellular $G_s\alpha$ levels, may alter the sensitivity of subsequent response to isoprenaline is unclear. Nantel et al. [9] have reported a 3-fold reduction in potency following a 30 min treatment with isoprenaline, whereas Liggett et al. [18] noted no alteration. In both cases, however, in contrast to the effects noted herein with long-term exposure to isoprenaline, no alterations in maximal responsiveness were observed. Thus it may be that further study is required to define clearly whether the reduction in agonist potency which we observe is indeed a reflection of down-regulation of $G_s\alpha$.

Whereas the majority of G-protein-linked receptors are down-regulated by maintained exposure to their agonists, the β3-adrenoceptor is not the only example of a receptor reported to be resistant to such a process. In studies comparing the desensitization characteristics of the α2C10, α2C4 and α2C2-adrenoceptors following transfection into CHO cells, it was noted that the α2C4 was not down-regulated but, as with the β3-adrenoceptor as noted herein, the G-protein (G_i) linked to the α2-adrenoceptors was down-regulated [26].

The physiological significance of the current findings remain to be explored. In 3T3-F442A cells, expressing endogenous β3-adrenoceptor, receptor density appears to be up-regulated after prolonged agonist exposure, possibly due to the presence of potential cAMP response elements (CREs) in the 5′ flanking region of the β3-adrenoceptor gene [27], whereas the co-expressed β1-adrenoceptor is down-regulated [28]. It is possible that, *in vivo*, at least two mechanisms operate to regulate the β3-adrenoceptor response in opposing directions, i.e. receptor up-regulation but G-protein down-regulation. The net effect on β3-adrenoceptor-mediated responses might conceivably be neutral (i.e. no long-term tolerance to the effect of β3-agonists).

Recently, a second form of the human β3-adrenoceptor has been identified. The two forms of the receptor arise from alternative splicing of the same primary transcript. The result is one form which is six amino acids longer at the C-terminus than the other [29]. The shorter form of the receptor was used in the present study. The additional six amino acids include residues which might potentially be susceptible to phosphorylation. We are currently investigating the effects of short- and long-term agonist exposure on cells expressing the longer form of the receptor.

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