Agonist regulation of adenylate cyclase activity in neuroblastoma × glioma hybrid NG108-15 cells transfected to co-express adenylate cyclase type II and the β_2-adrenoceptor

Evidence that adenylate cyclase is the limiting component for receptor-mediated stimulation of adenylate cyclase activity

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Stable cell lines, derived from NG108-15 cells and transfected to express both the β_2-adrenoceptor and adenylate cyclase type II, were produced and examined. The absence of adenylate cyclase type II in the parental cells and its presence in these clones was demonstrated by reverse transcriptase-PCR. Total cellular levels of adenylate cyclase were increased in a number of clones between 3- and 8-fold, as assessed by guanine nucleotide-stimulated specific high-affinity binding of [^{3}H]forskolin to cellular membranes. Basal adenylate cyclase activity was markedly elevated compared with a clone expressing similar levels of the β_2-adrenoceptor in the absence of adenylate cyclase type II. Each of NaF, forskolin and guanosine 5′-[β,γ-imido]triphosphate (a poorly hydrolysed analogue of GTP) produced substantially higher levels of adenylate cyclase activity in membranes of the clones positive for expression of adenylate cyclase type II than was achieved with the parental cells. Both isoprenaline, acting at the introduced β_2-adrenoceptor, and iloprost, acting at the endogenously expressed IP prostanoid receptor, stimulated adenylate cyclase activity to much higher levels in the clones expressing adenylate cyclase type II compared with the clone lacking this adenylate cyclase; however, the concentration–effect curves for adenylate cyclase stimulation by these two agonists were not different between parental cells and clones over-expressing adenylate cyclase type II. A maximally effective concentration of the β-adrenoceptor partial agonist ephedrine displayed similar intrinsic activity and potency to stimulate adenylate cyclase in membranes of clones both with and without adenylate cyclase type II. Both secretin and 5′-*N*-ethylcarboxamidoadenosine (acting at an endogenous A_2 adenosine receptor) were also able to produce substantially greater maximal activations of adenylate cyclase in the clones expressing excess adenylate cyclase type II, without alterations in agonist intrinsic activity or potency. These results demonstrate that the maximal output of the stimulatory arm of the adenylate cyclase cascade can be increased by increasing total levels of adenylate cyclase in the genetic background of NG108-15 cells.

INTRODUCTION

There has been enormous interest in the expression and function of the individual components of signal transduction cascades and in the mechanisms of generation of intracellular second messengers. Despite this, knowledge of the absolute levels of expression and the stoichiometry of the protein components of G-protein-linked cell signalling cascades is extremely limited. A series of recent studies has indicated, however, that the effector enzymes of such cascades appear to be expressed in substantially lower levels than the G-protein(s) which regulate them [1–3]. This might be viewed as surprising, given that such systems act as amplification mechanisms to allow cells to respond effectively to low concentrations of receptor ligands.

We have recently shown, in NG108-15 neuroblastoma × glioma hybrid cells transfected to express various levels of the human β_2-adrenoceptor, that maximal adenylate cyclase activity can be achieved by occupation by isoprenaline of only some 200 fmol of this receptor/mg of membrane protein [4]. Expression of higher levels of the receptor does not result in greater adenylate cyclase activity, but only in a progressive shift in the agonist-concentration–response curve to lower concentrations [5], indicative of the presence of spare receptors. Increased expression of $G_s\alpha$ in this genetic backgound, as anticipated from the basal stoichiometries noted above, does not result in any significant alteration in the maximal effectiveness of the adenylate cyclase cascade (I. Mullaney and G. Milligan, unpublished work), even though receptors can access and activate the introduced $G_s\alpha$ as effectively as the endogenous G-protein pool [6]. To further extend such analyses, in the present study we have isolated stable clonal cell lines following transfection of adenylate cyclase type II [7,8] into this genetic background and examined how this alters the effectivness of signal transduction from both endogenously expressed and stably transfected $G_s\alpha$-coupled receptors in clones in which the total adenylate cyclase content of the cell is increased by up to 6-fold.

EXPERIMENTAL

Materials

All reagents for tissue culture were purchased from Life Technologies, Paisley, Strathclyde, U.K. [^{3}H]Forskolin (36 Ci/mmol) was obtained from Dupont/New England Nuclear. [^{3}H]Dihydroalprenolol ([^{3}H]DHA) (56 Ci/mmol), [α-^{32}P]ATP and [^{3}H]cAMP were obtained from Amersham International. Isoprenaline and the other β-adrenoceptor compounds were purchased from

Abbreviations used: DHA, dihydroalprenolol; Gpp[NH]p, guanosine 5′-[β,γ-imido]triphosphate; NECA, 5′-*N*-ethylcarboxamidoadenosine.

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Research Biochemicals International (Natick, MA, U.S.A.). Iloprost was kindly donated by Schering Health Care, Burgess Hill, W. Sussex, U.K. All other chemicals were bought from Sigma or BDH and were of the highest purity available.

Generation and isolation of a clone of NG108-15 cells expressing the human β_2-adrenoceptor (clone βN22)

Plasmid pJM16 [9], which harbours a copy of the neomycin resistance gene, was cut with the restriction enzymes *Bam*H1 and *Xho*1 to allow a cDNA encoding the human β_2-adrenoceptor to be ligated downstream of the β-actin promoter of this plasmid [5,10]. Human β_2-adrenoceptor cDNA (treated with Geneclean; Life Technologies), with 5′ *Bam*H1 and 3′ *Xho*1 sites, was ligated into the digested pJM16. Competent *Escherichia coli* cells were transformed with DNA from the ligation reactions and coated on to ampicillin (25 μg/ml) plates, and ampicillin-resistant colonies were picked. A 10 μg sample of this DNA was transfected into NG108-15 cells using Lipofectin reagent (Gibco/BRL) according to the manufacturer's instructions. Clones that were resistant to geneticin sulphate (800 μg/ml) were selected and expanded. Expression of the β_2-adrenoceptor in membranes from these clones was assessed by the specific binding of the β-adrenoceptor antagonist [^{3}H]DHA (see below). The generation and characterization of receptor expression by clone βN22 has previously been described in detail [5,10].

Generation of clones expressing adenylate cyclase type II

Cells of clone βN22 were co-transfected with a 10:1 ratio of a cDNA encoding adenylate cyclase type II (a gift from Dr. H. R. Bourne, UCSF, CA, U.S.A.) in plasmid pcDNA1 and plasmid pBABEhygro, which is able to direct expression of the hygromycin B resistance marker, by using DOTAP transfection reagent (Boehringer Mannheim) according to the manufacturer's instructions. After transfection the cells were maintained in medium containing hygromycin B (200 μg/ml). Individual colonies were selected and expanded, and subsequently examined for the maintained expression of the β_2-adrenoceptor and novel expression of adenylate cyclase type II (see the Results section).

Cell growth

Transfected neuroblastoma × glioma hybrid NG108-15 cells were grown in tissue culture as previously described [5,10]. Prior to confluency they were either split 1:10 into fresh tissue-culture flasks or harvested.

Membrane preparation

Membrane fractions were prepared from cell pastes which had been stored at −80 °C following harvest essentially as described [11]. 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/glass homogenizer. The resulting homogenate was centrifuged at 500 ***g*** for 10 min in a Beckman L5-50B centrifuge with a Ti50 rotor to remove unbroken cells and nuclei. The supernatant fraction from this treatment was then centrifuged 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 between 1 and 3 mg/ml and stored at −80 °C until required.

Adenylate cyclase assays

These were performed as described by Milligan et al. [12]. Each assay contained 100 mM Tris/HCl, pH 7.5, 20 mM phosphocreatine, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM cAMP, 1 μM GTP, 10 units of creatine kinase and 0.2 mM ATP containing 1 μCi of [α-^{32}P]ATP. Separation of radiolabelled cAMP and ATP was achieved using the double-column method described by Johnson and Salomon [13].

[^{3}H]Forskolin binding experiments in membrane preparations

Binding experiments were performed routinely with 10–20 nM [^{3}H]forskolin in the absence and presence of 10 μM forskolin to define total and non-specific binding respectively [3,14,15]. Assays were performed in 20 mM Tris/HCl, 50 mM sucrose, 5 mM $MgCl_2$, pH 7.5 (buffer B), at 20 °C for 60 min. These studies also contained 100 μM guanosine 5′-[β,γ-imido]triphosphate (Gpp[NH]p) to promote the formation of a complex of $G_s\alpha$ and adenylate cyclase. No specific binding of [^{3}H]forskolin (as defined above) was observed in the absence of Gpp[NH]p. Incubations were terminated by filtration through Whatman GF/C filters followed by three washes (5 ml) with ice-cold buffer B. Assessment of the affinity of binding of [^{3}H]forskolin to the $G_s\alpha$–adenylate cyclase complex was assessed by performing displacement curves using non-radioactive forskolin. Data were analysed by non-linear least-squares analysis. Estimates of maximal binding capacity based on the specific binding of a submaximal concentration of [^{3}H]forskolin were produced by application of the saturation binding isotherm: $B/B_{max} = L/(L+K_d)$ (where B is the observed binding, B_{max} is the maximal number of binding sites, L is the concentration of radioligand used in the assay and K_d is its dissociation constant), as extended by DeBlasi et al. [16].

Binding experiments with [^{3}H]DHA

A single concentration (2 nM) of [^{3}H]DHA in the absence and presence of 10 μM propranolol was used to define maximal and non-specific binding respectively. Assays were performed at 37 °C for 30 min in 20 mM Tris/HCl (pH 7.5), 50 mM sucrose, 20 mM $MgCl_2$ (buffer C). Specific binding, defined as above, represented greater than 90 % of the total binding of [^{3}H]DHA. All binding experiments were terminated by rapid filtration through Whatman GF/C filters followed by three washes (5 ml) with ice-cold buffer C.

Reverse transcriptase-PCR

RNA extraction

Total RNA was extracted by the acid phenol/guanidinium thiocyanate method of Chomczynski and Sacchi [17] using RNAzol B (Biogenesis). The purity and quantification of RNA were assessed by A_{260}/A_{280} ratios.

Reverse transcription

The reverse transcriptase-PCR procedure was carried out as follows. Samples of 5 μg of RNA (8 μl) were denatured by incubation at 65 °C for 10 min followed by chilling on ice, and were then reverse-transcribed in 33 μl of reaction mixture using a first-strand cDNA synthesis kit (Pharmacia LKB Bio-

technology) as detailed by the manufacturer. Incubation was carried out at 37 °C for 1 h and the reaction was stopped by heating samples at 95 °C for 5 min, followed by chilling on ice.

PCR

PCR reactions on the reverse-transcribed samples or on 200 ng of appropriate cDNA species were carried out using the following primers: adenylate cyclase type I sense, 5′GATCCTGCTCT-CCGGGCTCA 3′; adenylate cyclase type I antisense, 5′CTTC-TCAGCAGCCGGTGGACT 3′; adenylate cyclase type II sense, 5′ GCTGTGCAAGGCTGTGCTCT 3′; adenylate cyclase type II antisense, 5′ CCAGACGATGACGAAGATGTG 3′; adenylate cyclase type VI sense, 5′ CGGAAAGTAGACCCTCG-TTTCCGA 3′; adenylate cyclase type VI antisense, 5′ GCCAA-GCCATGGACGCTAAGCA3′.

Amplifications were performed in 100 μl of buffer containing 20–40 pmol of primers and 2.5 units of *Taq* polymerase (Promega) in a HYBAID Omnigene temperature cycler. Cycles were as follows: 95 °C/5 min; 55 °C/1 min, 72 °C/1 min (one cycle); 95 °C/30 s, 55 °C/1 min, 72 °C/1 min (30 cycles); 95 °C/30 s, 55 °C/1 min, 72 °C/5 min (one cycle). Reaction products were separated by 1.50–1.75 % agarose gel electrophoresis.

Immunoblotting

Membranes were resolved by SDS/PAGE [10 % (w/v) acrylamide] and subsequently transferred to nitrocellulose for immunoblotting. For detection of $G_s\alpha$, antiserum CS was used. This antiserum, initially characterized by Milligan and Unson [18], was raised in a New Zealand White rabbit after immunization with a glutaraldehyde conjugate of keyhole limpet haemocyanin and a synthetic peptide, RMHLRQYELL, which corresponds to the C-terminal decapeptide common to all isoforms of $G_s\alpha$. Immunoblots were quantified using a Bio-Rad GS-670 Imaging Densitometer linked to an Apple Macintosh Quadra 800 microcomputer.

Data analysis

All binding data were analysed using the Kaleidograph curve-fitting programme (Version 2.1) driven by an Apple Macintosh computer.

RESULTS

A clone (βN22) derived from NG108-15 neuroblastoma × glioma hybrid cells following transfection with a cDNA encoding the human β_2-adrenoceptor was further transfected in a 10:1 ratio with a combination of a cDNA encoding adenylate cyclase type II in plasmid pcDNA1 and plasmid pBABEhygro, which allows expression of resistance to the antibiotic hygromycin B. Colonies were selected following exposure to hygromycin B (200 μg/ml) and expanded. The presence of mRNA encoding adenylate cyclase type II in both parental βN22 cells and a number of the isolated colonies was assessed by reverse transcriptase-PCR performed on RNA isolated from these cell lines with a primer pair (see the Experimental section) designed to amplify a 980 bp fragment specifically from the type II isoform of adenylate cyclase. At this level of sensitivity no adenylate cyclase type II mRNA could be detected in clone βN22 cells but a clear signal, of varying magnitude, was detected in a number of the clones isolated following transfection with the adenylate cyclase type II cDNA (Figure 1). As a positive control in these experiments, PCR was performed with the adenylate cyclase type II cDNA in plasmid pcDNA1 as template, and the same specific pair of primers. Such reactions also generated a 980 bp fragment (Figure 1). Equivalent PCR experiments performed with primer pairs designed to detect the presence and expression of either type I or type VI adenylate cyclase demonstrated the expression of mRNA encoding the type VI but not the type I adenylate cyclase in these cells (results not shown). Positive PCR controls utilizing either a cDNA encoding bovine type 1 adenylate cyclase or one encoding rat type VI adenylate cyclase confirmed that the selected primer pairs would be capable of specifically detecting the presence of reverse-transcribed mRNA corresponding to these species, as such reactions specifically generated fragments of the anticipated size (results not shown).

We have previously made use of the stimulation of the specific high-affinity binding of [^{3}H]forskolin to cell membrane preparations by the poorly hydrolysed analogue of GTP, Gpp[NH]p, to detect the formation of a complex of $G_s\alpha$ and adenylate cyclase in NG108-15 and βN22 cells [3,14]. Equivalent studies herein on membranes of clone βN22 and of clones designated AC2.4, AC2.5 AC2.7 and AC2.9 demonstrated that the guanine-nucleotide-stimulated specific binding of [^{3}H]forskolin to membranes of each of these cells was substantially higher than in clone βN22, with the highest levels of binding observed in clone AC2.7 (Table 1). Confirmation that this elevated specific binding of [^{3}H]forskolin truly represented an increase in the total number of $G_s\alpha$–adenylate-cyclase complexes which could be formed in clone AC2.7 by exposure to Gpp[NH]p was obtained by per-

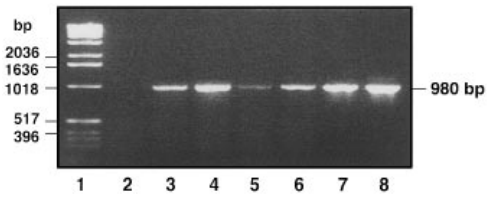

Figure 1 Isolation of clones derived from βN22 cells which express adenylate cyclase type II: reverse transcriptase-PCR detection of adenylate cyclase type II mRNA

RNA was isolated from clone βN22 cells (lane 2) and from clones AC2.5 (lane 3), AC2.7 (lane 4), AC2.9 (lane 5) and AC2.4 (lane 6). These RNA preparations were reverse-transcribed and PCRs were then performed using the primers for adenylate cyclase type II described in the Experimental section. These were designed to generate a 980 bp fragment from adenylate cyclase type II. As a positive control a cDNA encoding adenylate cyclase type II in plasmid pcDNA1 was also subjected to PCR under identical conditions (lanes 7 and 8). Size markers (bp) are displayed in lane 1.

Table 1 Adenylate cyclase type II-positive clones express elevated levels of guanine nucleotide-stimulated [^{3}H]forskolin binding

Membranes (100 μg) from each of clone βN22 and the adenylate cyclase type II mRNA-positive clones used in Figure 1 were examined for Gpp[NH]p (100 μM)-dependent specific high-affinity [^{3}H]forskolin binding as described in the Experimental section and in [14]. The data are means ± S.E.M. from three independent experiments.

Clone	Gpp[NH]p-stimulated [^{3}H]forskolin binding (fmol/mg)
βN22	48 ± 1
AC2.4	294 ± 3
AC2.5	292 ± 2
AC2.7	433 ± 7
AC2.9	161 ± 5

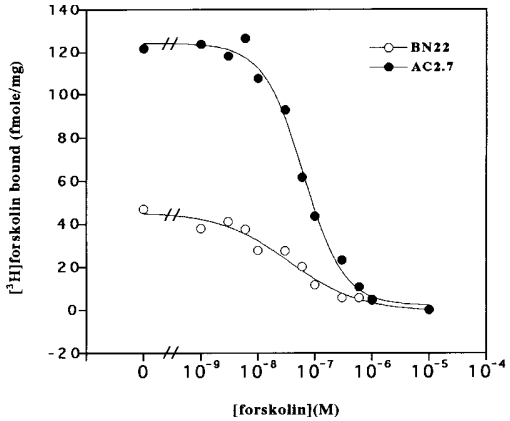

Figure 2 Adenylate cyclase type II-positive clone AC2.7 expresses higher levels of guanine-nucleotide-stimulated [^{3}H]forskolin binding than parental βN22 cells

Membranes from clones AC2.7 (●) and βN22 (○) were incubated with 11.4 nM [^{3}H]forskolin and Gpp[NH]p (100 μM), as described in the Experimental section, in the presence of various concentrations of non-radioactive forskolin as indicated. The data represent the means of triplicate determinations from a representative experiment of three performed. In the example displayed, the estimated IC_{50}, K_d and B_{max} values are 37 nM, 26 nM and 150 fmol/mg respectively for clone βN22, and 63 nM, 52 nM and 690 fmol/mg respectively for clone AC2.7.

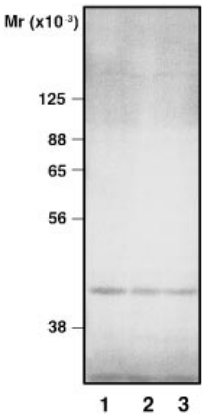

Figure 3 Clones βN22, AC2.4 and AC2.7 express similar levels of $G_s\alpha$

Membranes (25 μg) of clones βN22 (lane 1), AC2.4 (lane 2) and AC2.7 (lane 3) were resolved by SDS/PAGE [10% (w/v) acrylamide], transferred to nitrocellulose and immunoblotted to detect the presence of $G_s\alpha$ using antiserum CS [17] at a 1:500 dilution.

Table 2 Clones βN22, AC2.4 and AC2.7 express similar levels of the β_2-adrenoceptor

β_2-Adrenoceptor expression was measured in membranes (10 μg) of clones βN22, AC2.4 and AC2.7 as judged by the specific binding of a single concentration of [^{3}H]DHA (see the Experimental section). The data represent the means ± S.E.M. from three experiments performed with separate membrane preparations.

Clone	Specific [^{3}H]DHA binding (fmol/mg of protein)
βN22	2420 ± 200
AC2.4	2580 ± 80
AC2.7	2550 ± 255

Table 3 Increased basal and $G_s\alpha$-stimulated adenylate cyclase activity in membranes of adenylate cyclase type II-expressing cells

Adenylate cyclase activity was measured in membranes (10 μg) of clones βN22, AC2.4 and AC2.7 in the absence (basal) or presence of forskolin (100 μM), Gpp[NH]p (100 μM) or AlF_4^- [provided as NaF (10 mM)], isoprenaline (10 μM), iloprost (10 μM) or bradykinin (10 μM). The data represent means ± S.E.M., $n = 3$.

Stimulus	Adenylate cyclase activity (pmol/min per mg)		
	βN22	AC2.4	AC2.7
Basal	60 ± 4	690 ± 43	890 ± 25
Forskolin	270 ± 43	1300 ± 23	1820 ± 110
Gpp[NH]p	260 ± 6	2750 ± 160	4000 ± 250
AlF_4^-	240 ± 36	2610 ± 200	3780 ± 270
Isoprenaline	260 ± 12	1920 ± 58	3190 ± 140
Iloprost	230 ± 4	1580 ± 76	2880 ± 230
Bradykinin	43 ± 11	590 ± 91	850 ± 51

forming such binding assays on membranes of clones βN22 and AC2.7 in the presence of Gpp[NH]p (100 μM) with a fixed concentration of [^{3}H]forskolin and increasing concentrations of non-radioactive forskolin. Specific [^{3}H]forskolin binding to membranes from both clone βN22 and clone AC2.7 was displaced by self-competition with unlabelled forskolin in a concentration-dependent manner (Figure 2). Fitting of such data by non-linear least-squares analysis and application of the formalisms of DeBlasi et al. [16] to these results provided estimates of B_{max} and K_d respectively of 110 ± 20 fmol/mg of protein and 20 ± 7 nM (means ± S.E.M., $n = 4$) for clone βN22, and 580 ± 70 fmol/mg of protein and 44 ± 4 nM (means ± S.E.M., $n = 3$) for clone AC2.7. Although these results demonstrate a large increase in total levels of $G_s\alpha$–adenylate-cyclase complexes which can be formed in response to Gpp[NH]p in clone AC2.7 compared with clone βN22 ($P = 0.001$), potential differences in the affinity of [^{3}H]forskolin binding in the two clones could not be assessed adequately due to the relatively high error (35%) associated with estimates of the IC_{50} for forskolin in clone βN22.

Both clones AC2.4 and AC2.7 were demonstrated to have retained similarly high levels of the β_2-adrenoceptor as clone βN22, as measured by the specific binding of the β-adrenoceptor antagonist [^{3}H]DHA (Table 2). Immunoblotting analysis of the levels of the GLUT 1, GLUT 3 and GLUT 4 glucose transporter isoforms revealed no alteration in the levels of these proteins between clones βN22, AC2.4 and AC2.7 (results not shown). Furthermore, immunodetectable levels of the adenylate cyclase stimulatory G-protein $G_s\alpha$ (Figure 3) or, indeed, the phosphoinositidase C-linked G-proteins $G_q\alpha/G_{11}\alpha$ (results not shown) were not substantially different in membranes of clones AC2.4 and AC2.7 compared with clone βN22. These two clones were selected for more detailed analysis in comparison with their parental βN22 cells.

Basal adenylate cyclase activity in membranes of clones AC2.7 and AC2.4 was substantially elevated in comparison with that in clone βN22 (Table 3; see also Figure 4, upper panel), and addition of forskolin (100 μM), Gpp[NH]p (100 μM) or NaF (10 mM, as a source of AlF_4^-) produced markedly higher adenylate cyclase activities in each of these two clones compared with clone βN22 (Table 3). Isoprenaline (acting at the β_2-adrenoceptor) or iloprost (acting at the endogenously expressed IP prostanoid receptor) (both at 10 μM) resulted in activation of

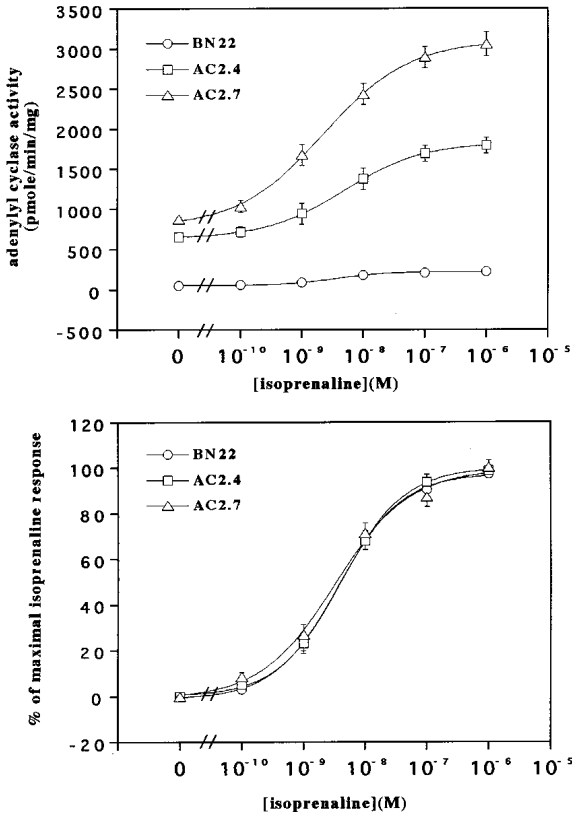

Figure 4 Isoprenaline-stimulated adenylate cyclase activity: concentration–effect analysis in membranes from clones βN22, AC2.4 and AC2.7

Upper panel: adenylate cyclase activity was measured in membranes (10 μg) of clones βN22, AC2.4 and AC2.7 in the absence or presence of various concentrations of isoprenaline. The data represent the means $\pm$ S.E.M., derived from three independent experiments. Lower panel: the data were then normalized relative to adenylate cyclase activity produced by a maximally effective concentration of isoprenaline (10 μM in each case).

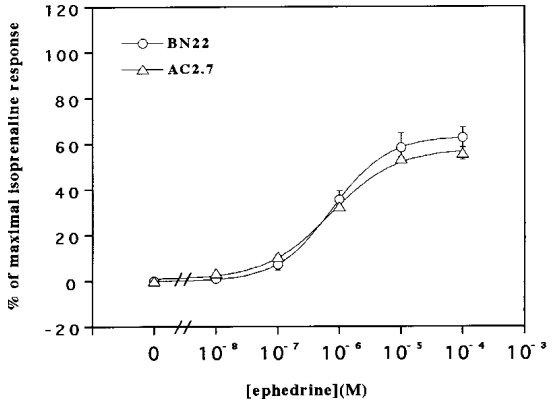

Figure 5 The intrinsic activity and potency of ephedrine to stimulate adenylate cyclase is not altered by overexpression of adenylate cyclase type II

Ephedrine concentration–response curves for adenylate cyclase activity in membranes (10 μg) of clones βN22 and AC2.7 were compared. Data are normalized to the response observed with a maximal concentration (10 μM) of isoprenaline. In a representative experiment from this series, basal adenylate cyclase activity was 54 ± 8 and 690 ± 20 pmol/min per mg and 10 μM isoprenaline-stimulated activity was 170 ± 7 and 3010 ± 45 pmol/min per mg in clones βN22 and AC2.7 respectively (means $\pm$ S.D. of triplicate determinations). The data represent the means $\pm$ S.E.M. from three independent experiments.

Table 4 The intrinsic activities of a range of β-adrenoceptor agonists are not altered by overexpression of adenylate cyclase type II

The effect on adenylate cyclase activity of a maximally effective concentration (10 μM) of compounds which display a range of intrinsic activities at the β_2-adrenoceptor [4] was compared in membranes (10 μg) of clones βN22 and AC2.7. Data are normalized to the response seen with isoprenaline (100%). In a representative experiment, basal adenylate cyclase activity was 33 ± 3 and 490 ± 14 pmol/min per mg, and 10 μM isoprenaline-stimulated activity was 160 ± 6 and 1750 ± 43 pmol/min per mg, in clones βN22 and AC2.7 respectively (means $\pm$ S.D. of triplicate determinations). The data represent means $\pm$ S.E.M., $n = 3$, from individual membrane preparations.

	Adenylate cyclase activity (% of isoprenaline response)	
Stimulus	βN22	AC2.7
Isoprenaline	100	100
Salbutamol	100 ± 8	97 ± 2
Ephedrine	58 ± 6	54 ± 1
Alprenolol	6 ± 3	12 ± 2
Sotalol	-5 ± 3	1 ± 1
Timolol	-2 ± 2	1 ± 0
ICI 118 551	13 ± 2	10 ± 2
Propranolol	0 ± 3	2 ± 1

adenylate cyclase activity in the membranes of both clones AC2.4 and AC2.7 far greater than that obtained in membranes from clone βN22 (Table 3). However, agonist occupancy of the endogenously expressed bradykinin B_2 receptor (a receptor which stimulates the activity of phosphoinositidase C) resulted in no stimulation of adenylate cyclase activity in membranes derived from any of the clones studied (Table 3). A similar pattern was observed by measuring the ability of isoprenaline or iloprost to stimulate the specific high-affinity binding of [^{3}H]forskolin in whole cells of these clones (results not shown). Concentration–response curves to isoprenaline indicated, however, little difference in the EC_{50} values for adenylate cyclase stimulation in clones βN22, AC2.4 and AC2.7 (EC_{50} values of 3.6, 4.0 and 3.3 nM respectively) (Figure 4).

Stimulation of adenylate cyclase activity by the β-adrenoceptor partial agonist ephedrine was observed in membranes of clones βN22, AC2.4 and AC2.7. This agent caused greater adenylate cyclase activity in clones AC2.4 and AC2.7 than in clone βN22. Despite this, when compared with the effect of isoprenaline, the measured intrinsic activity of ephedrine was unaltered between the clones (Figure 5). Furthermore, concentration–effect curves for adenylate cyclase stimulation by ephedrine were very similar in membranes of clone βN22 and clone AC2.7 (Figure 5). A variety of β-adrenoceptor ligands again showed little difference in their intrinsic activities when stimulating adenylate cyclase activity in membranes from clones βN22 and AC2.7 (Table 4).

Both secretin and adenosine A_2 receptors are expressed endogenously by NG108-15 cells. Agonists at each of these receptors [secretin and 5′-*N*-ethylcarboxamidoadenosine (NECA) respectively] were unable to cause as large a stimulation of adenylate cyclase activity in membranes of clone βN22 as either isoprenaline or iloprost. However, these agonists also produced substantially greater total adenylate cyclase activity in clone AC2.7 than in clone βN22 (Table 5). When compared with the effect of isoprenaline the activities of these two agonists were again very similar, and concentration–effect curves displayed no significant alterations in the EC_{50} values for these agents in stimulation of

Table 5 Stimulation of adenylate cyclase activity in clones βN22 and AC2.7 by secretin and NECA

Adenylate cyclase activity was measured in membranes (10 μg) of clones βN22 and AC2.7 in the absence (basal) or presence of a maximally effective concentration of secretin, NECA or isoprenaline. The data represent means ± S.E.M. from between two and four independent experiments.

Condition	Adenylate cyclase activity (pmol/min per mg)	
	βN22	AC2.7
Basal	68 ± 4	1020 ± 54
Secretin (30 μM)	160 ± 5	1960 ± 71
NECA (10 μM)	130 ± 16	2290 ± 150
Isoprenaline (10 μM)	270 ± 17	3430 ± 260

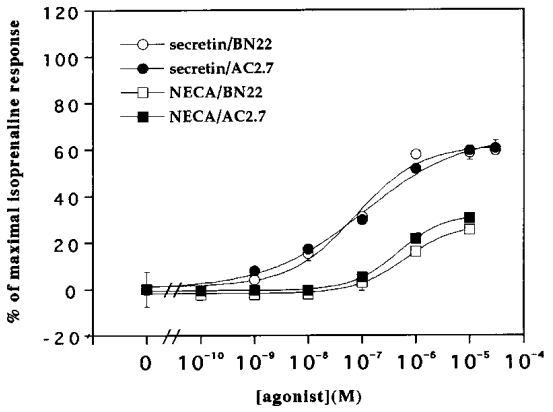

Figure 6 The intrinsic activity and potency of secretin and the adenosine receptor agonist NECA are not altered by overexpression of adenylate cyclase type II

Secretin and NECA concentration–response curves for adenylate cyclase activity in membranes (10 μg) of clones βN22 and AC2.7 were compared. Data are normalized to the response observed with a maximally effective concentration (10 μM) of isoprenaline. The data represent the means ± SD of triplicate determinations derived from a representative experiment of four performed.

adenylate cyclase activity (Figure 6). Interestingly, co-addition of maximally effective concentrations (10 μM) of both NECA and secretin to membranes of either clone βN22 or clone AC2.7 did not result in additivity of the stimulation of adenylate cyclase, with the activity being no greater than when secretin was added alone (results not shown).

DISCUSSION

The transfer of information across the plasma membranes of cells transduced by G-protein-coupled signalling systems provides an amplification cascade [1]. However, little is known about the absolute levels of expression of each protein component of these cascades in individual cells and how regulation of the levels of each individual component might alter the effectiveness of signal transduction. There is now considerable interest in this topic, as the technology of transgenic manipulation of proteins in a tissue-specific manner has started to allow an assessment of potential therapeutic uses of such regulation [19–21]. We have previously established that the levels of the α subunit of the stimulatory G-protein G_s are (at some 1.2×10^6 copies per cell) considerably higher than the levels of the best studied and most highly expressed endogenous receptor (the IP prostanoid receptor) that couples to this G-protein (1×10^5 copies per cell) [3]. This was not surprising, as it is widely accepted that G-protein-linked receptors can function catalytically to amplify the response induced by agonist binding to the receptor. More surprising was our observation that the total number of G_s–adenylate-cyclase complexes which could be formed in membranes of these cells was far lower [3]. This estimation was based on the ability of the poorly hydrolysed analogue of GTP, Gpp[NH]p, to form a long lasting complex between $G_s\alpha$ and adenylate cyclase in cell membranes which could be detected by the high-affinity binding of [^{3}H]forskolin [3], and by the use of the same reporter in whole cells in response to activation of the IP prostanoid receptor [14]. If this assay acts as a faithful reporter of the number of $G_s\alpha$-accessible adenylate cyclase catalytic enzymes, and by a number of criteria it does appear to (see [2], for example), then it seems that, at least in NG108-15 cells, the (G-protein-accessible) adenylate cyclase represents the least prevalent component of the stimulatory adenylate cyclase cascade and thus may represent the most useful component to regulate in order to alter the maximal effectiveness of the system.

In the present study we thus wished to alter the levels of expression of adenylate cyclase in this genetic background to evaluate whether this would result in a greater maximal output following agonist activation of the adenylate cyclase cascade and to examine how potencies of agonists might be regulated. At least nine individual adenylate cyclase isoforms have now been identified and isolated as distinct cDNA species [22,23], but little information is as yet available as to the cellular profile of expression of these different species. One of the most studied isoforms to date is adenylate cyclase type II [7,8,24–26] and thus for these studies we selected this isoform for transfection into clone βN22 (which was derived from NG108-15 cells by transfection of a β_2-adrenceptor cDNA and which expresses 2–3 pmol of this receptor/mg of membrane protein). βN22 cells do not express detectable adenylate cyclase type II mRNA (Figure 1). Although we did not perform an exhaustive analysis, we did detect the presence of mRNA encoding type VI but not that encoding type I adenylate cyclase in these cells (results not shown). We thus do not know at this stage whether adenylate cyclase type VI is the only, or indeed the most prevalent, adenylate cyclase isoform expressed by NG108-15-derived cells. Following transfection of clone βN22 cells with a cDNA for adenylate cyclase type II, we were able to identify a series of clones exhibiting stable expression of adenylate cyclase type II mRNA (Figure 1).

Use of a high-affinity [^{3}H]forskolin binding assay in the presence of Gpp[HN]p on membranes of these clones also demonstrated excess total stable expression of adenylate cyclase in these same clones compared with clone βN22 (Table 1 and Figure 2). Although we clearly have created cell lines expressing adenylate cyclase type II in this genetic background, we have so far been unable to isolate clones in which the levels of adenylate cyclase appear to be (based on the [^{3}H]forskolin binding assay) massively higher than in the parental βN22 cells.

It is possible, though yet to be explored in any significant detail, that individual adenylate cyclase isoforms will display different activities in the absence of receptor-mediated activation of $G_s\alpha$. In support of this concept, Iyengar and colleagues [27] have demonstrated type II adenylate cyclase to have a considerably higher basal activity than type VI adenylate cyclase. Our estimates of the quantitative increases in total cellular levels of adenylate cyclase in the type II-expressing clones were based on the maximal levels of high-affinity [^{3}H]forskolin binding that

could be achieved. Although it is appreciated that forskolin is able to interact with all of the adenylate cyclase isoforms currently known [23], it is possible that the individual isoforms may display rather different affinities for this ligand. We hoped to be able to assess this by analysis of the forskolin self-competition curves, but this was limited by the relatively low levels of specific [^{3}H]forskolin binding in membranes of clone βN22 cells, which resulted in relatively imprecise estimates in this clone. We thus must be cautious about absolute quantification of adenylate cyclase expression. It is also known that forskolin can interact with other cellular polypeptides [28], particularly those such as the GLUT family of facilitative glucose transporters which share certain topographical features with the adenylate cyclase polypeptides. However, the guanine-nucleotide-stimulated high-affinity binding of [^{3}H]forskolin appears to represent binding only to the active form of adenylate cyclase, and we demonstrated herein that levels of individual GLUT isoforms were not altered in the adenylate cyclase type II positive clones compared with the parental βN22 cells (results not shown). Analogues of forskolin which show higher selectivity between adenylate cyclase and the GLUT proteins have been described (see, e.g., [29]), but these are not widely available and certainly not in a radiolabelled form.

Activation of the β_2-adrenoceptor that was transfected into NG108-15 cells to generate clone βN22 resulted in markedly elevated adenylate cyclase activity in the adenylate cyclase type II-expressing clones compared with clone βN22 (Figure 4, upper panel), as did activation of the endogenously expressed IP prostanoid receptor (Table 3). These results demonstrate conclusively that the transfected adenylate cyclase type II can be activated in these clones by both of these receptors and that, in this genetic background, adenylate cyclase expression is indeed the limiting function for maximal output from this cascade. We have not, at this stage, examined the cellular distribution of the introduced adenylate cyclase type II compared with the endogenously expressed type VI isoform, but it clearly has access to the activated $G_s\alpha$ produced upon relevant receptor occupation. This was an important observation, given known constraints on the free mobilities of polypeptides involved in inhibitory regulation of adenylate cyclase in NG108-15 cells [30] and the emerging view that organizational structure exists in G-protein-coupled signalling systems (see [31] for a review). This will form the basis of further studies, as we were surprised to note that co-addition of NECA and secretin was unable to result in additivity of adenylate cyclase activation even though neither ligand was capable of causing the same degree of stimulation of activity as isoprenaline or iloprost.

Although greater receptor-mediated maximal output from the adenylate cyclase cascade could be produced in the adenylate cyclase type II-expressing cells, we noted that this did not result in any significant alterations in the concentration–effect curves for stimulation of adenylate cyclase activity by either the transfected β_2-adrenoceptor or any of the endogenously expressed (IP prostanoid, adenosine A_2 or secretin) $G_s\alpha$-linked receptors.

The levels of expression and activity state of downstream regulators of G-protein-linked signalling cascades such as the G-protein-linked receptor kinases are also likely to be able to modify the effectiveness of G-protein-linked receptor signalling. Indeed, in a recent report, transgenic overexpression of β-adrenoceptor kinase (BARK-1) [also called G-protein-coupled receptor kinase (GRK2)] [32] in the hearts of mice has been shown to be able to diminish the signalling capacity of β-adrenoceptors [20]. Further transfections to modify the levels of expression of these kinases in the cell lines utilized in the present work will be a target for subsequent studies.

The clear implication of the current studies is that targeted efforts to improve the maximal signalling capacity of the stimulatory arm of the adenylate cyclase cascade, as has been suggested, for example, as a strategy to combat the failing heart [19], is likely to be achieved most effectively by increasing levels of the adenylate cyclase polypeptide(s) rather than of other components of the cascade. Future studies will ascertain whether different adenylate cyclase isoforms will be more or less efficient in this regard. Informed choices of alterations in levels of receptors, G-proteins or effectors will clearly be dependent upon far greater knowledge of levels of expression of the components of signal transduction cascades in individual cells and tissues which might be targeted for modification.

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