Detection and analysis of agonist-induced formation of the complex of the stimulatory guanine nucleotide-binding protein with adenylate cyclase in intact wild-type and β 2-adrenoceptor-expressing NG108-15 cells

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Neuroblastoma $\times$ glioma hybrid, NG108-15, cells appear to express the α -subunit of the guanine nucleotide-binding protein G_s in a substantial molar excess over its effector adenylate cyclase [Kim, Adie and Milligan (1994) *Eur. J. Biochem.* **219**, 135–143]. Addition of the IP prostanoid receptor agonist iloprost to intact NG108-15 cells resulted in a dose-dependent increase in formation of the complex between $G_s\alpha$ and adenylate cyclase (GSAC) as measured by specific high-affinity binding of [3 H]-forskolin. NG108-15 cells transfected to express either relatively high (clone β N22) or low (clone β N17) levels of β 2-adrenoceptor both showed dose-dependent increases in specific [3 H]forskolin binding in response to the β -adrenoceptor agonist isoprenaline, and maximally effective concentrations of isoprenaline resulted in the generation of similar numbers of GSAC complexes in both clones. The dose–effect curve for clone β N22, however, was some

15-fold to the left of that for clone β N17, which is similar to that noted for isoprenaline-mediated stimulation of adenylate cyclase activity [Adie and Milligan (1994) *Biochem. J.* **303**, 803–808]. In contrast, dose–effect curves for iloprost stimulation of [3 H]-forskolin binding were not different in clones β N22 and β N17. Basal specific [3 H]forskolin binding in the absence of agonist was significantly greater in cells of clone β N22 than clone β N17. This was not a reflection of higher immunological levels of adenylate cyclase, indicating that the higher basal formation of GSAC probably reflects empty-receptor activation of G_s . This higher basal specific [3 H]forskolin binding was partially reversed by propranolol. The addition of the opioid peptide D-Ala-D-Leu-enkephalin to NG108-15 cells did not reduce iloprost-stimulated [3 H]forskolin binding even though this peptide inhibits stimulated adenylate cyclase activity by activation of a δ opioid receptor.

INTRODUCTION

It has been known for many years that receptors that mediate stimulation of adenylate cyclase do so via activation of the stimulatory guanine nucleotide-binding protein (G-protein) G_s . However, despite this, very little information is available on the relative or absolute levels of expression of G_s and adenylate cyclase in individual cell types, on what proportions of these proteins become activated after agonist occupation of a receptor and whether different G_s -linked receptors have access to the total cellular population of G-protein and effector enzyme or whether each receptor is restricted to utilizing different pools of either G_s and/or adenylate cyclase.

The diterpene, forskolin, binds with high affinity to the complex of $G_s\alpha$ and adenylate cyclase (GSAC) but with substantially lower affinity to isolated adenylate cyclase [1,2]. This property has been utilized to purify GSAC, after treatment with either fluoroaluminate or a poorly hydrolysed analogue of GTP to persistently activate $G_s\alpha$, by passage of cellular detergent extracts over a forskolin-affinity resin [3,4]. Adenylate cyclase in the absence of G_s has also been purified by use of a forskolin-affinity column to which membrane extracts have been applied without incubation with persistent G-protein activators [5].

We have recently used the stimulation of specific high-affinity (K_d approx. 20 nM) binding of [3 H]forskolin produced by either NaF or guanylyl 5'-[β , γ -imido]triphosphate (p[NH]ppG) to total cell membranes of neuroblastoma $\times$ glioma hybrid, NG108-15, cells to attempt to define the maximal number of GSAC complexes that can be formed in these membranes [6]. We have

calculated this to be about 17000 copies per cell which is substantially less than the number of molecules of $G_s\alpha$ per cell (some 1300000) [6].

In the present study we utilize both wild-type NG108-15 cells [7], which endogenously express a $G_s\alpha$ -linked IP prostanoid receptor [8], and clones derived from these cells transfected to express different levels of the human β 2-adrenoceptor [9–11], to examine agonist regulation of the interaction of $G_s\alpha$ and adenylate cyclase, as measured by agonist promotion of the high-affinity binding of [3 H]forskolin.

MATERIALS AND METHODS

Materials

All materials for tissue culture were from Gibco/BRL. [3 H]Forskolin (26–33 Ci/mmol) and [3 H]dihydroalprenolol (56 Ci/mmol) were from Amersham International. Iloprost was a gift from Schering Health Care (Burgess Hill, Sussex, U.K.). All other chemicals were from Sigma or BDH and were of the highest purity available.

Generation and isolation of clones of NG108-15 cells expressing the human β 2-adrenoceptor

Plasmid pJM16 [12] which harbours a copy of the neomycin-resistance gene was cut with the restriction enzymes *Bam*HI and

*Xho*I to allow a cDNA encoding the human β 2-adrenoceptor to be ligated downstream of the β -actin promoter of this plasmid. GeneCleaned (Gibco/BRL) human β 2-adrenoceptor cDNA, with 5' *Bam*HI and 3' *Xho*I sites, was ligated into the digested pJM16. Competent *Escherichia coli* were transformed with DNA from the ligation reactions, spread on ampicillin (25 μ g/ml) plates and ampicillin-resistant colonies were picked. DNA was extracted from bacterial cultures of these colonies using phenol/chloroform (1:1, v/v) and run on an agarose gel (1%, w/v) to demonstrate the presence of the cDNA as insert. DNA from a colony with the insert present was purified with CsCl_2 , and then 10 μ g of this purified DNA was stably transfected into NG108-15 cells using Lipofectin reagent (Gibco/BRL) according to the manufacturer's instructions. Clones that were resistant to geneticin sulphate (800 μ g/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 [^3H]dihydroalprenolol. The generation and characterization of receptor expression by clones β N17 and β N22 has previously been described in detail [10,11].

Cell growth

Wild-type and transfected NG108-15 cells were grown in tissue culture as previously described [10,11]. Transfected cells were also maintained in the presence of geneticin sulphate (800 μ g/ml). Before confluency they were either split 1:10 into fresh tissue culture flasks or harvested.

Membrane preparation

Membrane fractions were prepared from cell pastes that had been stored at -80°C after harvesting essentially as described previously [13]. Frozen cell pellets were suspended in 5 ml of buffer A (10 mM Tris/HCl, 0.1 mM EDTA, pH 7.5), and cells were ruptured by 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 Ti50 rotor to remove unbroken cells and nuclei. The supernatant was then centrifuged at 48000 g for 10 min, and the pellet washed and resuspended in 10 ml of buffer A. After a second centrifugation at 48000 g for 10 min the membrane pellet was resuspended in buffer A to a final protein concentration of between 1 and 3 mg/ml and stored at -80°C until required.

Preparation of cells for [^3H]forskolin-binding experiments

This was modified from the method of Alousi et al. [14]. After harvesting in growth medium, cells were centrifuged (1500 g , 5 min) and the growth medium was discarded; this was followed by two wash and centrifugation cycles with 20 ml volumes of Dulbecco's modified Eagle's medium buffered to pH 7.4 at 4°C with 20 mM Hepes (buffer B). Cells were finally resuspended at a density of 8×10^5 to 1.5×10^6 cells/ml in buffer B. Resuspended cells were maintained on ice for 20 min before use in intact-cell-binding assays. Details of cell numbers used in individual experiments can be found in the Figure legends.

Binding experiments with [^3H]forskolin

Binding experiments with membranes

These were carried out routinely with 20 nM [^3H]forskolin in the absence and presence of 10 μM forskolin to define total and non-specific binding respectively [6]. Assays were performed in buffer C (20 mM Tris/HCl, 50 mM sucrose, 5 mM MgCl_2 , pH 7.5) at 20°C for 60 min. In these studies 100 μM p[NH]ppG was

included to promote the formation of GSAC [6]. The difference between specific high-affinity [^3H]forskolin binding in the presence and absence of p[NH]ppG (100 μM) was used to define the GSAC complex. Incubations were terminated by filtration through Whatman GF/C filters followed by three washes (5 ml) with ice-cold buffer C. The affinity of [^3H]forskolin for the GSAC complex was assessed by producing displacement curves using the unlabelled drug and subsequently either correcting specific binding to account for dilution of the specific radioactivity and then plotting the saturation curve or by using the method of DeBlasi et al. [15].

Binding experiments with whole cells

Specific high-affinity binding of [^3H]forskolin to the GSAC complex in whole cells was measured essentially as described by Alousi et al. [14]. Cells harvested as described above were recovered by centrifugation, resuspended in buffer B and added to tubes containing [^3H]forskolin (approx. 10 nM) in either the absence or presence of 10 μM unlabelled forskolin to define non-specific binding. The tubes also contained various concentrations of either the β -adrenoceptor agonist isoprenaline or the IP prostanoid receptor agonist iloprost. They were incubated at 4°C for 60 min, and the contents then filtered through Whatman GF/B filters and subjected to liquid-scintillation counting.

In a number of experiments, [^3H]forskolin binding was measured in whole cells in the presence of a receptor-saturating dose of isoprenaline (10 μM), and displacement of binding achieved with different concentrations of forskolin was determined. The displacement data were analysed as described above to obtain an estimate of K_d for [^3H]forskolin binding to GSAC in whole cells.

All binding data were analysed using the Kaleidograph curve-fitting program on an Apple McIntosh computer.

Immunological detection of adenylate cyclase

This was performed using an anti-peptide serum raised against the sequence IGARKPQYDIWGNT which is highly conserved in all molecularly identified hormone-regulated adenylate cyclase isoforms [16]. The antiserum was a gift from Dr. Ravi Iyengar (Mount Sinai School of Medicine, New York, NY, U.S.A.). Membranes were resolved by SDS/PAGE [7.5% (w/v) acrylamide] and transferred to nitrocellulose. The nitrocellulose filter was subsequently treated as previously described [17] and immunopeptides detected by treatment with ^{125}I -labelled Protein A (0.04 $\mu\text{Ci}/\text{ml}$) followed by exposure to a phosphor storage plate.

Statistical analysis

The data were analysed by the use of Student's t test. Probability values (P) < 0.05 determined the validity or otherwise of the hypothesis.

RESULTS

The derivation of clone β N22 (which expresses 4000 fmol of human β 2 adrenoceptor/mg of membrane protein) and β N17 (which expresses 300 fmol of human β 2 adrenoceptor/mg of membrane protein) by transfection of NG108-15 cells has been described in detail previously [10,11]. Specific binding of [^3H]forskolin (approx. 20 nM) to total membrane preparations of these two cell lines was assessed in the presence of p[NH]ppG (100 μM), as previously performed with membranes of parental NG108-15 cells [6]. Displacement of p[NH]ppG-stimulated

Figure 1 Analysis of the affinity of the GSAC complex for [^3H]forskolin in membranes of an NG108-15-derived cell line

Displacement of guanine nucleotide-stimulated [^3H]forskolin binding to membranes of βN22 cells by forskolin is shown. Aliquots of a total (P1) membrane fraction of clone βN22 cells (250 μg) were incubated with 100 μM p[NH]ppG and [^3H]forskolin (15.8 nM) along with various concentrations of forskolin for 60 min as described in the Materials and methods section, and bound [^3H]forskolin was measured. In the example shown the estimated IC_{50} for forskolin was 42.8 nM. Application of the formalism of DeBlasi et al. [15] ($K_d = \text{IC}_{50} - [\text{H}]\text{ligand}$) gave an estimate for K_d for [^3H]forskolin of 27 nM.

specific [^3H]forskolin binding was achieved by forskolin in βN22 membranes (Figure 1) and βN17 membranes (results not shown) with high efficiency. Use of the formalism of DeBlasi et al. [15] ($K_d = \text{IC}_{50} - [\text{H}]\text{ligand}$ when the radioligand and displacing drug are one and the same) provided estimates of the affinity of binding of [^3H]forskolin (K_d) in these membranes in the presence of p[NH]ppG of between 20 and 30 nM.

Specific high-affinity binding of [^3H]forskolin and its regulation by G_s -linked receptor agonists were assessed in intact wild-type NG108-15 cells and in those of clones βN22 and βN17 using the method of Alousi et al. [14]. Preliminary time courses of agonist stimulation of [^3H]forskolin binding demonstrated that maximal levels of iloprost (10 μM)-stimulated [^3H]forskolin binding in NG108-15 cells were achieved by 60 min (Figure 2a), as was maximal stimulation of specific [^3H]forskolin binding by either iloprost (10 μM) or the β -adrenoceptor agonist isoprenaline (10 μM) in cells of clone βN22 (Figure 2b). This length of incubation was subsequently used for all studies. Time courses of specific [^3H]forskolin binding achieved in the absence of agonist also suggested that maximal levels were obtained within 60 min (Figures 2a and 2b), but these were not generally amenable to computer analysis because of the low level of specific binding obtained. Little specific binding of 10 nM [^3H]forskolin (1600 ± 800 sites per cell; mean $\pm$ S.E.M., $n = 3$) was observed at 60 min in wild-type NG108-15 cells in the absence of a receptor ligand but treatment with iloprost (10 μM) resulted in a large increase in specific [^3H]forskolin binding to 15000 ± 180 sites per cell (mean $\pm$ S.E.M., $n = 3$; $P = 0.04$). Addition of isoprenaline (10 μM), however, failed to increase specific [^3H]forskolin binding (1900 ± 1100 sites per cell; mean $\pm$ S.E.M., $n = 3$; $P = 0.86$). When similar experiments were performed on cells of clone βN17 , the specific binding of [^3H]forskolin in the absence of a receptor ligand was similar to that observed in wild-type NG108-15 cells (2700 ± 530 sites per cell; mean $\pm$ S.E.M., $n = 3$; $P = 0.34$) but iloprost (10 μM) produced a larger stimulation of specific [^3H]forskolin binding than observed in wild-type NG108-15 cells (31000 ± 3900 sites per cell; mean $\pm$ S.E.M.,

Figure 2 Time course of agonist stimulation of specific [^3H]forskolin binding in intact parental and transfected NG108-15 cells

(a) NG108-15 cells. The ability of iloprost (10 μM) (●) to promote specific [^3H]forskolin (13.2 nM) binding in intact NG108-15 cells (4.0×10^5 /assay) with time was assessed as described in the Materials and methods section as was the rate of specific binding of [^3H]forskolin in the absence of agonist (□). Results are means $\pm$ S.E.M. for three determinations. Maximal levels of binding were achieved by 60 min. (b) Clone βN22 cells. The ability of iloprost (10 μM) (◇) or isoprenaline (10 μM) (■) to promote specific [^3H]forskolin (11.0 nM) binding in intact clone βN22 cells (4.4×10^5 /assay) with time was assessed as described in the Materials and methods section, as was the rate of specific binding of [^3H]forskolin in the absence of agonist (●). Results are means $\pm$ S.E.M. for three determinations. Maximal levels of binding were achieved by 60 min.

Table 1 Agonist-stimulated maximal specific [^3H]forskolin binding in intact βN22 and βN17 cells

Specific binding of [^3H]forskolin (10.0 nM) to intact βN22 and βN17 cells was assessed as described in the Materials and methods section in the absence (basal) or presence of either iloprost (10 μM) or isoprenaline (10 μM). Data are presented as means $\pm$ S.E.M. $n = 3$ for the calculated number of high-affinity [^3H]forskolin-binding sites per cell detected by this concentration of ^3H -labelled ligand.

	[^3H]Forskolin bound (sites/cell)	
	βN17	βN22
Basal	2700 $\pm$ 530	11000 $\pm$ 1200
Isoprenaline (10 μM)	26000 $\pm$ 2500	35000 $\pm$ 4900
Iloprost (10 μM)	31000 $\pm$ 3900	34000 $\pm$ 4100

$n = 3$; $P = 0.047$) and now addition of isoprenaline (10 μM) resulted in a substantial increase in specific [^3H]forskolin binding (26000 ± 2500 sites per cell; mean $\pm$ S.E.M., $n = 3$; $P = 0.001$) (Table 1). A similar pattern was observed in βN22 cells except

Table 2 Effect of propranolol on basal specific [3 H]forskolin binding in NG108-15 and clone β N22 cells

The specific binding of [3 H]forskolin (13.0 nM) was determined in either parental NG108-15 cells or clone β N22 cells (2.2×10^5 per assay). Data are presented as means $\pm$ S.E.M. from triplicate determinations from a single experiment. Similar data were observed in a further experiment. N.D., Not determined.

	[3 H]Forskolin binding (d.p.m.)	
	NG108-15	β N22
Basal	110 $\pm$ 19	260 $\pm$ 11
Isoprenaline (10 μ M)	97 $\pm$ 3	840 $\pm$ 160
Propranolol (10 μ M)	100 $\pm$ 3	160 $\pm$ 26
Isoprenaline (10 μ M) + propranolol (10 μ M)	N.D.	430 $\pm$ 16

that the specific binding of [3 H]forskolin in the absence of receptor agonist (11 000 $\pm$ 1200 sites per cell; mean $\pm$ S.E.M., $n = 3$) was substantially higher than in both wild-type NG108-15 cells ($P = 0.009$) and β N17 cells ($P = 0.02$) (Table 1). However, the maximal levels of specific binding of [3 H]forskolin produced by isoprenaline (10 μ M) ($P = 0.21$) and iloprost ($P = 0.68$) were not significantly greater than in β N17 cells (Table 1).

To assess whether the elevated levels of basal specific binding observed in clone β N22 reflect spontaneous empty-receptor activation of GSAC formation by the expressed β_2 -adrenoceptor, we performed [3 H]forskolin-binding experiments with both NG108-15 cells and clone β N22 cells in the absence of agonist or in the presence of either isoprenaline (10 μ M) or propranolol (10 μ M). Propranolol has previously been shown to be an inverse agonist, able to cause a reduction in basal adenylate cyclase activity in membranes of β N22 cells [11]. It had no effect on basal specific [3 H]forskolin binding in parental NG108-15 cells but reduced that in β N22 cells (Table 2). Furthermore, although isoprenaline had no ability to stimulate specific [3 H]forskolin binding in NG108-15 cells, isoprenaline stimulation of specific [3 H]forskolin binding in β N22 cells was competed for by propranolol (Table 2).

To assess whether the K_d for specific binding of [3 H]forskolin was the same in whole cells as in cell membranes, the ability of forskolin to displace isoprenaline-stimulated [3 H]forskolin binding in intact β N22 cells was measured (Figure 3). Applying the formalism of DeBlasi et al. [15] to the data, a K_d for [3 H]forskolin of 29 ± 6 nM was determined.

Iloprost treatment of wild-type NG108-15 cells resulted in a dose-dependent increase in specific binding of [3 H]forskolin. A maximum was observed with 100 nM iloprost and this was not increased further by incubation with concentrations up to 10 μ M. Half-maximal stimulation of specific [3 H]forskolin binding was achieved with 5 nM iloprost (Figure 4a). Virtually identical dose-effect curves were recorded for both β N22 (EC_{50} 5.1 ± 1.5 nM) and β N17 (EC_{50} 2.5 ± 0.3 nM) cells and the parental NG108-15 cells (Figure 4b). Isoprenaline also stimulated specific high-affinity [3 H]forskolin binding in both clones β N22 and β N17 in a dose-dependent manner (Figure 5) which reached a similar maximum level. However, unlike the situation with iloprost, the dose-effect curves were substantially different in the two cell lines. In clone β N22 cells the EC_{50} for isoprenaline (1.5 ± 0.4 nM; mean $\pm$ S.E.M., $n = 3$) was 15-fold lower than in clone β N17 (20 ± 1.5 nM; mean $\pm$ S.E.M., $n = 3$).

We have previously demonstrated that the basal cellular levels of $G_i\alpha$ are the same in clones β N22 and β N17 as in the parental

Figure 3 Displacement of isoprenaline-stimulated [3 H]forskolin binding in intact clone β N22 cells by forskolin

Clone β N22 cells (3.1×10^5 /assay) were incubated with isoprenaline (10 μ M), [3 H]forskolin (11.1 nM) and various concentrations of forskolin for 60 min as described in the Materials and methods section and bound [3 H]forskolin subsequently measured. The estimated IC_{50} was 33.4 nM. Application of the formalism of DeBlasi et al. [15] as in Figure 1 resulted in an estimation of K_d for [3 H]forskolin of 22 nM.

Figure 4 Iloprost stimulation of specific [3 H]forskolin binding to intact wild-type NG108-15, β N22 and β N17 cells: dose-effect data

(a) Iloprost stimulation of specific [3 H]forskolin-binding data to intact NG108-15 cells. The specific binding of [3 H]forskolin (10.6 nM) and its regulation by various concentrations of iloprost were measured in NG108-15 cells (2.1×10^5 cells). In the example shown the EC_{50} for iloprost was 3.6 nM. (b) Iloprost stimulation of specific [3 H]forskolin-binding data to intact β N22 and β N17 cells. The specific binding of [3 H]forskolin (10.0 nM) and its regulation by various concentrations of iloprost were measured in both β N22 (2.3×10^5 cells) (■) and β N17 (2.5×10^5 cells) (○). In the example shown the estimated EC_{50} for iloprost in β N22 cells was 5.1 nM and in β N17 cells 2.5 nM.

Figure 5 Differences in dose-effect curves for isoprenaline stimulation of specific [^3H]forskolin binding to βN22 and βN17 cells

The specific binding of [^3H]forskolin (9.7 nM) and its regulation by various concentrations of isoprenaline were measured in both βN22 (2.3×10^5 cells) (■) and βN17 (1.9×10^5 cells) cells (○). In the example shown the EC_{50} for isoprenaline in βN22 cells was 0.8 nM and in βN17 cells 18 nM.

NG108-15 cells [10]. To examine whether the reason for the elevated levels of basal high-affinity [^3H]forskolin binding in βN22 cells is related to increased expression of the adenylate cyclase catalytic moiety specifically associated with this clone, we immunoblotted membranes of each of the cell types with an anti-peptide antibody raised against primary amino acid sequence that is highly conserved in all hormonally regulated adenylate cyclase isoforms identified. These immunoblots indicated a single 120 kDa polypeptide (Figure 6), the levels of which were not substantially different in the three cell lines.

To assess whether IP prostanoid receptor-mediated stimulation of high-affinity [^3H]forskolin binding in NG108-15 cells would be compromised by activation of a receptor that mediates inhibition of adenylate cyclase in these cells, we measured stimulation of high-affinity [^3H]forskolin binding by various concentrations of iloprost in the presence and absence of a receptor-saturating concentration (10 μM) of the peptide DADLE, which in NG108-15 cells mediates inhibition of adenylate cyclase via the δ opioid receptor and activation of G_{i2} [18]. The opioid peptide was unable to reduce iloprost-stimulated [^3H]forskolin binding (Table 3), even with submaximal stimulation of [^3H]forskolin binding by iloprost (results not shown), and DADLE had no effect in isolation on basal [^3H]forskolin binding. Similar results were obtained for both iloprost- and isoprenaline-stimulated high-

Figure 6 Immunological detection of adenylate cyclase in membranes of wild-type NG108-15 and clones βN22 and βN17

Membranes (100 μg) of NG108-15 (lanes 1 and 4) and clone βN17 (lanes 2 and 5) or βN22 (lanes 3 and 6) cells derived from untreated (lanes 1–3) or isoprenaline (10 μM , 16 h)-treated (lanes 4–6) cells were resolved by SDS/PAGE [7.5% (w/v) acrylamide], transferred to nitrocellulose and immunoblotted with the anti-(adenylate cyclase common peptide) serum as described in the Materials and methods section.

Table 3 Effect of the opioid agonist DADLE on agonist stimulation of specific [^3H]forskolin binding to NG108-15 cells

The specific binding of [^3H]forskolin (12.0 nM) was assessed in intact NG108-15 cells (2.0×10^5 cells/assay) as described in the Materials and methods section either in the absence of agonist or in the presence of the IP prostanoid receptor agonist iloprost (10 μM), the opioid receptor agonist DADLE (10 μM) or both. Data are presented as means $\pm$ S.D. of quadruplicate determinations from a single experiment. Similar results were obtained in two further experiments.

	[^3H]Forskolin binding to NG108-15 cells (d.p.m.)
Basal	79 $\pm$ 4
Iloprost (10 μM)	320 $\pm$ 8
DADLE (10 μM)	85 $\pm$ 11
Iloprost (10 μM) + DADLE	310 $\pm$ 14

affinity [^3H]forskolin binding in βN17 and βN22 cells (results not shown).

DISCUSSION

The absolute levels and the stoichiometry of interactions between polypeptides that comprise signal-transduction cascades across the plasma membrane of cells have hardly been investigated despite enormous interest in the mechanisms of transmembrane signalling. Indeed, data on the levels of an adenylate cyclase stimulatory receptor, G_s , and adenylate cyclase have been presented concurrently only for two cell lines. Alousi and co-workers [14] have demonstrated that, in S49 lymphoma cells, the absolute numbers of β_2 -adrenoceptor/ $\text{G}_s\alpha$ /adenylate cyclase are 1500:100000:3500, and Kim et al. [6] have shown that, in neuroblastoma $\times$ glioma hybrid cells, levels of IP prostanoid receptor/ $\text{G}_s\alpha$ /adenylate cyclase are 113000:1275000:17000. In both cases the measured levels of the adenylate cyclase catalytic subunit are very much lower than those of the corresponding G-protein, suggesting that the adenylate cyclase is likely to be the limiting component for information transfer. If this is so, receptor activation of the cascade might be expected to result in virtually complete activation of the theoretically available adenylate cyclase. In the present study we have examined the ability of receptors to activate adenylate cyclase in NG108-15 cells and in clones generated from these cells that have been transfected to express different levels of the human β_2 -adrenoceptor. The strategy of Alousi et al. [14] was adopted in which the ability of the agonist to promote the formation and maintenance of a high-affinity binding site for the diterpene [^3H]forskolin provides a measure of the formation of the physical complex between $\text{G}_s\alpha$ and adenylate cyclase (GSAC). As this fails to occur in a variant cell line that has a mutation in $\text{G}_s\alpha$ that prevents coupling of the receptor to the G-protein and also in a second mutant in which the G-protein fails to become activated after stimulation with agonist but is seen in a third form of the cell line that lacks the downstream effector for cyclic AMP, protein kinase A, then agonist enhancement of high-affinity binding of [^3H]forskolin does appear to provide a good assay for this interaction in intact cells [14]. Furthermore, we have previously demonstrated in membranes of NG108-15 cells that both NaF and p[NH]ppG stimulation of high-affinity [^3H]forskolin binding (K_d approx. 20 nM) have similar dose-effect curves to those of stimulation of adenylate cyclase activity by these two agents [6].

In the present study we also demonstrate that the K_d measured for binding of [^3H]forskolin to the GSAC complex is similar

whether it is assessed by forskolin competition for the [3 H]forskolin-binding site after activation of the complex in membranes with p[NH]ppG or in whole cells by addition of receptor agonist (Figures 1 and 3). Although there are at least eight known molecular forms of mammalian hormonally regulated adenylate cyclase (see refs. [16,19] for reviews), all are targets for regulation by forskolin [16,19]. Both of these points indicate that the approach we have used in this study is appropriate no matter the cellular expression profile of the adenylate cyclase isoforms, a matter which remains virtually uncharted.

Incubation of parental NG108-15 cells with [3 H]forskolin in the absence of receptor agonists resulted in very little specific binding of this ligand (Figure 2a). However, addition of the IP prostanoid receptor agonist iloprost produced a substantial increase in specific [3 H]forskolin binding. Time courses of these effects indicated that maximum levels of binding were obtained by 60 min, both in these cells and in the clonal cell lines transfected to express the β 2-adrenoceptor (Figure 2b). Dose-effect curves for iloprost demonstrated an EC_{50} for this effect of approx. 5 nM (Figure 4). The lack of significant high-affinity [3 H]forskolin binding in the absence of agonist probably indicates that there is little preformed precoupled GSAC complex in NG108-15 cells. The question of whether receptors and G-proteins and G-proteins and effectors exist in some form of precoupled arrangement has caused controversy for years (see refs. [20,21] for examples in favour of precoupling of G_{α} and adenylate cyclase). The ability to co-purify [3,4] or co-immunoprecipitate [22] a complex containing both adenylate cyclase and G_s in an apparently 1:1 stoichiometric ratio would appear to provide evidence for the precoupling of these polypeptides. These experiments, however, have usually been performed on samples after preactivation of the G-protein with either a poorly hydrolysed guanine nucleotide (e.g. p[NH]ppG or guanosine 5'-[γ -thio]triphosphate) or a mixture of Al^{3+} , Mg^{2+} and F^- ions. These agents are all predicted to promote the essentially irreversible formation of a GSAC complex, as they persistently activate adenylate cyclase by producing a quasiconstitutive activation of G_{α} . Furthermore, it is after such pretreatments that forskolin-affinity columns have been noted to be most useful for the retention and hence purification of adenylate cyclase [3,4]. Other studies that have detected the presence of preformed GSAC complex have been carried out at low ionic strength, an inappropriate approach compared with the intact-cell studies reported here. It has also been argued from kinetic considerations that a GSAC complex must exist in the absence of hormonal stimulation (see refs. [20,21] for reviews). However, if the high-affinity state for [3 H]forskolin binding indeed represents this complex, then it cannot exist to any significant degree in either unstimulated whole NG108-15 cells or membranes from these cells, as we observed little high-affinity binding in the absence of hormonal or direct stimulation in the parental cells. An alternative possibility is that the high-affinity state for [3 H]forskolin is only produced by a complex of GTP-GSAC and not by GDP-GSAC. It is difficult, however, to devise experiments in whole cells that would allow these possible models to be differentiated.

Whereas few specific high-affinity [3 H]forskolin-binding sites could be measured either in the absence of hormonal stimulation in intact wild-type NG108-15 cells or in cells of clone β N17, which express relatively low levels of the β 2 adrenoceptor, this was not the case in cells of clone β N22, in which levels of this receptor are considerably higher [9,10]. The basal level of high-affinity [3 H]forskolin binding was substantially (some 3-fold) higher than in the other two cell lines (Tables 1 and 2). This clearly indicates that there is either a greater preponderance of preformed

GSAC complex in the absence of hormonal stimulation in these cells or that more of this complex, if it exists preformed, is in the GTP-occupied state. These findings are entirely in accord with our previous observation that membranes of β N22 cells have a substantially increased basal adenylate cyclase activity compared with those of either wild-type NG108-15 or clone β N17 cells [11], an effect that we have interpreted as reflecting the spontaneous intrinsic activation of a fraction of the cellular G_s population in clone β N22 as the result of the high level expression of β 2-adrenoceptor [11]. This is a general concept that has found support in a number of other recent studies of 'negative agonism' at the β 2-adrenoceptor after its expression in either mammalian cell lines or insect Sf9 cells [23,24]. Furthermore, after expression of the human β 2-adrenoceptor in *E. coli* and reconstitution of membranes of these cells with G_s , the β -adrenoceptor antagonist propranolol has been shown to reduce the rate of binding of ^{35}S -labelled guanosine 5'-[γ -thio]triphosphate to below basal levels [25]. Studies of transgenic mice that express the β 2-adrenoceptor in high levels in a cardiac-tissue-specific manner have also provided data consistent with such a model, as in these animals a spontaneous increase in β 2-adrenoceptor function was observed [26]. This observation is consistent with propranolol either displaying 'negative intrinsic activity' [26] or simply suppressing the spontaneous activity resulting from the high levels of expression of the receptor (see above). An alternative explanation for these observations might be that the cells generate sufficient endogenous catecholamine during the incubations to activate the receptor population. If this were so, it would be expected to result in elevated levels of 'basal' specific [3 H]forskolin binding in clone β N22 cells but not in the parental cells, which is indeed the effect we observed. Furthermore, we would expect propranolol to inhibit basal specific [3 H]forskolin binding in clone β N22 cells by competing with the endogenously produced ligand. Although such a hypothesis is indeed consistent with the observations made, there are a number of reasons why such reasoning is incorrect in this case. (1) If propranolol were competing with endogenously generated catecholamine for the β 2-adrenoceptor ligand-binding site, then it would be expected to totally reverse the elevated 'basal' level if present in sufficiently high concentration. This was not observed; high concentrations of propranolol were used in these assays specifically to address this point. Basal specific [3 H]forskolin binding in clone β N22 cells was reduced by propranolol but not to the levels observed in the parental NG108-15 cells. (2) Exactly the same partial reversal of elevated activity was observed in our previous studies on adenylate cyclase activity in membranes derived from these cells [11]. (3) We have previously attempted to measure levels of endogenously generated catecholamines in the tissue culture medium of β N22 cells at the moment of cell harvest to assess whether carryover from this could account for the increased adenylate cyclase activity [11] via agonist-mediated stimulation of the receptor: both adrenaline and noradrenaline were below detectable levels, whereas standards at concentrations as low as 125 pM were easily detected [11]. (4) We have extended these studies in this report to determine whether the cells produce detectable levels of catecholamines during the period of the [3 H]forskolin binding assay. This seemed unlikely as exocytotic and secretory processes are not likely to function effectively at 4 °C, and indeed we have failed to detect measurable levels of catecholamine in the assay medium after such incubations (results not shown).

Clearly a trivial explanation of our data in β N22 cells, which has not been investigated in the other mammalian expression systems noted above, is that these cells endogenously express higher levels of the adenylate cyclase catalytic moiety. To exclude

this possibility, we immunoblotted membranes from β N22, β N17 and parental NG108-15 cells with an antiserum raised against a sequence predicted to be highly conserved among the known mammalian adenylate cyclase isoforms [16]. This antiserum identified an apparently single 120 kDa polypeptide which was present at the same level in the three cell lines (Figure 6).

In both β N22 and β N17 cells, but not parental NG108-15 cells which do not express the β 2-adrenoceptor, the addition of the β -adrenoceptor agonist isoprenaline resulted in a substantial increase in high-affinity [3 H]forskolin binding. Maximally effective concentrations of isoprenaline caused similar maximal stimulation in both β N17 and β N22 cells, but the dose-effect curve indicated an EC_{50} of only 1.5 nM in β N22 cells compared with 20 nM in β N17 cells. Interestingly, we have noted previously that the EC_{50} for isoprenaline stimulation of adenylate cyclase in membranes of β N22 cells is substantially less than that observed in β N17 cells [11]. The absolute EC_{50} values noted in the [3 H]forskolin-binding assay were somewhat lower than those recorded for stimulation of adenylate cyclase but this may be a reflection of the different assay conditions used (whole cells compared with membranes, different temperatures).

The difference in isoprenaline dose-response curves for stimulation of high-affinity [3 H]forskolin binding between these cells indicates that a lower fractional occupancy of the receptor population is required to cause stimulation of GSAC complex-formation in clone β N22 than in clone β N17. This is not surprising given the absolute levels of expression of the receptor in the two clones, but it does provide clear evidence for a large β 2-adrenoceptor reserve for activation of the adenylate cyclase cascade in clone β N22 cells.

The addition of iloprost (acting at the endogenously expressed IP prostanoid receptor) also led to a dose-dependent increase in formation of the GSAC complex in both clones (Figure 4b). Iloprost produced a similar maximum activation of [3 H]forskolin binding in the two cell lines but, in contrast with the situation for isoprenaline, similar dose-effect curves were observed. This is not surprising as we have noted previously that clones β N17 and β N22 express similar levels of the IP prostanoid receptor [10]. A similar dose-effect curve for iloprost stimulation of [3 H]forskolin binding was also observed in wild-type NG108-15 cells (Figure 4a).

One potential concern with these studies is that agonist regulation of [3 H]forskolin binding had to be performed at 4 °C. We have attempted to measure agonist stimulation of [3 H]forskolin binding in both membranes and whole cells at higher temperatures, but with a conspicuous lack of success. Such a limitation was also noted by Alousi et al. [14]. This is probably because, as noted by a variety of workers, G-protein GTPase activity is negligible at 4 °C, and therefore, after stimulation, the GSAC complex will be maintained because the G-protein will remain in the active GTP-liganded form, whereas at higher temperatures the G-protein will be deactivated and the high-affinity complex for [3 H]forskolin will thus not be stably maintained.

Analysis of the mechanism(s) of receptor-mediated inhibition of adenylate cyclase has led to a variety of proposals (see ref. [27] for review), but recent studies have indicated that at the least both a direct inhibition by $G_i\alpha$ and an inhibition via receptor-generated free G-protein $\beta\gamma$ -subunits may occur in specific situations. One model for direct inhibition envisages competition between $G_s\alpha$ and $G_i\alpha$ for a common site on adenylate cyclase. To assess this we added the opioid agonist DADLE, which inhibits

adenylate cyclase in NG108-15 cells via binding to the δ opioid receptor and subsequent activation of G_{i2} [18], and determined whether this altered the increase in specific [3 H]forskolin binding induced by various concentrations of iloprost. The failure of DADLE either to alter iloprost stimulation of [3 H]forskolin binding or itself to promote [3 H]forskolin binding may indicate that $G_s\alpha$ and $G_i\alpha$ do not share a common or overlapping site on adenylate cyclase.

The results presented here demonstrate that agonist stimulation of high-affinity binding of [3 H]forskolin in whole cells can provide a good measure of GSAC formation and can be used to evaluate the stoichiometry of receptor activation of adenylate cyclase. This approach has also been demonstrated to provide useful and novel information on the molecular nature of spare receptors. The data also argue strongly that previously observed elevated levels of basal adenylate cyclase activity in cells expressing high levels of the β 2-adrenoceptor reflect empty-receptor stimulation of the cascade, as these cells basally contain more preformed activated copies of the GSAC complex. Such an approach should be amenable for analysis in a range of cell systems.

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