Regulation of basal adenylate cyclase activity in neuroblastoma × glioma hybrid, NG108-15, cells transfected to express the human $\beta 2$ adrenoceptor: evidence for empty receptor stimulation of the adenylate cyclase cascade

Elaine J. ADIE and Graeme MILLIGAN*

Molecular Pharmacology Group, Departments of Biochemistry and Pharmacology, University of Glasgow, Glasgow G12 8QQ, Scotland, U.K.

Clones of neuroblastoma × glioma hybrid, NG108-15, cells expressing differing levels of the human $\beta 2$ adrenoceptor were isolated. Two clones were examined in detail, β N22 which expressed some 4000 fmol/mg of membrane protein and clone β N17 which expressed approx. 300 fmol/mg of membrane protein of the receptor. In β N22 cells 'basal' adenylate cyclase activity measured in the presence of Mg^{2+} was significantly greater than that in wild-type NG108-15 or β N17 cells. Both isoprenaline and iloprost were able to stimulate adenylate cyclase activity in each of β N22 and β N17 membranes. However, the EC_{50} for isoprenaline stimulation of adenylate cyclase in membranes of β N22 cells (6 nM) was significantly lower than that in membranes of β N17 cells (80 nM), whereas the EC_{50} for iloprost stimulation of adenylate cyclase (approx. 25 nM) was the same in

the two clones and in parental NG108-15 cells. The high basal adenylate cyclase activity of β N22 cell membranes was not a reflection of higher levels of expression of the adenylate cyclase catalytic unit, as adenylate cyclase activity measured in the presence of Mn^{2+} was equivalent in membranes of each of wild-type NG108-15 cells and clones β N22 and β N17. Basal adenylate cyclase activity measured in the presence of Mg^{2+} in clone β N22 was significantly reduced, however, by the β -receptor antagonist propranolol, whereas this agent was without effect on basal adenylate cyclase activity in membranes of wild-type NG108-15 cells. These data indicate that the elevated basal adenylate cyclase cascade in NG108-15 cells expressing high levels of the $\beta 2$ adrenoceptor represents empty receptor activation of the signalling cascade.

INTRODUCTION

Information transfer from the outside of a cell produced via a guanine-nucleotide-binding protein (G-protein)-linked cellular signalling cascade requires the co-ordinated regulation of members of at least three classes of proteins: receptors, G-proteins and effectors [1,2]. While agonist occupation of the receptor leads to activation of the G-protein and subsequently an alteration in the activity of the effector species, recent evidence has indicated that absence of agonist does not necessarily define the minimum activity of the cascade [3–10]. Experiments performed with drugs described as possessing negative intrinsic activity have shown that the 'basal' activity can be further reduced [3,4] (see [5] for review). If an empty receptor can stimulate its relevant G-protein such that a signalling cascade is partially operative in the absence of an agonist then it must be anticipated that the presence of a greater number of receptors should potentially lead to a further 'basal' stimulation of the G-protein population and thus of the effector. The logical conclusion of such an argument is that in the presence of a sufficiently great number of receptors, effector stimulation would occur to a maximal level in the absence of agonist and thus provide constitutive regulation of the signalling cascade. Constitutive activation of G-protein-linked signalling cascades have been reported in situations in which either the receptor has a mutation such that the conformational switch which functions to allow activation of the G-protein (which is normally triggered by agonist binding to the receptor) is overridden [7–10] or in which the G-protein has a mutation which prevents or hinders the GTPase activity which acts as its normal turn-off mechanism [11–14].

The hypothesis that high level expression of a receptor could result in elevation of the basal activity of an effector without alterations occurring in the levels or intrinsic activity of either the G-protein or the effector has not been examined in detail. To do so, in this study, we have used clones of NG108-15 cells expressing either low or high levels of the human $\beta 2$ adrenoceptor following transfection of the cells with a plasmid containing a $\beta 2$ adrenoceptor cDNA. We demonstrate that high level, but not low level, expression of the $\beta 2$ adrenoceptor in NG108-15 cells results in a substantially elevated basal adenylate cyclase activity and that this elevated basal activity is not a reflection of higher levels of expression or function of G_{α} or of a higher level of expression of the adenylate cyclase catalytic moiety by this clone. Furthermore, the high basal adenylate cyclase activity is partially reversed by addition of the β -receptor antagonist propranolol, which is without effect on basal adenylate cyclase activity in membranes of wild-type NG108-15 cells.

MATERIALS AND METHODS

Materials

All materials for tissue culture were from GIBCO. [α - ^{32}P]ATP and [3H]cyclic AMP were from Amersham International. All fine chemicals were either from Sigma or BDH.

Generation and isolation of clones of NG108-15 cells expressing the human $\beta 2$ adrenoceptor

The generation of the clones used in this study has been detailed previously [15]. Briefly, a cDNA encoding the human $\beta 2$ adreno-

Abbreviation used: DHA, dihydroalprenolol.

* To whom correspondence should be addressed.

ceptor was ligated downstream of the β actin promoter in plasmid pJM16 [16]. This plasmid also harbours a copy of the neomycin resistance gene. This construct was used to transfect NG108-15 cells using the Lipofectin reagent (Gibco) according to the manufacturer's instructions. Clones which were resistant to geneticin sulphate (800 μ g/ml) were selected and expanded [15]. Expression of the β 2 adrenoceptor in membranes from these clones was assessed by the specific binding of the β -adrenoceptor antagonist [3 H]dihydroalprenolol (DHA) [15].

Cell growth

Wild-type and transfected neuroblastoma $\times$ glioma hybrid, NG108-15, cells were grown in tissue culture as previously described [15] except that the 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. Cells were harvested following removal from the tissue-culture flasks by centrifugation (5 min, 500 g). The medium was then either discarded or reserved for analysis of catecholamine levels (see later) and the cell pellet resuspended in 10 ml of phosphate-buffered saline (PBS; 2.7 mM KCl, 137 mM NaCl, 1.5 mM KH_2PO_4 , 8 mM Na_2HPO_4 , pH 7.4). The cells were recentrifuged, the supernatant discarded and the wash step with PBS repeated. The cell pellet was then stored as a paste at -80°C until membranes were prepared (see below). S49 lymphoma *cyc*⁻ cells [17], which were a kind gift from Dr. K. P. Ray (Glaxo Group Research Ltd., Greenford, Middlesex, U.K.), were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) heat-inactivated horse serum in logarithmic phase and maintained at between 5×10^5 and 2×10^6 cells/ml. S49 *cyc*⁻ cell pastes were prepared as above. Membrane fractions were prepared from cell pastes which had been stored at -80°C following harvesting essentially as in [18]. Frozen cell pellets were resuspended in 5 ml of 10 mM Tris/HCl, 0.1 mM EDTA, pH 7.5, (buffer A) and rupture of the cells 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 centrifuged at 48000 g for 10 min and the pellet from this treatment 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.

Adenylate cyclase assays

These were performed as described by Milligan et al. [19]. Each assay contained 100 mM Tris/HCl, pH 7.5, 20 mM creatine phosphate, 50 mM NaCl, 5 mM MgCl_2 , 1 mM cyclic AMP, 1 μ M GTP, 10 units of creatine phosphokinase and 0.2 mM ATP containing 1 μ Ci of α -[32 P]ATP. Separation of radiolabelled cyclic AMP and ATP was achieved using the double-column method described by Johnson and Salomon [20].

Reconstitution protocols

Reconstitution assays in S49 lymphoma *cyc*⁻ membranes were performed essentially as described by Milligan and Klee [21]. Sodium cholate (1%, w/v) was used (1 h, 4°C) to extract protein from membrane preparations of wild-type or clone β N22 NG108-

15 cells. Samples were then centrifuged at 172.5 kPa (150000 g) in an airfuge (Beckman Instruments) and the supernatant fraction was taken as the soluble extract. Up to 5 μ l of extract (containing protein equivalent to 5 μ g of NG108-15 membrane protein) was added to a final volume of 100 μ l containing 10 mM NaF or 10 mM NaCl, in 50 mM Hepes, pH 8.0, 10 μ g of BSA, 10 units of creatine phosphokinase, 6 mM MgCl_2 , 0.2 mM EGTA, 2 mM 2-mercaptoethanol, 1 mM [3 H]cyclic AMP (15000 c.p.m.), 0.2 mM [α - 32 P]ATP (2×10^6 c.p.m.) and 10 μ g of S49 *cyc*⁻ membranes. Samples were incubated at 30°C for 60 min and the [32 P]cyclic AMP generated was assayed as above. Differences in the generation of [32 P]cyclic AMP in response to NaF and NaCl were taken to represent a measure of the relative activities of G_{α} . Preliminary experiments demonstrated that fluoride-mediated stimulation of [32 P]cyclic AMP production was linear with time under these conditions (results not shown but see [22] for example).

Binding experiments with [3 H]DHA

In experiments designed to assess the maximal binding capacity of membranes for [3 H]DHA concentrations of this ligand were varied between 0.1 and 5 nM in the absence and presence of 10 μ M propranolol to define maximal and non-specific binding respectively. Assays were performed at 30°C for 30 min in 20 mM Tris/HCl (pH 7.5) containing 50 mM sucrose and 20 mM MgCl_2 (buffer B). Specific binding, defined as above, represented more 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 B.

All binding data were analysed by a non-linear least-squares curve-fitting programme. See [15] for a detailed analysis of the binding characteristics of β 2 adrenoceptors in the β N17 and β N22 cell membranes used for these studies.

Miscellaneous

The presence and levels of adrenaline and noradrenaline in the medium of both β N17 and β N22 cells at the time of cell harvesting was assessed by comparison with standards of these catecholamines using h.p.l.c. with electrochemical detection as described in [23]. Protein was measured as in [24].

RESULTS

Neuroblastoma $\times$ glioma hybrid, NG108-15, cells were transfected as described in [15] with cDNA encoding a human β 2 adrenoceptor cDNA. Clones β N22 and β N17 from this transfection have been described previously [15]. Clone β N22 expresses high levels of the β 2 adrenoceptor ($B_{\text{max}} = 3994 \pm 700$ fmol/mg of membrane protein; mean $\pm$ S.E.M., $n = 6$) as assessed by the specific binding of [3 H]DHA ($K_d = 0.32 \pm 0.10$ nM) whereas clone β N17 expresses much lower levels of the β 2 adrenoceptor ($B_{\text{max}} = 292 \pm 62$ fmol/mg of membrane protein, $K_d = 0.52 \pm 0.20$ nM; mean $\pm$ S.E.M., $n = 3$) [15].

Both isoprenaline (10 μ M) and iloprost (an agonist at the IP prostanoid receptor which is expressed endogenously by NG108-15 cells) (1 μ M) were able to cause stimulation over basal adenylate cyclase activity in membranes of clone β N22 cells (Table 1). In contrast, iloprost, but not isoprenaline, caused a robust stimulation of adenylate cyclase in membranes of wild-type NG108-15 cells (Table 1). Furthermore, both iloprost and isoprenaline produced stimulation of adenylate cyclase activity in membranes of clone β N17 cells (Table 1). However, while

Table 1 Basal and agonist stimulation of adenylate cyclase in wild-type NG108-15 cells and β 2-adrenoceptor-expressing clones of these cells

Basal adenylate cyclase activity and its modulation by receptor-saturating levels of either isoprenaline (10 μ M) (β 2 adrenoceptor) or iloprost (1 μ M) (IP prostanoid receptor) were assessed in membranes from each of wild-type NG108-15 cells and cells of both clone β N22 and clone β N17. Data are mean $\pm$ S.E.M. derived from three independent experiments performed on different membrane preparations.

	Adenylate cyclase activity (pmol/mg of membrane protein per min)		
	NG108-15	β N17	β N22
Basal	29.4 $\pm$ 4.0	20.9 $\pm$ 0.1	73.1 $\pm$ 7.7
Isoprenaline	26.7 $\pm$ 2.7	140.6 $\pm$ 2.5	217.3 $\pm$ 14.5
Iloprost	236.0 $\pm$ 19.9	201.0 $\pm$ 18.0	225.7 $\pm$ 17.0

Table 2 Adenylate cyclase activity in wild-type NG108-15 cells and β 2-adrenoceptor-expressing clones: the effect of Mn^{2+}

'Basal' adenylate cyclase activity was measured in the presence of Mg^{2+} as described in the Materials and methods section or in the presence of Mn^{2+} (20 mM). Data are mean $\pm$ S.E.M. of data derived from three independent experiments. In the presence of Mn^{2+} there was no statistical difference between the adenylate cyclase activities in any of the membrane preparations.

	Adenylate cyclase activity (pmol/mg of membrane protein per min)			
	NG108-15	β N22	β N17	β N34
Basal	34.0 $\pm$ 1.7	72.1 $\pm$ 2.0	33.0 $\pm$ 1.3	69.2 $\pm$ 1.9
+ Mn^{2+}	12.4 $\pm$ 1.3	11.6 $\pm$ 2.6	10.1 $\pm$ 1.3	11.8 $\pm$ 0.8

membranes of clone β N17 had a basal adenylate cyclase activity which was not statistically different ($P = 0.31$) from that in membranes of wild-type NG108-15 cells, the basal adenylate cyclase activity of membranes of clone β N22 was significantly ($P = 0.007$) higher than that of wild-type membranes (Table 1). In support of the concept that the high basal adenylate cyclase activity might be related to the expression of high levels of the β 2 adrenoceptor, a further clone (β N34) which also expressed high levels of this receptor ($B_{max} = 6425 \pm 525$ fmol/mg of membrane protein, K_d for [3 H]DHA 0.73 ± 0.21 nM; mean $\pm$ range, $n = 2$) also exhibited a markedly higher basal adenylate cyclase activity compared with wild-type NG108-15 cells (β N34, 69.2 ± 1.9 pmol/mg of membrane protein per min; wild-type NG108-15, 34.0 ± 1.3 pmol/mg of membrane protein per min; means $\pm$ S.E.M., $n = 3$ when experiments were performed in parallel on membranes from the two cell lines).

Adenylate cyclase activity in membranes from wild-type NG108-15 cells and each of the clones β N22, β N34 and β N17 was similar, however, when measured in the presence of high concentrations (20 mM) of Mn^{2+} (Table 2), a condition believed to provide information on the activity of adenylate cyclase in the absence of G-protein regulation [25].

Analysis of dose-effect curves indicated that although iloprost stimulated basal adenylate cyclase activity with similar EC_{50} values in membranes from β N22 (13.6 ± 6.3 nM) and β N17 (23.2 ± 2.4 nM) (Figure 1a), which in each case was not sub-

Figure 1 Differences in potency of isoprenaline but not iloprost for stimulation of adenylate cyclase activity in membranes of β N22 and β N17 cells

The ability of iloprost (a) and isoprenaline (b) to stimulate adenylate cyclase was measured in membranes of β N22 (■) and β N17 (●) cells. Results are presented as percentages of the maximal effect observed, i.e. the stimulation recorded over basal levels in the presence of 10 μ M of either agonist. Data are from a single experiment, representative of three performed on separate membrane preparations.

stantially different from that produced in membranes of wild-type NG108-15 cells (21.4 ± 2.9 nM; mean $\pm$ S.E.M., $n = 3$ in each case), isoprenaline stimulated adenylate cyclase activity in membranes of β N22 cells significantly ($P < 0.001$) more potently ($EC_{50} = 6.1 \pm 2.2$ nM; mean $\pm$ S.E.M., $n = 5$) than in membranes of β N17 cells ($EC_{50} = 80.8 \pm 15.1$ nM; mean $\pm$ S.E.M., $n = 3$) (Figure 1b).

In an attempt to assess whether the apparent partial constitutive activity of the adenylate cyclase cascade in clone β N22 membranes might be related to either altered levels or to an intrinsic modification of $G_s\alpha$, sodium cholate (1%, w/v) extracts of membranes of wild-type and clone β N22 NG108-15 cells were added to membranes of S49 lymphoma *cyc*⁻ cells (which fail to

Figure 2 Levels of $G_s\alpha$ in wild-type NG108-15 cells and in clone β N22

Membranes (40 μ g) of wild-type NG108-15 (lane 1) and clone β N22 (lane 2) cells were resolved by SDS/PAGE [10% (w/v) acrylamide] and immunoblotted for $G_s\alpha$ using antiserum CS1 [32]. No significant differences in levels were noted. The Figure is representative of three experiments performed on different membrane preparations.

Table 3 The effect of propranolol on basal adenylate cyclase activities in membranes of wild-type NG108-15 and clone β N22 cells

Basal adenylate cyclase activity and modulation of this activity by the β -adrenoceptor antagonist propranolol was measured in membranes of both wild-type NG108-15 and clone β N22 cells. Data are mean $\pm$ S.E.M. derived from three separate experiments performed on separate membrane preparations. This effect of propranolol was highly significant ($P < 0.012$) in β N22 but not NG108-15 cell membranes.

	Adenylate cyclase activity (pmol/mg of membrane protein per min)	
	NG108-15	β N22
Basal	29.7 $\pm$ 3.4	79.5 $\pm$ 4.8
+ Propranolol (10 μ M)	31.7 $\pm$ 1.7	57.0 $\pm$ 1.7

express $G_s\alpha$). The detergent extracts from the two cell lines were able to reconstitute equal amounts of adenylate cyclase activity to the *cyc*⁻ membranes, demonstrating that there were equivalent amounts of $G_s\alpha$ activity in the detergent extracts from each membrane. In both cases this reconstitutive activity was dependent upon the addition of fluoroaluminate [provided as NaF (10 mM) to the assay], thus demonstrating that the $G_s\alpha$ in membranes of clone β N22 was not constitutively active. The stimulation of adenylate cyclase activity by NaF was 2.2 $\pm$ 0.3-fold when using extracts from NG108-15 cells and 2.4 $\pm$ 0.2-fold (means $\pm$ S.E.M., $n = 7$ in each case) when using extracts from β N22 cells. In addition, immunoblotting membranes from both wild-type NG108-15 cells and β N22 cells with an antiserum (CS1) directed against the C-terminal decapeptide of forms of $G_s\alpha$ identified equivalent amounts of a 45 kDa polypeptide in each (Figure 2). We have previously demonstrated this to co-migrate in SDS/PAGE with *Escherichia coli*-expressed re-

combinant $G_s\alpha$ (long form) and to be the major isoform of $G_s\alpha$ expressed by NG108-15 cells [26].

Addition of the β -adrenoceptor antagonist propranolol (10 μ M) to membranes of clone β N22 cells reduced basal adenylate cyclase activity substantially, although not to the levels of wild-type NG108-15 cell membranes (Table 3). This effect represented a 42 $\pm$ 3 % reduction of the observed basal adenylate cyclase activity when compared with the basal activity recorded in parental NG108-15 cells. By contrast, this agent was without effect on the basal adenylate cyclase activity of membranes from wild-type NG108-15 cells (Table 3) or from clone β N17 (results not shown).

As NG108-15 and other neuroblastoma-derived cell lines have the capacity to synthesize and secrete neurotransmitters including adrenaline and noradrenaline [27] we wished to assess that the elevated basal adenylate cyclase activity noted in membranes of clone β N22 was not a reflection that catecholamines present in the growth medium of the cells was carried through in the membrane preparation and into the adenylate cyclase assays. The levels of both adrenaline and noradrenaline in the medium of each of β N22 and β N17 cells at the time of cell harvest was, however, below the levels of detection using h.p.l.c. with electrochemical detection, while adrenaline (125 pM) and noradrenaline (312 pM) at the lowest concentrations assayed were easily detected in parallel experiments (results not shown).

DISCUSSION

Neuroblastoma $\times$ glioma hybrid, NG108-15, cells [27] have been widely used to examine G-protein-mediated signal-transduction processes as they express a wide range of G-proteins and the receptor and effector species which interact with them. In this study we have used clones of NG108-15 cells, which express either relatively high (clone β N22) or more modest levels (clone β N17) of this receptor, isolated following transfection of the parental cells with a plasmid containing the human β 2 adrenoceptor [15]. These clones have allowed us to explore the concept that 'empty' receptors, i.e. in the absence of agonist, may be able to activate their cellular signalling cascade if they are present in high levels [5]. NG108-15 cells also express endogenously other receptors able to cause stimulation of adenylate cyclase. Of these the most fully studied has been an IP prostanoid receptor at which iloprost acts as a full agonist (see [15,26] for example). Isoprenaline was able to stimulate adenylate cyclase activity in membranes isolated from both clone β N22 and β N17 but not wild-type NG108-15 cells, while iloprost stimulated adenylate cyclase activity in all three cell lines. Moreover, maximal isoprenaline and iloprost stimulation of adenylate cyclase in the two clones was similar (Table 1). The high level of expression of the β 2 adrenoceptor in clone β N22 versus clone β N17 was noted to result in differences in the potency of isoprenaline for stimulation of adenylate cyclase in membranes from these cells. The measured EC_{50} for this effect in membranes of clone β N22 (6 nM) was some 10–15-fold to the left of the dose–effect curve in membranes of β N17 cells (Figure 1) whereas the measured EC_{50} values for iloprost stimulation of adenylate cyclase was the same in the two clones and in wild-type NG108-15 cells (Figure 1). This is not surprising given the fact noted above that maximally effective concentrations of iloprost and isoprenaline were able to stimulate adenylate cyclase activity to similar levels. We have noted previously that levels of the IP prostanoid receptor expressed in β N17 and β N22 cells are similar, whereas the level of expression of the β 2 adrenoceptor in the two clones differs by some 13-fold [15]. It would thus be anticipated that occupation of only a fraction of the expressed β 2 adrenoceptors in membranes of

clone β N22 would be required to activate the same total amount of $G_s\alpha$ and hence adenylate cyclase as achieved by the full β 2-adrenoceptor population in clone β N17 cell membranes. There will thus appear to be 'spare' β 2 adrenoceptors for adenylate cyclase stimulation in clone β N22 in comparison with clone β N17. Moreover, receptors may not have to activate the entire pool of theoretically available G-protein to produce a maximal response. We have recently calculated there to be some 70-fold molar excess of $G_s\alpha$ over the maximal number of $G_s\alpha$ -adenylate cyclase complexes which can be formed in membranes of NG108-15 cells [28]. Although initially unanticipated, we noted that the basal adenylate cyclase activity in membranes of clone β N22 was substantially (some 3-fold) higher than those in either the parental NG108-15 cells or in clone β N17 (Table 1). High basal adenylate cyclase activity was also present in membranes of a second clone, β N34, which expresses high levels (some 6 pmol/mg of membrane protein) of the β 2 adrenoceptor (results not shown). These observations clearly suggest that empty β 2 adrenoceptor stimulation of G_s may be responsible for this high basal activity. The 'basal' activity of the effector enzyme of a signalling cascade represents the activity noted without the addition of receptor agonist. However, this is not necessarily the same as the activity independent of receptor regulation. Empty-receptor regulation of G-proteins has been recorded (see [5] for a review of this area). Evidence in favour of such a model includes the ability of agents possessing negative intrinsic activity to reduce basal high-affinity GTPase activity [3,4]. We were unable to ascertain whether a β -receptor antagonist would be able to reduce either basal or agonist stimulation of GTPase activity in membranes of β N22 cells because of the well-established technical difficulties in recording receptor-mediated stimulation of G_s GTPase activity within the background of the basal activity of other high-affinity GTPases (see [29] for review). However, addition of the β -adrenoceptor antagonist propranolol resulted in a substantial reduction in basal adenylate cyclase activity in membranes of β N22 cells (Table 3) without altering the basal adenylate cyclase activity in membranes from wild-type NG108-15 cells or from clone β N17. This is not the only report to note that propranolol can hinder empty (i.e. agonist unoccupied) β 2 adrenoceptor activation of G_s and thus potentially adenylate cyclase activation. Freissmuth et al. [6] noted that propranolol would inhibit [35 S]GTP[S] binding to $G_s\alpha$ produced simply by reconstitution of recombinantly expressed β 2 adrenoceptor, $G_s\alpha$ and G-protein $\beta\gamma$ subunit complex. Furthermore in a more physiological system the β -adrenoceptor antagonist bisoprolol has been reported to decrease basal contractile force in electrically driven atrial muscle strips [30]. While there must be a concern in some systems that a noted reduction of basal activity by an antagonist represents simply a competition between the antagonist and endogenously produced agonist for the receptor this is clearly not the case either in reconstitution systems [6] or, as in this report, in highly washed membranes derived from tissue-culture cells. Indeed in this report we demonstrate that the endogenous levels of catecholamines in the cell culture medium at the time of cell harvest was below the levels of detection when using h.p.l.c. with electrochemical detection. Furthermore, antagonist inhibition of basal or empty receptor G-protein activation has also been recorded for other G-protein-linked receptors such as the muscarinic receptor in pig atrial membranes [31].

Addition of agonist stabilizes or promotes the probability of a receptor adopting an active conformation which can then stimulate a signalling cascade. An antagonist, by contrast, must limit or deny the receptor such a conformation. However, this conformation must thermodynamically be attainable in the absence of receptor occupancy. As such, in cells expressing high levels of

a receptor it may be anticipated that the number of receptors stochastically anticipated to be in the 'agonist' conformation at any instant may be sufficient to stimulate the signal pathway significantly even in the absence of agonist. We believe that we have demonstrated this to be the case for the adenylate cyclase cascade in β N22 cells.

Clearly a simple explanation for the elevated basal adenylate cyclase activity in β N22 membranes might relate to an enhanced expression of the adenylate cyclase catalytic moiety in the cells associated either with the transfection process or simply within the clones isolated. The basal adenylate cyclase activities in membranes of wild-type NG108-15 cells and from each of clones β N22, β N34 and β N17 were not different, however, when the assays were performed in the presence of a high concentration of Mn^{2+} (Table 2) rather than in the presence of Mg^{2+} . This condition is believed to provide the best indication of the basal activity of the catalytic moiety of adenylate cyclase without regulation of this activity by G-proteins [25]. Such results further indicate that the enhanced basal activity of adenylate cyclase in β N22 membranes results from G-protein stimulation of the effector rather than elevated levels of the adenylate cyclase catalytic moiety resulting artefactually from the transfection process. The other potential level at which the observed high basal adenylate cyclase activity could be explained, other than being due to the high levels of expression of the receptor, was that $G_s\alpha$ was either substantially upregulated in these cells or had become persistently activated. An example of a system in which agonist-independent, constitutive activation of adenylate cyclase has been observed are cells and tumours harbouring a *gsp* oncogene mutation of $G_s\alpha$ [11,12]. To exclude the possibility that clone β N22 contained significantly greater levels of $G_s\alpha$ than either the parental cells or clone β N17, membranes of these lines were immunoblotted to detect the presence of $G_s\alpha$. In these cells $G_s\alpha$ is represented primarily by a 45 kDa polypeptide which co-migrates in SDS/PAGE with the long form of $G_s\alpha$ expressed recombinantly in *E. coli* [24]. No differences in amounts of this polypeptide were noted between these membranes (Figure 2). To assess whether $G_s\alpha$ in clone β N22 had become activated by some mechanism we used sodium cholate extracts of membranes from β N22 and wild-type NG108-15 cells to reconstitute adenylate cyclase activity to S49 lymphoma *cyc⁻* membranes. The reconstitution was equally effective with G_s from each source. In both the wild-type and β N22 samples it was necessary to add fluoroaluminate (as NaF) to the assay to detect the stimulation of adenylate cyclase, indicating that $G_s\alpha$ from clone β N22 is not constitutively active.

The studies reported in this paper demonstrate that high level expression of the β 2 adrenoceptor in NG108-15 cells results in an elevation of the 'basal' activity of adenylate cyclase. Based on the unaltered level and activity of $G_s\alpha$, on unaltered G-protein-independent activity of adenylate cyclase and on the ability of a β -adrenoceptor antagonist to partially reverse the elevated 'basal' adenylate cyclase activity we conclude that this is due to empty receptor stimulation of the adenylate cyclase cascade.

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