Analysis of inverse agonism at the δ opioid receptor after expression in Rat 1 fibroblasts

Ian MULLANEY, I. Craig CARR and Graeme MILLIGAN*

Molecular Pharmacology Group, Division of Biochemistry and Molecular Biology, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ, Scotland, U.K.

A cDNA encoding the mouse δ opioid receptor was expressed stably in a Rat 1 fibroblast cell line. Expression of this receptor was demonstrated both in ligand binding studies and by reverse transcriptase–PCR. In membranes of clone D2 cells the opioid peptide [D-Ala^2]-leucine enkephalin (DADLE) produced a robust, concentration-dependent, stimulation of basal high-affinity GTPase activity; the prototypic opioid antagonist naloxone and the highly selective and potent δ opioid ligands H-Tyr-Tic-Phe-Phe-OH (TIPP) and H-Tyr-Tic[CH_2-NH]Phe-Phe-OH (TIPP[ψ]) had little effect but *N*,*N*-diallyl-Tyr-Aib-Aib-Phe-Leu (ICI174864) caused a marked dose-dependent inhibition of this activity (Tic, 1,2,3,4-tetrahydroisoquinolin-2-yl-carbonyl; Aib, α-aminobutyric acid). This effect of ICI174864 was reversed by TIPP[ψ] and attenuated after treatment of the cells with pertussis toxin. No stimulation by DADLE or inhibition by ICI174864 was observed in Rat 1 fibroblasts that did not express the δ opioid receptor. Basal binding of [^{35}S]guanosine 5′-*O*-(3-thiotriphosphate) to membranes of clone D2 cells was also stimulated by DADLE and inhibited by ICI174864; both of these effects were reversed by co-incubation with TIPP[ψ]. When cholera toxin-catalysed [^{32}P]ADP-ribosylation was performed on membranes of clone D2 cells in the absence of guanine nucleotides, a 40 kDa G_i-family polypeptide was labelled in addition to both the long and short isoforms of $G_s\alpha$. Labelling of the 40 kDa polypeptide was enhanced by addition of DADLE and fully attenuated by addition of ICI174864. In contrast, labelling of the isoforms of $G_s\alpha$ was unaffected by either opioid ligand. Again, both the positive effect of DADLE and the inhibitory effect of ICI174864 were prevented by co-incubation with TIPP[ψ] which, in isolation, had little effect on cholera toxin-catalysed [^{32}P]ADP-ribosylation of either G_s or G_i. These data demonstrate that the δ opioid receptor displays a spontaneous activity when expressed in this genetic background. Attenuation of this activity is produced by ICI174864, which by acting as an 'inverse agonist' in this system, functionally uncouples the expressed receptor from the cellular G-protein population. The complete attenuation of agonist-independent cholera toxin-catalysed [^{32}P]ADP-ribosylation of G_i demonstrates that ICI174864 acts as an inverse agonist with high intrinsic activity at this receptor.

INTRODUCTION

The basal activity of a signal transduction cascade in the absence of a receptor agonist is likely to represent 'empty receptor' activation of the cascade. Receptor agonists regulate signal generation by stabilizing the active conformation of the receptor and thus altering the equilibrium between inactive and active receptor [1–3]. Antagonists bind but do not alter this equilibrium, whereas ligands that preferentially stabilize the inactive conformation of the receptor would be expected to regulate the basal activity in the opposite direction to an agonist. Such agents are described as inverse agonists [4–7].

Considerable interest has been accorded to observations that in membranes of neuroblastoma × glioma hybrid, NG108-15, cells that express a δ opioid receptor, the high-affinity GTPase activity is reduced below basal levels by the opioid ligand *N*,*N*-diallyl-Tyr-Aib-Aib-Phe-Leu (ICI174864) [4,8] (Aib, α-aminobutyric acid). Recently, cDNA species encoding each of the δ, μ and κ opioid receptors have been isolated [9–14]. Expression of these species in heterologous systems would allow a detailed examination of the signalling characteristics of these receptors and the potential ability of ligands to act as inverse agonists at these receptors. In the present study we examine the mouse δ opioid receptor after stable expression in Rat 1 fibroblasts. Using a range of assay end points we demonstrate that this receptor displays a spontaneous ability to activate pertussis toxin-sensitive G-proteins in the absence of opioid ligands and record that this spontaneous activity can be inhibited fully by ICI174864, which acts as an inverse agonist with high intrinsic activity.

MATERIALS AND METHODS

Materials

[^{35}S]Guanosine 5′-*O*-(3-thiotriphosphate) (GTP[S]) (1026 Ci/mmol) and [^{32}P]NAD^+ (800 Ci/mmol) were obtained from DuPont/New England Nuclear. [γ-^{32}P]GTP (more than 10 Ci/mmol) and [^{3}H]diprenorphine (50 Ci/mmol) were obtained from Amersham International. Cholera toxin was from Sigma and pertussis toxin was from Porton Products (Porton Down, Wiltshire, U.K.). All reagents for tissue culture were from Life Technologies (Paisley, Strathclyde, U.K.). Hygromycin B was from Boehringer Mannheim (Mannheim, Germany). Opioid

Abbreviations: Aib, α-aminobutyric acid; Tic, 1,2,3,4-tetrahydroisoquinolin-2-yl-carbonyl; DADLE, [D-Ala^2]-leucine enkephalin; DMEM, Dulbecco's modified Eagle's medium; ICI174864, *N*,*N*-diallyl-Tyr-Aib-Aib-Phe-Leu; DAMGO, H_2N-Tyr-D-Ala-Gly-*N*-Me-Phe-Gly-ol; DSLET, [D-Ser^2]-leucine enkephalin-Thr; U-50488, *trans*-(±)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]-benzene acetamide; TIPP, H-Tyr-Tic-Phe-Phe-OH; TIPP[ψ], H-Tyr-Tic[CH_2-NH]Phe-Phe-OH; RT–PCR, reverse transcriptase–PCR; GTP[S], guanosine 5′-*O*-(3-thiotriphosphate).

* To whom correspondence should be addressed.

ligands were from Research Biochemicals Incorporated, Tocris Cookson or Sigma except H-Tyr-Tic-Phe-Phe-OH (TIPP) and H-Tyr-Tic[CH_2-NH]Phe-Phe-OH (TIPP[ψ]) [15], which were gifts of Dr. Peter Schiller, Clinical Research Institute of Montreal, Montreal, Canada (Tic, 1,2,3,4-tetrahydroisoquinolin-2-yl-carbonyl). The cDNA encoding the mouse δ opioid receptor was a gift from Dr. Graeme Bell, Howard Hughes Medical Institute, University of Chicago, Chicago, Illinois, U.S.A.

Generation and isolation of clones of Rat-1 fibroblasts expressing the mouse δ opioid receptor

A cDNA encoding the mouse δ opioid receptor [14] was ligated into plasmid pCMV-ms12. Rat 1 fibroblasts were co-transfected with this cDNA (5 μg) and with the plasmid pBABE hygro (0.5 μg), which is able to direct expression of the hygromycin B resistance marker, using Lipofectin reagent (Life Technologies) in accordance with the manufacturer's instructions. Clones that demonstrated resistance to hygromycin B (200 μg/ml) were selected and expanded. Expression of the δ opioid receptor polypeptide in membranes from these clones was assessed initially by the specific binding of the opioid receptor ligand [^{3}H]diprenorphine.

Cell culture

Cells of clone D2 were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (w/v) donor calf serum, penicillin (100 i.u./ml) and streptomycin (100 μg/ml) in air/CO_2 (19:1) at 37 °C. Cells were grown in 75 cm^2 tissue culture flasks and were harvested just before confluency. In a number of experiments, cells were treated with pertussis toxin (25 ng/ml) for 16 h before cell harvesting. Membranes were prepared from the cells by homogenization with a Teflon/glass homogenizer and differential centrifugation as described for a variety of other cells [16].

[^{3}H]Diprenorphine-binding experiments

In experiments designed to define ligand specificity, membranes (10 μg) were incubated at 30 °C for 45 min in 20 mM Tris/HCl, pH 7.5, 50 mM sucrose, 20 mM $MgCl_2$ (Buffer A) containing [^{3}H]diprenorphine in the absence or presence of 10 μM [D-Ala^2]-leucine enkephalin (DADLE) to define maximal and non-specific binding of the δ opioid receptor respectively. Specific binding, defined as above, represented more than 80% of the total binding of radioligand. The binding of radioligand was competed for by addition of various concentrations of each of DADLE, [D-Ser^2]-leucine enkephalin-Thr (DSLET), H_2N-Tyr-D-Ala-Gly-*N*-Me-Phe-Gly-ol (DAMGO) or *trans*-(±)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]-benzene acetamide (U-50488). Experiments designed to assess the maximal binding capacity of membranes for [^{3}H]diprenorphine were performed routinely with concentrations of [^{3}H]diprenorphine between 0.1 and 15 nM in Buffer A. All binding experiments were terminated by rapid filtration through Whatman GF/C filters followed by three washes (5 ml each) with ice-cold buffer A. Filters were maintained overnight in 10 ml Ultima-Flo AF scintillant before liquid-scintillation counting. In a number of experiments the ability of naloxone, ICI174864 and TIPP[ψ] to compete for the specific binding of [^{3}H]diprenorphine was also assessed to provide estimates of the relative affinity of these ligands for the δ opioid receptor binding site.

Reverse transcriptase–PCR (RT–PCR)

RNA extractions

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

Reverse transcription

Samples (5 μg; 8 μl) of RNA were denatured by incubation at 65 °C for 10 min followed by chilling on ice and reverse transcribed in 33 μl of reaction mixture with a first-strand cDNA synthesis kit (Pharmacia LKB Biotechnology) as detailed by the manufacturer. Incubation was performed at 37 °C for 1 h and the reaction was stopped by heating samples at 95 °C for 5 min and then chilling on ice.

PCR

PCR reactions on the reverse-transcribed samples or on 200 ng of appropriate cDNA species were performed with the following primers: mouse δ sense, 5′-GCTGTGCAAGGCTGTGCTCT-3′; mouse δ antisense, 5′-CCAGACGATGACGAAGATGTG-3′. Amplifications were performed in 100 μl of buffer containing 20–40 pmol of primers, 2.5 units of Taq polymerase (Promega) in a HYBAID Omnigene temperature cycler. Cycles were as follows: 95 °C for 5 min, 55 °C for 1 min, 72 °C for 1 min (1 cycle); 95 °C for 30 s, 55 °C for 1 min, 72 °C for 1 min (30 cycles); 95 °C for 30 s, 55 °C for 1 min, 72 °C for 5 min (1 cycle). Reaction products were separated by 1.5–1.75% agarose gel electrophoresis..

High-affinity GTPase assays

These were performed essentially as described [18,19], in the presence or absence of the opioid ligands described in the text.

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

[^{32}P]ADP-ribosylation of membranes of cells of clone D2 was performed in the absence of added guanine nucleotides basically as described [20,21], except that sodium phosphate, pH 7.0, replaced potassium phosphate, pH 7.0. Further additions to the assays were as detailed in the text. In the relevant ADP-ribosylation assays, pertussis toxin was present at 10 μg/ml and cholera toxin at 50 μg/ml. Dried gels were exposed to a phosphor storage plate for 24 h and then analysed with a FUJIX BAS1000 image analyser.

[^{35}S]GTP[S] binding assays

These were performed according to the principles outlined in [22]. Briefly, membranes were incubated at either 4 °C for 1 h or 30 °C for 30 min in a final assay volume of 100 μl. For the experiments performed at 30 °C the reaction mixture consisted of 20 mM Hepes, pH 7.4, containing 3 mM $MgCl_2$, 100 mM NaCl, 10 μM GDP and 0.2 mM ascorbate [7]. For the experiments performed at 4 °C the reaction mixture comprised 50 mM triethanolamine hydrochloride, pH 7.4, containing 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol (DTT), 150 mM NaCl and

100 μM GDP. All assays also contained 0.3–0.5 nM [^{35}S]GTP[S] (50 nCi) and the presence or absence of opioid ligands as described in the text. The experiments performed at 4 °C were terminated by the addition of 2.5 ml of ice-cold washing buffer B (50 mM Tris/HCl, pH 7.5, containing 5 mM $MgCl_2$); the experiments conducted at 30 °C were terminated by the addition of 2.5 ml ice-cold buffer C (20 mM Hepes, pH 7.4, containing 3 mM $MgCl_2$). All assays were then rapidly filtered through Whatman GF/C filters followed by three washes (5 ml each) with their respective washing buffers. Filters were maintained overnight in 10 ml Ultima-Flo AF scintillant before liquid-scintillation counting.

RESULTS

Rat 1 fibroblast cells, which do not endogenously express an opioid receptor, were co-transfected in a 1:10 ratio with the plasmid pBABE hygro, which allows expression of resistance to hygromycin B, and plasmid pCMV5 into which a cDNA encoding the mouse δ opioid receptor had been ligated [14]. Colonies displaying resistance to hygromycin B (200 μg/ml) were selected, expanded and examined for both mRNA corresponding to the δ opioid receptor by RT–PCR (Figure 1a) and expression of a high-affinity [^{3}H]diprenorphine binding site (Figure 1b) displaying high affinity for the δ opioid receptor selective agonist DSLET but markedly lower affinities for the μ opioid receptor agonist DAMGO and the κ opioid receptor agonist U-50488 (Figure 1c). Clone D2 displayed each of these features with a B_{max} for specific [^{3}H]diprenorphine binding to an apparent single site of 6.1 ± 1.2 pmol/mg of membrane protein and a K_d for this ligand of 7.3 ± 1.5 nM (means ± S.E.M.; $n = 3$ in each case).

Membranes derived from clone D2 displayed a basal high-affinity GTPase activity of 31.2 ± 7.7 pmol/min per mg of membrane protein when measured in the presence of 100 mM Na^+ and this was stimulated markedly by the synthetic opioid peptide DADLE, little affected by the prototypic opioid antagonist naloxone or the potent and highly δ opioid receptor selective

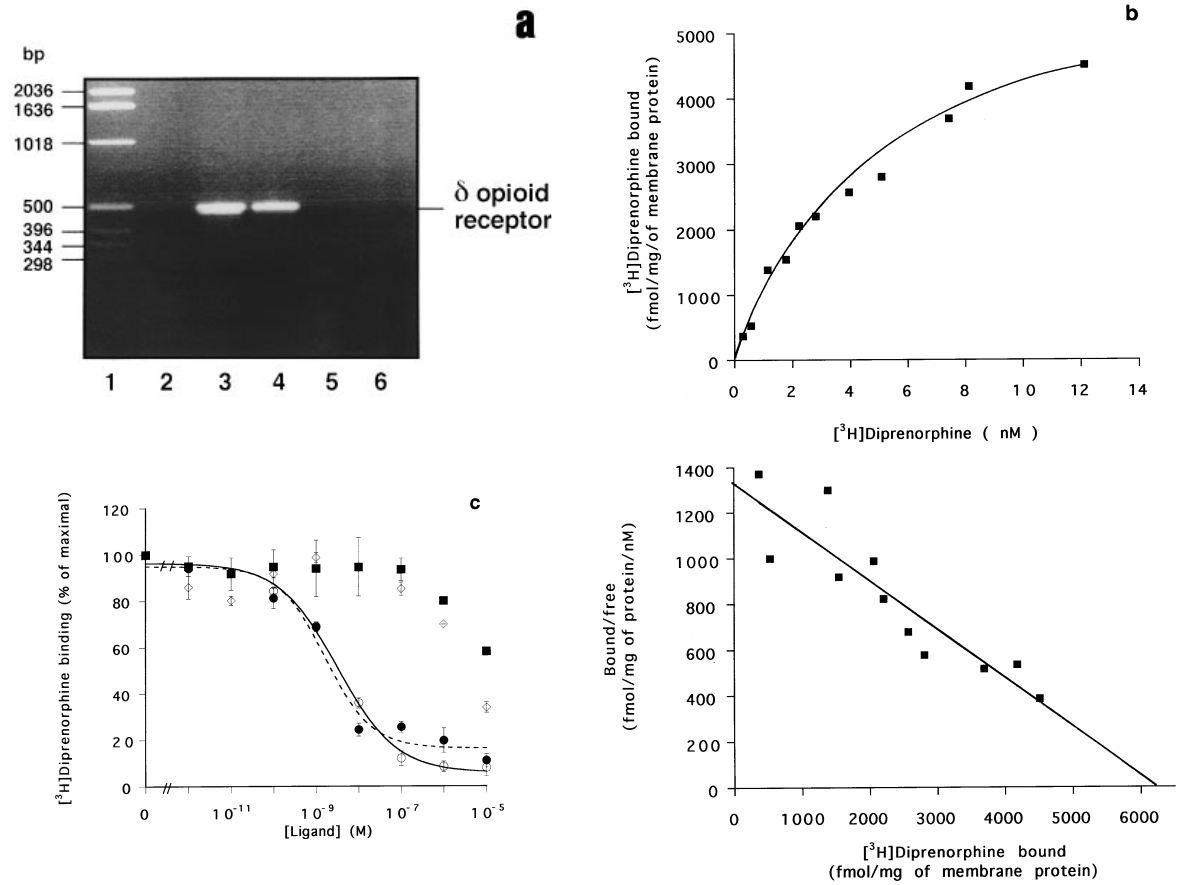

Figure 1 Expression of the δ opioid receptor in Rat-1 fibroblast clone D2

(**a**) RNA (5 μg) prepared from untransfected Rat-1 fibroblasts (lane 2) or clone D2 (lane 3) was reverse-transcribed as described in the Materials and Methods section and these products, along with cDNAs (200 ng) encoding δ (lane 4), κ (lane 5) and μ (lane 6) opioid receptors, were subjected to PCR (30 cycles) as described in the Materials and Methods section. PCR-amplified product (5 μl) was electrophoresed through a 1.75% (w/v) agarose gel containing 1 μg/ml ethidium bromide and viewed on a 300 nm UV transilluminator. The size of the product (495 bp) was exactly that expected for the δ opioid receptor with the selected primers. Molecular size markers are shown in lane 1. (**b**) The specific binding of various concentrations of [^{3}H]diprenorphine was measured in membranes of clone D2 (upper panel). These data were transformed to generate a Scatchard plot (lower panel). In the example displayed B_{max} was estimated to be 6250 fmol/mg of membrane protein and the K_d for [^{3}H]diprenorphine was 4.7 nM. (**c**) Membranes of clone D2 were incubated with 3.1 nM [^{3}H]diprenorphine and the radioligand was competed for by various concentrations of ligands known to be selective for individual opioid receptors. DSLET (●) is highly selective for δ opioid receptor, DAMGO (◇) is selective for μ opioid receptor, and U-50488 (■) is selective for κ opioid receptor. DADLE (○) has similar affinities at the δ and μ opioid receptors. Results are from a single representative experiment and are expressed as percentage of maximal binding. Two further experiments generated similar data.

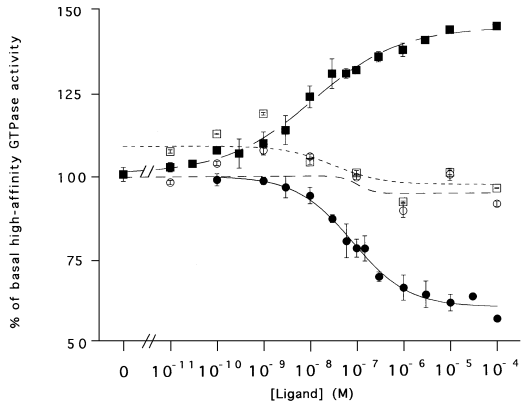

Figure 2 DADLE and ICI174864 affect GTPase activity in a concentration-dependent manner

Membranes (5 μg) from clone D2 were incubated with various concentrations of DADLE (■), TIPP[ψ] (○), naloxone (□) or ICI174864 (●) and high-affinity GTPase activity was assessed as described in the Materials and Methods section. Results are expressed as means ± S.E.M. for three separate preparations. The EC_{50} for DADLE was 11.5 nM and for ICI174864 81 nM.

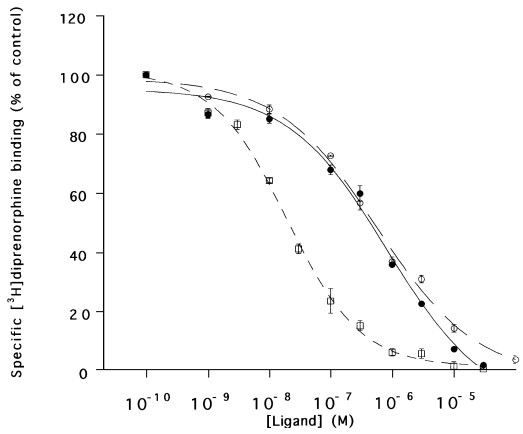

Figure 3 TIPP[ψ] displays higher affinity for the δ opioid receptor than does either naloxone or ICI174864

The specific binding of [^{3}H]diprenorphine (3 nM), measured as in the Materials and Methods section, was competed for by varying concentrations of TIPP[ψ] (□), ICI174864 (●) or naloxone (○). Results are the means ± S.E.M. for three independent experiments.

Table 1 High-affinity GTPase activity in membranes of clone D2 cells: regulation by opioid ligands

High-affinity GTPase activities were assessed as in the Materials and Methods section in membranes (5 μg) prepared from clone D2 cells in the absence or presence of various opioid ligands. The results are presented as means ± S.E.M. derived from three separate experiments. The basal activity in these experiments ranged between 19.9 and 32.4 pmol/min per mg of membrane protein.

Ligand	High-affinity GTPase activity (% of basal value)
DADLE (1 μM)	192.2 ± 3.8
TIPP[ψ] (10 μM)	99.2 ± 0.2
ICI174864 (10 μM)	70.3 ± 1.3
DADLE (1 μM) + TIPP[ψ] (10 μM)	107.8 ± 2.2
ICI174864 (10 μM) + TIPP[ψ] (10 μM)	91.6 ± 6.3

Table 2 The effects of both DADLE and ICI174864 require the presence of the δ opioid receptor

High-affinity GTPase activity was measured in membranes of parental Rat 1 fibroblasts. Results are presented as means ± S.E.M. for three separate experiments. Basal high-affinity GTPase activity in these experiments varied between 10.8 and 16.0 pmol/min per mg of protein.

Ligand	High-affinity GTPase activity (% of basal value)
DADLE (10 μM)	93.3 ± 6.7
ICI174864 (10 μM)	105.9 ± 3.4

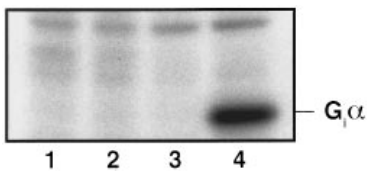

Figure 4 Pretreatment of clone D2 cells with pertussis toxin causes full ADP-ribosylation of the G_i-like G-proteins

Membranes (25 μg) from pertussis toxin-pretreated D2 cells (lanes 1 and 2) and untreated cells (lanes 3 and 4) were incubated with [^{32}P]NAD^+ in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of thiol-activated pertussis toxin (10 μg/ml, 2 h) as described in the Materials and Methods section. The membranes were resolved on SDS/PAGE [10% (w/v) acrylamide] and subjected to autoradiography. Prior treatment of the cells with pertussis toxin prevented subsequent [^{32}P]ADP-ribosylation of G_i *in vitro*. The experiment displayed is representative of four performed.

antagonists TIPP and TIPP[ψ], but inhibited by the δ opioid receptor ligand ICI174864 (Figure 2, Table 1 and results not shown). The stimulatory effect of DADLE (Figure 2) (EC_{50} 11.5 nM) and the inhibitory effect of ICI174864 (Figure 2) (IC_{50} 81 nM) were dose-dependent and the actions of both DADLE and of ICI174864 were prevented by co-incubation with naloxone, TIPP (results not shown) or TIPP[ψ] (Table 1). TIPP[ψ] was used as the ligand of choice for these competition studies as it was shown to possess more than 25-fold higher affinity than either naloxone or ICI174864 to compete with [^{3}H]diprenorphine for the δ opioid receptor binding site in membranes of clone D2 (Figure 3).

No ability of DADLE to stimulate high-affinity GTPase activity or of ICI174864 to inhibit this activity could be measured in membranes of untransfected parental Rat 1 fibroblasts (Table 2). The same was true for membranes prepared from a clone of Rat 1 fibroblasts that had been transfected to express the α_{2A}-adrenoceptor (results not shown), although the α_2-adrenoceptor agonist UK14304 stimulated high-affinity GTPase activity in the α_{2A}-adrenoceptor expressing cells (results not shown but see [21] for example). These results demonstrate clearly the requirement for the presence of the δ opioid receptor for observation of the effects of both DADLE and ICI174864.

Pretreatment of clone D2 cells with pertussis toxin (25 ng/ml, 16 h) before cell harvesting and membrane preparation prevented the ability of fresh thiol-activated pertussis toxin to catalyse [^{32}P]ADP-ribosylation of a $G_i\alpha$-like polypeptide in these membranes (Figure 4). This pretreatment resulted in a reduction in basal high-affinity GTPase activity to a level close to that

Table 3 The effect of pretreatment with pertussis toxin on basal and ligand-regulated high-affinity GTPase activity

High-affinity GTPase activity was measured in membranes of untreated and pertussis toxin-pretreated (25 ng/ml, 16 h) clone D2 cells. Results are presented as means ± S.E.M. for four separate experiments. Basal high-affinity GTPase activity is routinely observed to be lower in membranes of pertussis toxin-treated cells compared with untreated controls. Basal high-affinity GTPase activity in these experiments varied between 10.8 and 32.4 pmol/min per mg of protein in the control D2 membranes and between 6.4 and 13.6 pmol/min per mg of protein in the membranes derived from the pertussis toxin-treated cells. In each individual preparation, treatment with ICI174864 was able to decrease the basal high-affinity GTPase activity of control clone D2 membranes to a level close to that observed after pertussis toxin treatment.

	High-affinity GTPase activity (% of basal value)	
Ligand	Control	Pertussis toxin-pretreated
DADLE (10 μM)	185.4 ± 8.4	109.0 ± 17.7
ICI174864 (10 μM)	71.9 ± 1.9	97.8 ± 7.4

produced in untreated cells by the addition of maximally effective concentrations of ICI174864 (Table 3). Pertussis toxin (25 ng/ml, 16 h) pretreatment resulted in a large reduction of the stimulation of high-affinity GTPase by DADLE and in attenuation of its inhibition by ICI174864 (Table 3), indicating that these ligand effects were produced at the level of G_i-like G-proteins.

Thiol-activated cholera toxin was able to catalyse [^{32}P]ADP-ribosylation of both long (45 kDa) and short (42 kDa) isoforms of $G_s\alpha$ in membranes of clone D2 cells. When the assay was performed in the absence of guanine nucleotides, labelling of a 40 kDa polypeptide ($G_i\alpha$) was also observed (Figure 5a). Incorporation of [^{32}P]ADP-ribose into this 40 kDa polypeptide was markedly enhanced by inclusion of DADLE (10 μM) in the assay whereas the [^{32}P]ADP-ribosylation of the isoforms of $G_s\alpha$ was unaffected by the presence of this ligand (Figure 5a). Inclusion of ICI174864 (10 μM) into such assays nearly abolished the ligand-independent incorporation of [^{32}P]ADP-ribose into the 40 kDa polypeptide without affecting the labelling of the $G_s\alpha$ isoforms (Figure 5a). Both the basal labelling of the 40 kDa polypeptide and the ability of DADLE to stimulate incorporation of [^{32}P]ADP-ribose into the G_i polypeptide were prevented by pretreatment of the cells with pertussis toxin (Figure 5a).

As with ligand regulation of high-affinity GTPase activity, the effects of both DADLE (Figure 5b) (EC_{50} 8.8 ± 2.7 nM) and ICI174684 (Figure 5c) (IC_{50} 7.2 ± 1.3 nM) (means ± S.E.M.; $n = 7$ in each case) on [^{32}P]ADP-ribosylation of the 40 kDa G_i polypeptide were dose-dependent. TIPP[ψ] (10 μM) did not significantly alter the basal incorporation of [^{32}P]ADP-ribose into the 40 kDa G_i polypeptide but prevented both the stimulation produced by DADLE and the inhibition produced by ICI174864 (Figure 5d).

The binding of [^{35}S]GTP[S] to membranes of clone D2 cells in the presence of 150 mM Na^+ was unaffected by the presence of DADLE unless GDP was also included in the assay (results not shown). In the presence of 10 μM GDP the observed binding of GTP[S] was substantially lower than in the absence of GDP but now it was stimulated appreciably by the addition of DADLE (Table 4). DADLE stimulation of [^{35}S]GTP[S] binding was observed when the assay was performed at either 4 or 30 °C. ICI174864 was able to mediate inhibition of basal [^{35}S]GTP[S] binding (Table 4) in a concentration-dependent manner (Figure 6) (IC_{50} 26 ± 3.8 nM; mean ± S.E.M.; $n = 4$) and this was most noticeable when the assay was performed at 4 °C in the presence of 10 μM GDP (Figure 6). Inclusion of TIPP[ψ] (10 μM) had no significant effect on the basal levels of binding of [^{35}S]GTP[S] but again was able to reverse the effects of both DADLE and ICI174864 (Table 4).

DISCUSSION

Spontaneous or constitutive activity of G-protein-linked receptors in the absence of an agonist ligand has been observed both after designed mutagenesis [1,23] and in disease states that result from mutation of the receptor [24,25]. However, on thermodynamic grounds it must be expected that native G-protein-linked receptors will also display a level of spontaneous activity determined by the equilibrium position between the inactive state of the receptor (R) and its active conformation (R*) [1–3]. Agonist ligands stabilize R* preferentially and hence the position of the equilibrium R—R* is shifted to the right in their presence. Drugs (called inverse agonists) able to stabilize R preferentially would thus be expected to move the equilibrium to the left and hence reduce the observed spontaneous activity of the receptor.

The basal activity of an effector cascade in a cell or cell membrane will thus reflect, at least in part, the sum of the spontaneous activities of the various receptors present that function to both activate and inactivate the signal pathway. Clearly, a high level of expression of a receptor would be expected to result in a higher degree of basal regulation of a cascade in the absence of a receptor ligand, as in this situation a greater number of copies of the receptor are in the active conformation than with expression of lower levels of the receptor [5]. Furthermore it has recently become obvious that certain receptors display greater spontaneous activity than others. For example, expression of the D_{1B} (D5) dopamine receptor results in higher agonist-independent adenylate cyclase activity than expression, at equivalent levels, of the D_{1A} (D1) dopamine receptor [26].

The phenomenon of inverse agonism of ligands at G-protein-linked receptors has now been observed for a variety of receptors (see [3] and [27] for reviews) including the ability of the synthetic enkephalin ICI174864 to reduce basal high-affinity GTPase activity in membranes of neuroblastoma × glioma hybrid NG108-15 cells, which express a δ opioid receptor endogenously [4,8]. In this study we have examined this property in a rat fibroblast cell line that we have transfected to express stably the mouse δ opioid receptor, and we extend previous studies by examining this phenomenon in a range of distinct assays that provide information on the interactions between receptors and G-proteins and allow us to conclude that ICI174864 acts as an inverse agonist with high intrinsic activity [3]. Whether measuring receptor regulation of high-affinity GTPase activity [17,18], the binding of [^{35}S]GTP[S] to G-proteins regulated by the receptor [21] or using an assay in which pertussis toxin-sensitive G-proteins become substrates for cholera toxin-catalysed [^{32}P]ADP-ribosylation only when they are activated by a receptor [19,20], we demonstrate that expression of the mouse δ opioid receptor in this cellular environment results in agonist-independent activation of $G_i\alpha$ and that this spontaneous activity can be nearly attenuated by addition of ICI174864. The effects of ICI174864 were observed only in cells expressing the δ opioid receptor (Table 2), confirming that the effects of this ligand are due to interaction with the receptor.

In a number of studies, workers have altered the ionic enviroment of assays substantially from those most usually used to examine agonist activation to be able to see the greatest effects of inverse agonists. For example, opioid inverse agonism in

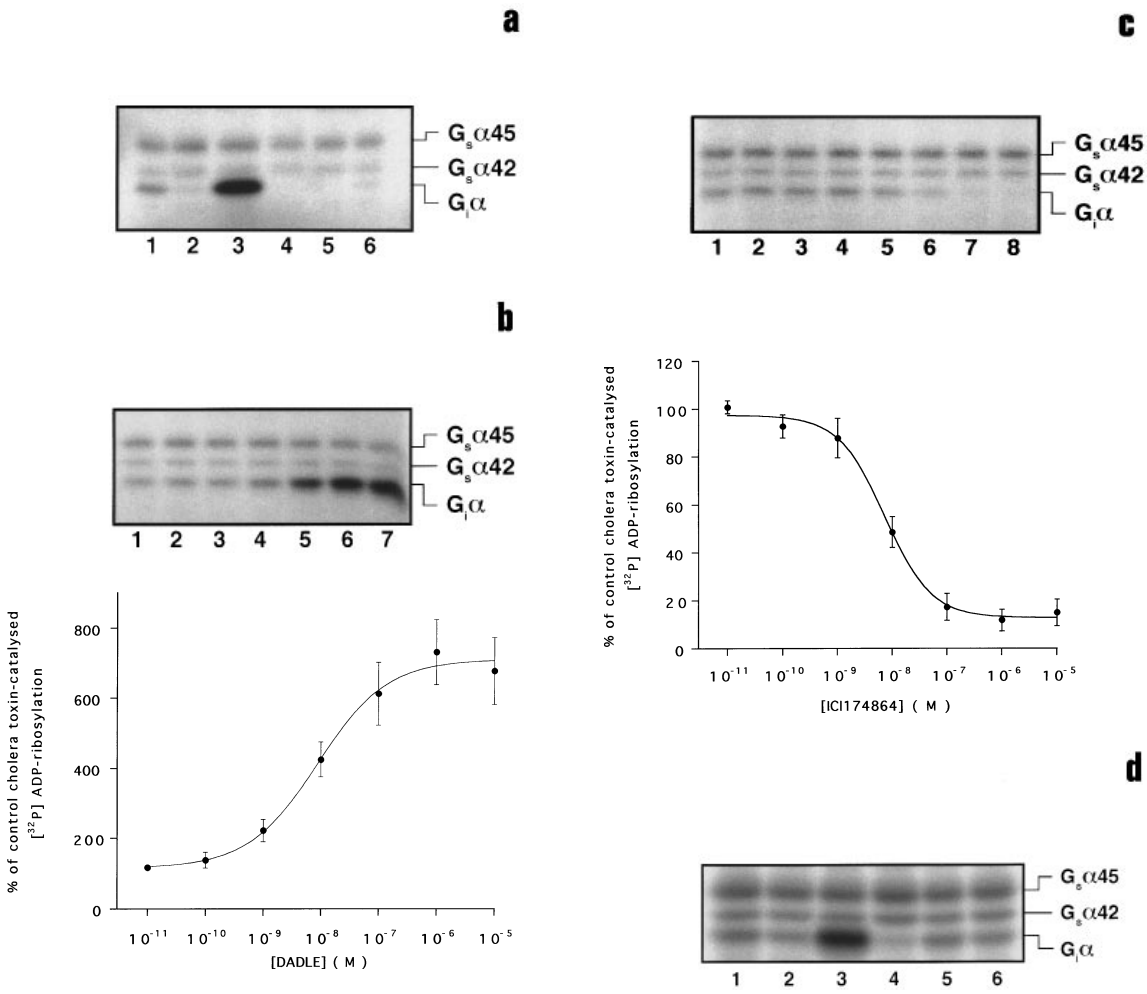

Figure 5 Cholera toxin-catalysed [^{32}P]ADP-ribosylation of G_i in membranes of clone D2 cells: regulation by opioid ligands

(**a**) Membranes (25 μg) from untreated clone D2 cells (lanes 1–3) and pertussis toxin-pretreated (25 ng/ml, 16 h) cells (lanes 4–6) were incubated with [^{32}P]NAD$^+$ and thiol-activated cholera toxin (50 μg/ml, 2 h) in the absence of ligand (lanes 1 and 4) or in the presence of 10 μM ICI174864 (lanes 2 and 5) or 10 μM DADLE (lanes 3 and 6) as described in the Materials and Methods section. The samples were then resolved on SDS/PAGE [10% (w/v) acrylamide] and subjected to autoradiography. (**b**) Membranes (25 μg) prepared from D2 cells were incubated with [^{32}P]NAD$^+$ and thiol-activated cholera toxin (50 μg/ml, 2 h) in the presence of various concentrations of DADLE (lane 1, zero; lane 2, 10 pM; lane 3, 100 pM; lane 4, 1 nM; lane 5, 10 nM; lane 6, 100 nM; lane 7, 1 μM). The samples were then resolved on SDS/PAGE [10% (w/v) acrylamide] and subjected to autoradiography (upper panel). Autoradiographs, derived from six such independent experiments, were scanned densitometrically to analyse the concentration dependence of DADLE-induced stimulation of labelling of $G_i\alpha$ (lower panel). Results are presented as means $\pm$ S.E.M. (**c**) Membranes (25 μg) prepared from clone D2 cells were incubated with [^{32}P]NAD$^+$ and thiol-activated cholera toxin (50 μg/ml, 2 h) in the presence of various concentrations of ICI174864 (lane 1, zero; lane 2, 10 pM, lane 3, 100 pM; lane 4, 1 nM; lane 5, 10 nM; lane 6, 100 nM; lane 7, 1 μM; lane 8, 10 μM). The samples were then resolved on SDS/PAGE [10% (w/v) acrylamide] and subjected to autoradiography (upper panel). Autoradiographs derived from seven independent experiments were scanned densitometrically to analyse the concentration-dependence of ICI174864-induced inhibition of labelling of $G_i\alpha$ (lower panel). Results are presented as means $\pm$ S.E.M. (**d**) Membranes (25 μg) were subjected to cholera toxin-catalysed [^{32}P]ADP-ribosylation as in (**a**) in the absence of ligand (lane 1) or in the presence of TIPP[ψ] (10 μM) (lane 2), DADLE (10 μM) (lane 3), ICI174864 (lane 4), DADLE (10 μM) plus TIPP[ψ] (10 μM) (lane 5) or ICI174864 (10 μM) plus TIPP[ψ] (10 μM) (lane 6). The samples were subsequently treated as in (**a**). Similar results were obtained in two further experiments.

membranes of NG108-15 neuroblastoma $\times$ glioma hybrid cells can be most easily measured in high-affinity GTPase assays that replace the normal levels of Na^+ with K^+ [4]. We have confirmed that even greater effects of ICI174864 can be observed in high-affinity GTPase assays performed on membranes of clone D2 cells if K^+ is substituted for Na^+ (results not shown) but we have resisted the temptation to use this approach here as we wished to demonstrate effects of both positive and inverse agonists under the same assay conditions and because we wished to demonstrate that inverse agonism can be observed in a range of assays in conditions that are widely used by investigators to examine the function of agonist ligands.

Pertussis toxin causes ADP-ribosylation of the cysteine residue located four amino acids from the C-terminus of G-protein α subunits of the G_i family. This agent has routinely been noted to cause a reduction in basal high-affinity GTPase activity of cell membranes and a reasonable explanation of these observations is that it attenuates agonist-independent activation of G_i-family G-proteins by receptors as it is known to prevent receptor activation of these G-proteins. Importantly for these studies

Table 4 The effect of opioid ligands on binding of [^{35}S]GTP[S] to membranes of clone D2 cells

The binding of [^{35}S]GTP[S] (30 °C, 30 min) to membranes (10 μg) of clone D2 cells and its regulation by opioid ligands was assessed as described in the Materials and Methods section. Results are presented as means ± S.E.M. for four independent experiments. *Values significantly ($P < 0.05$) different from the basal levels of binding.

Ligand(s) added	Binding of [^{35}S]GTP[S] (fmol/mg of protein)
None	678 ± 58
DADLE (1 μM)	1178 ± 98*
ICI174864 (10 μM)	469 ± 61*
TIPP[ψ] (10 μM)	590 ± 56
DADLE (1 μM) + TIPP[ψ] (10 μM)	633 ± 50
ICI174864 (10 μM) + TIPP[ψ] (10 μM)	620 ± 54

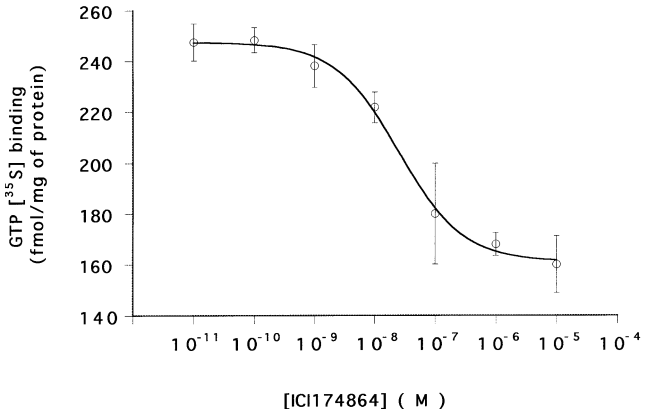

Figure 6 ICI174864 reduces the binding of [^{35}S]GTP[S] to membranes of clone D2 cells in a concentration-dependent manner

The ability of ICI174864 to affect binding of [^{35}S]GTP[S] to membranes prepared from clone D2 cells was assessed. Membranes (5 μg) were incubated (4 °C, 1 h) with 50 nCi of [^{35}S]GTP[S] as described in the Materials and Methods section in the presence of various concentrations of ICI174864. Results are expressed as means ± S.E.M. for percentage of control for four separate experiments.

both the stimulatory effect of DADLE and the inhibitory effect of ICI174864 on high-affinity GTPase activity were blocked by treatment of the cells with pertussis toxin and the level of high-affinity GTPase observed after pertussis toxin treatment was very similar to that produced by inclusion of a maximally effective concentration of ICI174864. Naloxone acted as a neutral antagonist in these studies, unable to regulate high-affinity GTPase activity significantly in either a positive or a negative fashion. The same was true of the highly selective δ opioid ligands TIPP and TIPP[ψ] [15] and, as expected for a competitive interaction, TIPP[ψ] was able to block the inhibitory effect of ICI174864 (Tables 1 and 4). Others have reported a small degree of inverse agonism to be produced by naloxone at the δ opioid receptor by measurement of alteration of GTPase activity [4] but we were unable to replicate these findings in membranes of clone D2; if naloxone does display features of an inverse agonist it clearly does not possess the high intrinsic activity of ICI174864.

To extend the analysis of the inverse agonist effect of ICI174864 on spontaneous function of the expressed δ opioid receptor in membrane systems we used two further assays. The first of these, namely ligand regulation of the binding of [^{35}S]GTP[S] to cellular G-proteins, is conceptually similar to regulation of high-affinity GTPase activity. In these assays we also observed stimulation of binding of the radiolabel by inclusion of DADLE and an inhibition on addition of ICI174864 (Table 4 and Figure 6). The other assay relies on the ability of a guanine nucleotide-depleted pertussis toxin-sensitive G-protein to become a substrate for cholera toxin-catalysed [^{32}P]ADP-ribosylation [19,20]. As receptor activation markedly enhances the release of GDP from a G-protein α subunit [28], then in the absence of exogenous guanine nucleotides to fill the empty site, agonist causes accumulation of a form of the G-protein that can act as a substrate for cholera toxin. This assay has previously been used to examine the coupling of the δ opioid receptor to G_i-like proteins in NG108-15 cells [17,29] as well as the coupling of a number of other receptors to G-proteins of this sub-family [20,30,31]. In membranes of clone D2 cells in the absence of receptor ligand, in addition to the expected [^{32}P]ADP-ribosylation of the long and short isoforms of $G_s\alpha$, cholera toxin was able to catalyse the [^{32}P]ADP-ribosylation of a 40 kDa $G_i\alpha$. This basal labelling was enhanced markedly by DADLE and eliminated by ICI174864 (Figure 5). Again, both of these effects were prevented by the presence of TIPP[ψ].

In the same manner that agonist ligands may range in intrinsic activity from partial to full agonists, it is expected that inverse agonists should also display a range of intrinsic activities [3,32] but in most assays it is difficult to establish the intrinsic activity of an inverse agonist. Results that support the high intrinsic activity of ICI174864 in this system as an inverse agonist are that the ligand was able to reduce basal high-affinity GTPase activity in membranes of clone D2 to essentially the same extent as pretreatment of the cells with pertussis toxin (Table 3) and that addition of this ligand completely attenuated the spontaneous activation of $G_i\alpha$ that was measured by this polypeptide acting as a substrate for cholera toxin in the absence of agonist occupancy of the receptor (Figures 5a and 5c). This indicates that ICI174864 should be viewed as an inverse agonist with high intrinsic activity [3].

The results provided in this manuscript analyse in detail features of inverse agonism and the role of empty receptor spontaneous activation of G-proteins by the δ opioid receptor when expressed in Rat 1 fibroblasts. By the use of a range of assay end points not only could the basic features of inverse agonism be observed but estimates of the intrinsic activity of such agents could also be examined. These approaches are likely to be useful in the identification of potential ligands for a range of receptors because in a variety of situations inverse agonists could be more appropriate for therapeutic intervention than antagonists [3].

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REFERENCES

1 Chidiac, P., Hebert, T. E., Valiquette, M., Dennis, M. and Bouvier, B. (1994) Mol. Pharmacol. **45**, 490–499
2 Samama, P., Pei, G., Costa, T., Cotecchia, S. and Lefkowitz, R. J. (1994) Mol. Pharmacol. **45**, 390–394
3 Milligan, G., Bond, R. A. and Lee, M. (1995) Trends Pharmacol. Sci. **16**, 10–13
4 Costa, T. and Herz, A. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 7321–7325
5 Adie, E. J. and Milligan, G. (1994) Biochem. J. **303**, 803–808
6 Barker, E. L., Westphal, R. S., Schmidt, D. and Sanders-Bush, E. (1994) J. Biol. Chem. **269**, 11687–11690
7 Thomas, D. R., Faruq, S. A., Balcarek, J. M. and Brown, A. M. (1995) J. Receptor Res. **15**, 199–211

8 Costa, T., Ogino, Y., Munson, P. J., Onoran, H. O. and Rodbard, D. (1990) Mol. Pharmacol. **41**, 549–560
9 Reisine, T. and Bell, G. I. (1993) Trends Neurosci. **16**, 506–510
10 Evans, C. J., Keith, D. E., Jr., Morrison, H., Magendzo, K. and Edwards, R. H. (1992) Science **258**, 1952–1955
11 Kieffer, B. L., Befort, K., Gaveriaux-Ruff, C. and Hirth, C. G. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 12048–12052
12 Chen, Y., Mestek, A., Liu, J., Hurley, J. A. and Yu, L. (1993) Mol. Pharmacol. **44**, 8–12
13 Fukada, K., Kato, S., Mori, K., Nishi, M. and Takeshima, H. (1993) FEBS Lett. **327**, 311–327
14 Yasuda, K., Raynor, K., Kong, H., Breder, C. D., Takeda, J., Reisine, T. and Bell, G. I. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 6736–6740
15 Schiller, P. W., Nguyen, T. M. D., Weltrowska, G., Wilkes, B. C., Marsden, B. J., Lemieux, C. and Chung, N. N. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 11871–11875
16 Milligan, G. (1987) Biochem. J. **245**, 501–505
17 Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. **162**, 156–159
18 Koski, G. and Klee, W. A. (1981) Proc. Natl. Acad. Sci. U.S.A. **78**, 4185–4189
19 McKenzie, F. R. and Milligan, G. (1990) Biochem. J. **267**, 391–398
20 Milligan, G. and McKenzie, F. R. (1988) Biochem. J. **252**, 369–373
21 Milligan, G., Carr, C., Gould, G. W., Mullaney, I. and Lavan, B. E. (1991) J. Biol. Chem. **266**, 6447–6455
22 Wieland, T. and Jakobs, K.-H. (1994) Methods Enzymol. **237**, 3–13
23 Allen, L. F., Lefkowitz, R. J., Caron, M. G. and Cotecchia, S. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 11354–11358
24 Coughlin, S. R. (1994) Curr. Opin. Cell Biol. **6**, 191–197
25 Raymond, J. R. (1994) Am. J. Physiol. **266**, F163–F174
26 Tiberi, M. and Caron, M. G. (1994) J. Biol. Chem. **269**, 27925–27931
27 Schutz, W. and Freissmuth, M. (1992) Trends Pharmacol. Sci. **13**, 376–380
28 Gilman, A. G. (1987) Annu. Rev. Biochem. **56**, 615–649
29 Roerig, S. C., Loh, H. H. and Law, P. Y. (1992) Mol. Pharmacol. **41**, 822–h831
30 Gierschik, P., Sidiropoulos, D. and Jakobs, K. H. (1989) J. Biol. Chem. **264**, 21470–21473
31 Remaury, A., Larrouy, D., Daviaud, D., Rouot, B. and Paris, H. (1993) Biochem. J. **292**, 283–288
32 Eason, M. G., Jacinto, M. T. and Liggett, S. B. (1994) Mol. Pharmacol. **45**, 696–702

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