Cholera toxin impairment of opioid-mediated inhibition of adenylate cyclase in neuroblastoma × glioma hybrid cells is due to a toxin-induced decrease in opioid receptor levels

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Cholera toxin treatment (up to 1 µg/ml, 16 h) of neuroblastoma × glioma hybrid NG108-15 cells produced a decrease of some 35 % in both δ opioid receptor-mediated stimulation of high-affinity GTPase activity and inhibition of forskolin-amplified adenylate cyclase. Coincident with these decreases was a down-regulation of some 35 % in the δ opioid receptor population. A similar pattern of a decrease in signalling capacity was noted for the α_{2B}-adrenergic receptor in these cells after cholera toxin treatment. Half-maximal effects of cholera toxin on all of the parameters assayed were noted at concentrations between 2 and 5 ng/ml. Neither levels of G_{i2}, as assessed by immunoblotting with specific antisera, nor the intrinsic activity of the α subunit of the guanine-nucleotide-binding protein which acts as the inhibitory G-protein of the adenylate cyclase in these cells, as assessed by guanosine 5'-[βγ-imido]triphosphate (Gpp[NH]p)-mediated inhibition of adenylate cyclase, was lowered by cholera toxin treatment. Furthermore, levels of another pertussis toxin-sensitive G-protein (G_o) expressed by these cells was also not lowered by cholera toxin treatment. However, as previously noted in other cells [Milligan, Unson & Wakelam (1989) *Biochem. J.* **262**, 643–649], marked down-regulation of the α subunit of the stimulatory G-protein (G_s) of the adenylate cyclase cascade was observed in response to cholera toxin treatment. Previous studies [Klee, Milligan, Simonds & Tocque (1985) *Mol. Aspects Cell Regul.* **4**, 117–129] have shown that cholera toxin treatment can result in a decrease in the maximal effectiveness of agonists which function to inhibit adenylate cyclase. These data have been used as evidence to suggest a functional interaction between G_s and 'G_i'. The results provided herein demonstrate that such effects of the toxin can be explained adequately by a decrease in the number of receptors that function to produce inhibition of adenylate cyclase.

INTRODUCTION

The neuroblastoma × glioma hybrid cell line NG108-15 has been used widely to examine transmembrane signalling cascades, as it expresses a wide range of receptors able to either stimulate or inhibit adenylate cyclase [1]. One of the most studied receptors on these cells is an opioid receptor of the δ subtype [2–4]. Agonist activation of this receptor results in both stimulation of high-affinity GTPase activity [3] and inhibition of adenylate cyclase [4]. Pretreatment of the cells with pertussis toxin attenuates the effects of opioids on both of these enzymic activities [5–7] and thus demonstrates that the guanine-nucleotide-binding protein (G-protein) that couples the δ opioid receptor to adenylate cyclase is a substrate for pertussis toxin-catalysed ADP-ribosylation. We have recently demonstrated, by using immunological probes able to interfere with the coupling of receptors and G-proteins, that this G-protein ('G_i') is the product of the G_{i2}α gene [8].

Whereas pertussis toxin is able to catalyse the ADP-ribosylation of the α subunits of a considerable number of G-proteins, including G_{i1}, G_{i2}, G_{i3} and G_o, cholera toxin has a much more limited range of G-protein substrates. Apart from forms of transducin and G_{olf} (a G-protein which regulates odorant stimulation of adenylate cyclase in olfactory neuroepithelium) [9], which are expressed in only very limited tissue locations, the only well-characterized G-protein that is modified by cholera toxin-catalysed ADP-ribosylation is the stimulatory G-protein (G_s) of the adenylate cyclase cascade [10].

Cholera toxin can catalyse the ADP-ribosylation of the α subunits of a number of G-proteins which are traditionally viewed as being substrates only for pertussis toxin-catalysed ADP-ribosylation, but this can be achieved only when using cell membranes under artificial conditions which produce a state of the G-protein that does not have either GDP or GTP in the nucleotide-binding pocket [10]. These conditions are unlikely to occur in a whole cell. Based on the known activity of cholera toxin and the inability of cholera toxin treatment of whole cells to produce covalent modification of pertussis toxin-sensitive G-proteins, it was therefore somewhat surprising that cholera toxin treatment of NG108-15 cells was observed to produce a decrease in the maximal degree of opioid-mediated inhibition of adenylate cyclase [7]. The present paper examines the mechanism of this effect of cholera toxin. We demonstrate that it can be accounted for by a down-regulation of the opioid receptor by cholera toxin. Similar effects were noted for the α_{2B}-adrenergic receptor on these cells, which also functions to produce an inhibition of adenylate cyclase.

MATERIALS AND METHODS

Materials

[³H]DADLE ([³H][D-Ala²,D-Leu⁵]enkephalin) (39.3 Ci/mmol) and [³H]yohimbine (80.0 Ci/mmol) were purchased from New England Nuclear (Darmstadt, Germany). [γ-³²P]GTP (> 10 Ci/mmol) and [α-³²P]ATP (30 Ci/mmol) were from Amersham

Abbreviations used: G-protein, guanine-nucleotide-binding protein; DADLE, [D-Ala²,D-Leu⁵]enkephalin; TBS, Tris-buffered saline; NP40, Nonidet P40; Gpp[NH]p, guanosine 5'-[βγ-imido]triphosphate; EC₅₀, concn. causing half-maximal stimulation.

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Table 1. Generation and specificities of anti-G-protein antisera

The antisera were generated in New Zealand White Rabbits using a conjugate of the synthetic peptide and keyhole limpet haemocyanin as detailed [11,12]. G₁α and G₂α have identical C-terminal decapeptides, and thus antiserum AS7 identifies each of these polypeptides equally. Transducin α is also identified by antiserum AS7, but as this G-protein is restricted in distribution to photo-receptor-containing tissues then the antiserum can be used as a probe for G₁ or G₂ in all other locations. NG108-15 cells express high levels of G₂ but do not express detectable levels of G₁ [8]. Antiserum I3B can be demonstrated to display weak cross-reactivity with G_oα from rat brain; however, the amounts of G_oα expressed in NG108-15 cells are sufficiently low that we have been unable to identify G_oα in membranes of these cells with this antiserum (results not shown). See [26] for detailed characterization of the reactivity of antiserum I3B against G₁3α. Comparisons of degrees of immunological staining and thus of the relative levels of G-protein subunits in membrane fractions were achieved either densitometrically as detailed in [27] or after exposure of the developed immunoblots to ¹²⁵I-labelled anti-rabbit IgG as detailed in [13].

Antiserum	Peptide	Antiserum identifies:
AS7	Transducin α-(341–350)	Transducin, G ₁ , G ₂
OC1	G _o α-(345–354)	G _o
I3B	G ₁ 3α-(345–354)	G ₁ 3
CS1	G _s α-(385–394)	G _s

International (Cardiff, Wales, U.K.). Cholera toxin was from Sigma. Horseradish peroxidase-coupled donkey anti-rabbit IgG was from the Scottish Antibody Production Unit (Carlisle, Scotland, U.K.). All other reagents were from Sigma or BDH, and were of the highest grade available.

Cell culture

Neuroblastoma × glioma hybrid NG108-15 cells were grown in tissue culture as described previously [8], except that foetal bovine serum was replaced by controlled process serum replacement-type 3 (CPSR-3) hybri-max (Sigma), as this has less potential batch-to-batch variation than foetal bovine serum. Cells were harvested when some 80 % confluent, and membrane fractions were prepared as in [8]. In some cases the cells were treated with cholera toxin (up to 1 µg/ml, 16 h) before harvest. Membranes were stored at –70 °C before use.

Immunological studies

The generation and specificities of the antisera used in this study are defined in Table 1. Each antiserum was produced in a New Zealand White rabbit using a conjugate of a synthetic peptide and keyhole limpet haemocyanin (Calbiochem) as antigen. The details of this process have previously been recorded [11].

Membrane samples were resolved by SDS/PAGE [12.5 % (w/v) acrylamide/0.0625 % (w/v) bisacrylamide] in 20 cm × 20 cm slab gels overnight at 100 V. Proteins were transferred to nitrocellulose (Schleicher and Schuell) and blocked for 2 h at 37 °C with 3 % gelatin in Tris-buffered saline (TBS) (20 mM-Tris/HCl, pH 7.5, and 500 mM-NaCl). Primary antiserum (1:200 dilution) in 1 % gelatin/TBS was then added and left overnight. The primary antiserum was then removed and the blot was washed extensively with distilled water followed by washes with TBS containing 0.2 % (v/v) Nonidet P40 (NP40) and then TBS. Secondary antiserum (donkey anti-rabbit IgG coupled to horseradish peroxidase) in 1 % gelatin/TBS was added and left for 3 h. Removal of the secondary antiserum was followed by the same series of extensive washes of the nitro-

cellulose, as detailed for after removal of the primary antiserum [12]. *o*-Dianisidine hydrochloride (Sigma) was employed as the substrate for detection of the antibody complex, as previously described [8].

Quantification of immunoblots

In a number of cases the developed immunoblots were subsequently treated with an ¹²⁵I-labelled donkey anti-rabbit IgG (Amersham International) (0.1 µCi/ml) in 1 % gelatin/TBS/0.2 % NP40 for 2 h. After extensive washing with TBS/0.2 % NP40 and then TBS the blot was air-dried and the coloured bands were excised, and radioactivity was assessed in a γ-radiation counter. In such cases preliminary experiments were performed to assess the range of linearity of the assay for each antiserum (see [13] for an example). Such experiments involved immunoblotting and subsequent treatment of various amounts of NG108-15 membranes with ¹²⁵I-labelled anti-rabbit IgG as described above. Amounts of membranes used to assess the effects of cholera toxin treatment on levels of the various G-proteins were, in all cases, within the observed linear region.

Adenylate cyclase assays

Forskolin (10 µM)-amplified adenylate cyclase activities and opioid peptide and α₂-adrenergic modulation of this activity were measured as described [8]. Resolution of [α-³²P]ATP and cyclic [γ-³²P]AMP was achieved by the method of Salomon *et al.* [14].

Regulation of adenylate cyclase by G_i in the absence of hormonal input was assessed by the ability of low concentrations of guanosine 5'-[βγ-imido]triphosphate (Gpp[NH]p) to produce inhibition of forskolin-amplified adenylate cyclase [15,16].

High-affinity GTPase assays

These were performed as in [17] using [γ-³²P]GTP (0.5 µM, 60 000 c.p.m.) and various concentrations of the synthetic opioid peptide DADLE. Non-specific GTPase activity was assessed in parallel assays containing 100 µM-GTP.

Binding experiments

These were performed at 25 °C for 30 min in 10 mM-Tris/HCl (pH 7.5)/50 mM-sucrose/20 mM-MgCl₂ (buffer B). In saturation experiments using [³H]DADLE the concentration of ligand was varied between 0.5 and 20 nM. Non-specific binding was defined in all cases by parallel assays containing 10 µM-DADLE. Non-specific binding increased with [³H]ligand concentration in a linear manner, but was never greater than 20 % of the total binding. Binding experiments were terminated by rapid filtration through Whatman GF/C filters followed by three washes of the filter with ice-cold buffer B (5 ml). Specific binding data were manipulated according to Scatchard [18]. The binding of [³H]yohimbine to α₂-adrenergic receptors was assessed in similar fashion. Non-specific binding of [³H]yohimbine was assessed in the presence of 100 µM-noradrenaline.

RESULTS

The synthetic enkephalin DADLE stimulated high-affinity GTPase activity in membranes of neuroblastoma × glioma NG108-15 cells in a dose-dependent manner (Fig. 1). Maximal stimulation, which in these experiments was 11.5 ± 0.5 pmol/min per mg of protein (mean ± S.E.M., *n* = 4) (basal, 20.8 ± 0.4; stimulated, 32.3 ± 0.5 pmol/min per mg of protein) was achieved with 1 µM-DADLE. Cholera toxin pretreatment (1 µg/ml, 16 h) of these cells resulted in a decrease in maximal stimulation by DADLE of high-affinity GTPase activity of some 37 % (7.2 ± 0.4 pmol/min per mg of protein; mean ± S.E.M., *n* = 4)

Fig. 1. Stimulation by DADLE of high-affinity GTPase activity in membranes of untreated and cholera toxin-treated NG108-15 cells

The effects of various concentrations of DADLE on high-affinity GTPase activity in membranes of both untreated (●) and cholera toxin (1 μ g/ml, 16 h)-treated (○) NG108-15 cells were assessed. Results represent means $\pm$ S.E.M. ($n = 4$) taken from a single experiment which was representative of three.

Fig. 2. Stimulation by DADLE of high-affinity GTPase activity in membranes of NG108-15 cells: effects of pretreatment with different concentrations of cholera toxin

The ability of a receptor-saturating concentration of DADLE (10 μ M) to modulate high-affinity GTPase activity in membranes of NG108-15 cells which had been treated with a range of concentrations of cholera toxin (0–1 μ g/ml, 16 h) was assessed. Results, which are means $\pm$ S.E.M. ($n = 4$), represent the stimulation of activity over that recorded in the absence of DADLE. Two further experiments produced similar data.

(basal, 20.0 ± 0.1 ; stimulated, 27.2 ± 0.4 pmol/min per mg of protein) compared with that in membranes from untreated cells (Fig. 1). No alteration in the EC_{50} (concentration producing half-maximal stimulation) (7 nM) for DADLE-stimulation of high-affinity GTPase activity was noted between the untreated and

Table 2. Cholera toxin pretreatment of NG108-15 cells lowers the maximal ability of DADLE to inhibit forskolin-amplified adenylate cyclase activity

Membranes were prepared from untreated and cholera toxin-treated (1 μ g/ml, 16 h) NG108-15 cells. Basal and forskolin-amplified adenylate cyclase activities were assessed, as was the ability of a receptor-saturating dose of DADLE to inhibit forskolin-amplified adenylate cyclase. Data, which are means $\pm$ S.E.M. ($n = 4$), are taken from a single experiment which was representative of three. Values in parentheses indicate percentage inhibition of forskolin-stimulated activity by DADLE.

	Adenylate cyclase activity (pmol/min per mg of protein)	
	Untreated	Cholera toxin-treated
Basal	49.5 ± 2.2	63.0 ± 2.3
+ Forskolin (10 μ M)	584.2 ± 13.7	723.3 ± 29.9
+ Forskolin (10 μ M) + DADLE (10 μ M)	381.6 ± 9.3 (34.7 %)	571.8 ± 12.8 (21.0 %)

Table 3. Effects of cholera toxin pretreatment of NG108-15 cells on levels of α_2B -adrenergic receptors and α_2 -adrenergic inhibition of adenylate cyclase

Membranes were prepared from either untreated or cholera toxin (1 μ g/ml, 16 h)-treated NG108-15 cells and levels of the α_2 -adrenergic receptor were measured, as assessed by the specific binding (subsequently corrected for receptor occupancy; K_d for [3 H]yohimbine = 2.5 nM) of a single concentration of [3 H]yohimbine (10 nM). The ability of a receptor-saturating concentration of the α_2 -adrenergic receptor agonist UK 14304 to mediate inhibition of forskolin-amplified adenylate cyclase was also assessed. Results are presented as means $\pm$ S.E.M. ($n = 4$). Values in parentheses indicate the percentage decrease due to cholera toxin treatment.

	Untreated	Cholera toxin-treated
Inhibition of forskolin (10 μ M)- amplified adenylate cyclase activity by UK 14304 (10 μ M) (%)	33.7 ± 3.2	23.1 ± 1.5 (31.5 %)
Specific binding of [3 H]yohimbine (fmol/mg of protein)	114.6 ± 1.5	69.4 ± 2.5 (39.5 %)

cholera toxin-treated cells, and cholera toxin pretreatment of the cells had no substantial effect on the basal high-affinity GTPase activity of the membranes. Variations in the concentration (0–1 μ g/ml) of cholera toxin used to pretreat NG108-15 cells (for 16 h) demonstrated that essentially maximal effects of the toxin on DADLE-stimulation of high-affinity GTPase activity were produced by 100 ng of cholera toxin/ml, and that half-maximal effects were produced at some 3 ng/ml (Fig. 2). After pretreatment of NG108-15 cells with cholera toxin (1 μ g/ml, 16 h) the basal adenylate cyclase activity in the membranes of these cells was only slightly higher than that in membranes from untreated cells (Table 2). We have previously noted this to be so and have demonstrated that it is a reflection of cholera toxin-induced down-regulation of most of the cellular levels of G_{α} over this time period. As with opioid-stimulation of high-affinity GTPase activity, DADLE-mediated inhibition of forskolin (10 μ M)-amplified adenylate cyclase activity was also lowered substantially in membranes from the cholera toxin-treated cells (maximal inhibition 21 %) compared with those from untreated cells (maximal inhibition 35 %) (Table 2).

Fig. 3. Membrane-associated levels of $G_s\alpha$, but not $G_{i2}\alpha$, are substantially decreased by treatment of NG108-15 cells with cholera toxin

Membranes (100 μ g) from NG108-15 cells which were untreated (lane a) or treated with 0.1 (b), 1.0 (c), 10.0 (d), 100 (e) or 1000 (f) ng of cholera toxin/ml for 16 h were resolved on SDS/PAGE (10% acrylamide) and immunoblotted with either antiserum CS1 (anti- $G_s\alpha$, 1:200 dilution) (a) or antiserum AS7 (anti- $G_{i2}\alpha$; 1:200 dilution) (b).

Table 4. Treatment of NG108-15 cells with cholera toxin does not decrease membrane-associated levels of the α subunits of G_o or G_i3

NG108-15 cells were treated or not with cholera toxin (1 μ g/ml) for 16 h. Membranes (60 μ g) prepared from these cells were immunoblotted for each of $G_o\alpha$ and $G_{i3}\alpha$ as described in the Materials and methods section. The developed immunoblots were quantified following overlay with an 125 I-anti-rabbit IgG, as described in the Materials and methods section. Results are from a typical experiment; two further experiments on membranes prepared from separate cell cultures produced qualitatively similar results.

Antibody	125 I bound to G-protein α subunit (c.p.m.)		Ratio (cholera toxin treated/untreated)
	Untreated	Cholera toxin-pretreated	
OC1 (anti- $G_o\alpha$)	753	862	1.14
I3B (anti- $G_{i3}\alpha$)	430	407	0.95

The α_2 -adrenergic receptor agonist UK 14304 also produced inhibition of forskolin-amplified adenylate cyclase activity in both untreated and cholera toxin-treated NG108-15 cells. However, as with the opioid agonist, the maximal effectiveness of this ligand was decreased in membranes from cholera toxin-pretreated cells (23%) compared with membranes from untreated cells (34%) (Table 3).

As effects of both opioid and α_2 -adrenergic agonists were decreased in membranes from cholera toxin-pretreated cells, we examined the levels and activity of $G_{i2}\alpha$ under each condition, as we have recently demonstrated that opioid regulation of adenylate cyclase is controlled by this G-protein [8]. A decrease in either of these parameters might be expected to produce the observed results.

Immunoblotting of membranes derived from NG108-15 cells treated with various concentrations of cholera toxin (0–1 μ g/ml) for 16 h with antiserum AS7 (which, of the various G-proteins expressed by these cells, identifies only $G_{i2}\alpha$ [8]) indicated no decrease in membrane-associated levels of this polypeptide (Fig. 3b). NG108-15 cells also express both $G_{i3}\alpha$ and $G_o\alpha$. Levels of these polypeptides were also not decreased by pretreatment of the cells with cholera toxin (1 μ g/ml) (Table 4). By contrast, as we have previously noted, chronic exposure of cells to cholera toxin produced a marked decrease in membrane-associated levels

Fig. 4. G_i is functional in both untreated and cholera toxin-treated NG108-15 cells

Membranes prepared from (a) untreated and (b) cholera toxin-pretreated (1 μ g/ml) NG108-15 cells were assessed for adenylate cyclase activity. Basal activity was amplified by forskolin (10 μ M) and the regulation of this activity by low concentrations (0–30 nM) of Gpp[NH]p was assessed.

Fig. 5. Cholera toxin treatment of NG108-15 cells produces a decrease in δ opioid receptor number

Saturation binding studies were performed on membranes from both untreated (O) and cholera toxin (1 μ g/ml, 16 h)-treated (●) NG108-15 cells using [3 H]DADLE (0.35–20.10 nM). Non-specific binding was assessed in parallel incubations by the inclusion of 10 μ M-DADLE. Data were manipulated according to [18].

of $G_s\alpha$ (Fig. 3a), as assessed by immunoblotting membranes from these cells with antiserum CS1. Indeed, at concentrations of cholera toxin above 10 ng/ml used for pretreatment of the cells

Fig. 6. Concentration-dependence of cholera toxin-mediated loss of δ opioid and α_{2B} -adrenergic receptors

NG108-15 cells were treated for 16 h with various concentrations of cholera toxin (0–1000 ng/ml). The specific binding of a single concentration (10 nM) of either [3 H]DADLE (a) or [3 H]yohimbine (b) to membranes of the cells was assessed, and this binding was corrected subsequently for receptor occupancy. Results are presented as a percentage of the number of receptors present in membranes from the untreated cells.

it was difficult to detect $G_s\alpha$ immunologically, which in these cells is primarily expressed as a 44 kDa polypeptide, in the membrane fraction. We have previously shown that antibody CS1 identifies equally well both ADP-ribosylated and non-modified forms of $G_s\alpha$ [19].

To assess if the activity of G_i was decreased by cholera toxin pretreatment of the cells, we measured forskolin-amplified adenylate cyclase activity in the presence of low concentrations of the poorly hydrolysed analogue of GTP, Gpp[NH]p, which has been demonstrated to display greater affinity for G_i than for G_s , and hence can be used to selectively activate the inhibitory arm of adenylate cyclase [15,16]. In membranes from both untreated and cholera toxin-treated cells, Gpp[NH]p was able to produce maximal inhibition of forskolin-activated adenylate cyclase at some 3 nM, and in both cases the degree of inhibition at this concentration was some 15% (Fig. 4). As such we were able to conclude that the intrinsic activity of G_i was not attenuated in membrane preparations from cells which had been pretreated with cholera toxin.

In the presence of 20 mM-Mg²⁺, saturation analysis of [3 H]DADLE binding to δ opioid receptors in membranes of untreated NG108-15 cells demonstrated that the [3 H]ligand interacts with an apparent single class of sites which displayed a

K_d for the ligand of approx. 2.5 nM and were present at some 590 fmol/mg of protein (Fig. 5). Similar binding studies in membranes from cholera toxin (1 μ g/ml, 16 h)-treated NG108-15 cells demonstrated a decrease in the levels of receptor binding sites to some 430 fmol/mg of protein in the experiment displayed in Fig. 5. The decrease in δ opioid receptor levels varied between 27 and 40% in a range of experiments performed on separate cultures of NG108-15 cells. The affinity of the remaining receptors for [3 H]DADLE (K_d = 2.4 nM), however, was the same as in membranes from the untreated cells (Fig. 5). The decrease in δ opioid receptor levels in membranes of NG108-15 cells produced by cholera toxin treatment over a 16 h period was half-maximal when 2.5 ng of toxin/ml was used, and was essentially maximal at 100 ng/ml (Fig. 6a). An equivalent (40%) decrease in maximal binding capacity was noted for α_2 -adrenergic receptors in NG108-15 membranes after treatment of the cells with cholera toxin (1 μ g/ml, 16 h), either when analysis was based upon the binding of a single, receptor-saturating, concentration of [3 H]yohimbine (Table 3) or when saturation binding analyses for this [3 H]ligand were performed (results not shown). The degree of receptor loss was similar to the decrease in the maximal inhibition of adenylate cyclase produced by a receptor-saturating concentration of the α_2 agonist UK14304 (33%) (Table 3). The concentration of cholera toxin producing half-maximal loss of α_2 -adrenergic receptors was approx. 3 ng/ml (Fig. 6b).

DISCUSSION

Cholera toxin, because of its ability to catalyse the mono-ADP-ribosylation of the α subunit(s) of G_s , has been of major importance in attempts to elucidate the mechanistic details of receptor regulation of the stimulation of adenylate cyclase. Although the persistent activation of G_s , and hence of adenylate cyclase, produced by this toxin is understood to result from an inhibition of the GTPase reaction which would normally function to de-activate $G_s\alpha$ [20], it has recently become apparent that the effects of cholera toxin treatment of cells on the adenylate cyclase cascade are not limited to this single manifestation. In terms of the stimulatory arm, cholera toxin-catalysed ADP-ribosylation of $G_s\alpha$ leads to a down-regulation of steady-state levels of this polypeptide at the plasma membrane [19,21]. Cholera toxin treatment of cells has also been noted to alter the effectiveness of receptor regulation of the inhibition of adenylate cyclase [7], but it has been uncertain at which level this effect is produced. In the present paper we demonstrate that a cholera toxin-mediated decrease in the function of receptors which produce inhibition of adenylate cyclase in NG108-15 cells can be accounted for by a cholera toxin-induced loss of plasma membrane receptors. After treatment of NG108-15 cells with cholera toxin (up to 1 μ g/ml, 16 h) levels of the δ opioid receptor were lowered by approx. 35% (Fig. 6). The remaining receptors displayed an identical affinity for [3 H]DADLE as those in membranes from untreated cells. These remaining receptors must, as such, still be coupled to the G-protein signalling apparatus, as we have previously noted that, in conditions in which the δ opioid receptors are uncoupled from the G-protein signalling system, for example after pretreatment of the cells with pertussis toxin or when the binding assay is performed in the presence of Gpp[NH]p, the poorly hydrolysed analogue of GTP, then the affinity of the receptors for [3 H]DADLE is lowered, such that the K_d is close to 6 nM [8].

The decrease in both maximal opioid-stimulation of high-affinity GTPase activity and inhibition of adenylate cyclase was of a similar magnitude to the loss of receptor number (in all cases 30–40%). Further, the concentration-dependence for stimulation of GTPase activity, which represents the most direct assay of

receptor activation of the G-protein, was identical in untreated and cholera toxin-treated cells, even though the maximal effect of the opioid agonist was decreased by 35% (Fig. 1), suggesting that the effect was produced at the level of receptor number. A similar decrease in degree both of the levels of the α_2 -adrenergic receptor, as assessed by the binding capacity for the α_2 -adrenergic antagonist [3 H]yohimbine, and of the ability of the α_2 -adrenergic-selective agonist UK 14304 to inhibit forskolin-amplified adenylate cyclase activity (Table 3), indicated further that the effect of cholera toxin on the inhibitory control of adenylate cyclase was not limited to the opioid receptor.

A decrease in the ability of both opioid peptides and an α_2 -adrenergic agonist to inhibit adenylate cyclase could potentially be produced if either the absolute levels or the activity of G_i were lowered by cholera toxin treatment, and indeed this could act as a convenient site for the induction of a heterologous form of control of the inhibition of adenylate cyclase. Such a scenario has recently been reported to occur in rat adipocytes in tissue culture, where incubation of the cells with an adenosine A1 receptor agonist produced marked down-regulation of each of G_{i1} , G_{i2} and G_{i3} , but not of G_s [22]. Such down-regulation is accompanied by the development of a heterologous form of desensitization at receptors which produce inhibition of adenylate cyclase (A. Green, G. Milligan & S. E. Belt, unpublished work).

We have recently taken advantage of the ability of antisera directed against the C-terminal regions of the α subunits of various G-proteins to prevent contact of the G-protein with receptors in order to demonstrate that the $G_{i2\alpha}$ gene product represents the true inhibitory G-protein of the adenylate cyclase cascade, at least in NG108-15 cells [8]. A similar conclusion as to the molecular nature of G_i has been reached by Simonds *et al.* [23] in studies on human platelets. By immunoblotting membranes of NG108-15 cells, which had been treated with various concentrations of cholera toxin, with an antiserum (AS7) which in these cells identifies only $G_{i2\alpha}$, we have demonstrated that steady-state levels of $G_{i2\alpha}$ were not decreased by cholera toxin treatment (Fig. 3). Further, no alteration in the mobility of G_{i2} in SDS/PAGE was observed after cholera toxin treatment (Fig. 3). This provides supportive evidence that cholera toxin treatment did not produce ADP-ribosylation of $G_{i2\alpha}$, as we have previously shown that the addition of ADP-ribose to a G-protein α subunit decreases its mobility through SDS/PAGE [11], presumably due to the extra molecular mass provided by the ADP-ribose. We have also demonstrated that levels of the other pertussis toxin-sensitive G-proteins expressed by these cells, $G_{i3\alpha}$ and $G_{o\alpha}$, were not decreased by cholera toxin treatment of the cells (Table 4).

A decrease in the functional activity of G_i in membranes of cholera toxin-pretreated NG108-15 cells might also have been sufficient to account for the results produced. We have also been able to demonstrate, however, that G_i (G_{i2}) is equally active in membranes from untreated and cholera toxin-pretreated cells by assessing the effectiveness of this G-protein in producing inhibition of adenylate cyclase independently of receptor activation. To do this we have taken advantage of the fact that Gpp[NH]p, the poorly hydrolysed analogue of GTP, has a higher affinity for G_i than for G_s [15,16]. As such, low concentrations of Gpp[NH]p selectively activate the inhibitory arm of the adenylate cyclase cascade. Maximal inhibition of forskolin-amplified adenylate cyclase was achieved with 3 nM-Gpp[NH]p in membranes from both untreated and cholera toxin-treated NG108-15 cells, and inhibition of adenylate cyclase at this concentration was approx. 15% in each case (Fig. 4). Higher concentrations of Gpp[NH]p did not produce a greater degree of inhibition of cyclase activity, due to the nucleotide causing activation of both G_s and G_i at such concentrations.

Effects of cholera toxin treatment of cells on levels of various receptors have previously been recorded. The decrease in the ability of vasopressin to stimulate production of inositol phosphates in L6 skeletal myoblasts [24] and of both vasopressin and angiotensin to do the same in renal glomerulosa cells [25] has been demonstrated to be due to a cyclic AMP-dependent down-regulation of the relevant receptor, presumably subsequent to activation of protein kinase A. Indeed, it is likely that the effects of cholera toxin on cellular levels of δ opioid and α_2 -adrenergic receptors in NG108-15 cells are produced in a similar manner and are dependent upon the generation of cyclic AMP. Stimulation of cyclic AMP production in NG108-15 cells either by direct activation of adenylate cyclase with forskolin or by activating a prostanoid receptor on these cells with prostaglandin E_1 also produces a decrease in the level of the δ opioid receptor (F. R. McKenzie, S. J. McClue & G. Milligan, unpublished work). It is unclear if the effect of cyclic AMP on δ opioid receptor number is related to phosphorylation of the receptor or is produced at some other level. As the δ opioid receptor has not yet been cloned and suitable antisera are not available, information relating to this point is unlikely to be obtained easily. The only alteration in levels, activities or interactions of the components of the inhibitory arm of adenylate cyclase that we have been able to measure after cholera toxin treatment of NG108-15 cells is the number of receptor-binding sites detectable in the membrane. We must conclude that the decreased maximal activity of opioid agonists in stimulating high-affinity GTPase activity, and subsequently in inhibiting adenylate cyclase, which is observed subsequent to cholera toxin treatment of NG108-15 cells is due entirely to the decrease in opioid receptor number.

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