A DNA encoding the human α2-C10 adrenergic receptor was transfected into Rat 1 fibroblasts and clones selected on the basis of resistance to G418 sulfate. Two clones, one of which (1C) expressed some 3.5 pmol/mg membrane protein of the receptor as assessed by the specific binding of [3H]yohimbine and one (4D) which did not express detectable amounts of the receptor were selected for further study. When cholera toxin-catalyzed ADP-ribosylation was performed with [32P]NAD on membranes of these cells in the absence of added guanine nucleotides, radioactivity was incorporated into a polypeptide(s) of 40 kDa in addition to the 45- and 42-kDa forms of Giα. Addition of the selective α2 receptor agonist U.K.14304 enhanced markedly, in a dose-dependent manner, the cholera toxin-catalyzed [32P]ADP-ribosylation of the 40-kDa polypeptide(s), but not the 45- or 42-kDa polypeptides, in membranes of the 1C cells. Dose response curves for U.K.14304 enhancement of cholera toxin-labeling of the 40-kDa polypeptide(s) and stimulation of high affinity GTPase activity were identical. By contrast, U.K.14304 was ineffective in either assay in membranes from the 4D cells, demonstrating this effect to be dependent upon receptor activation. Furthermore, the α2 receptor antagonist yohimbine blocked all effects of U.K.14304. The agonist promotion of chola toxin-catalyzed ADP-ribosylation of Gi, was completely blocked by guanine nucleotides. Whether GDP or GDP + fluoroaluminate (as a mimic of GTP) was used, blockade of the agonist effect was complete and indeed both conditions prevented agonist-independent labeling by cholera toxin of the 40-kDa polypeptide(s).

Mg2+ produced an agonist-independent cholera toxin-catalyzed [32P]ADP-ribosylation of the 40-kDa polypeptide(s) but even in the presence of [Mg2+], agonist-stimulation of cholera toxin-labeling of the 40-kDa polypeptide(s) was observed and was additive with the effect of [Mg2+].

Agonist stimulation of cholera toxin-catalyzed ADP-ribosylation of Gi, was completely attenuated by pretreatment of the cells with pertussis toxin, which prevents contact between receptors and G-proteins which are substrates for this toxin. By contrast, pretreatment of the cells with concentrations of cholera toxin able to "down-regulate" essentially all of the membrane-associated Gα did not prevent agonist stimulation of cholera toxin-catalyzed ADP-ribosylation of Gi.

Immunoprecipitation of the 40-kDa polypeptide(s) radiolabeled by cholera toxin, with selective anti-G-protein antisera, demonstrated that both G2 and G3 were modified in an agonist-dependent manner. These results demonstrate that agonist stimulation of the cholera toxin-catalyzed ADP-ribosylation of "inappropriate" G-proteins can define interactions of receptors with pertussis toxin-sensitive G-proteins and that to achieve such results the guanine nucleotide-binding pocket of the G-protein must lack either GDP or GTP. Furthermore, these results indicate that the α2-C10 adrenergic receptor can interact functionally in these membranes with both G2 and G3. This is the first demonstration that a single molecularly defined receptor can activate multiple pertussis toxin-sensitive G-proteins in a native membrane.

α2-Adrenergic receptors represent the most extensively studied examples of receptors which function to produce inhibition of adenylyl cyclase (1). While all receptors with α2 pharmacology produce inhibition of adenylyl cyclase (2) it is clear that multiple forms of α2-adrenergic receptors are expressed and that these are the protein products of distinct genes (3–5). cDNAs corresponding to two distinct α2-adrenergic receptors have been isolated. While the α2-C10 receptor corresponds to the form of α2 receptor in human platelets (6) and demonstrates all of the pharmacological characteristics expected for the α2A subtype, the α2-C4 receptor appears to display a number of pharmacological facets which are not those anticipated for the α2B receptor (2) and it is thus possible that these two species are not identical. As such, a more complex pattern may yet await definition.

The α2A receptor, in common with other receptors which produce inhibition of adenylyl cyclase, does so via the activation of a perussis toxin-sensitive guanine nucleotide-binding protein (G-protein), G, (7). A considerable range of perussis toxin-sensitive G-proteins are known to be expressed, including G1, G2, G3, and G4 (8), and each of these, while highly homologous, is transcribed from a separate gene (9). In the case of both the α2A receptor of human platelets (10) and the α2B receptor of neuroblastoma × glioma hybrid, NG108-15, cells (11, 12) G, appears to be the product of the G2α gene. Such conclusions have been based on experiments in which a series of antipeptide antisera directed against the

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extreme C-terminal decapeptide of the α subunits of various G-proteins have been used to either block receptor-mediated inhibition of adenylate cyclase (10, 11) or to uncouple the receptor from the G-protein signaling system such that the receptor adopts a conformation which displays a reduced affinity to bind agonists but not antagonists (11, 12).

Cholera toxin has been an invaluable pharmacological tool to study stimulation of adenylate cyclase as it is able to catalyze the ADP-ribosylation of the stimulatory G-protein of the adenylate cyclase cascade on a specific arginine residue (8). The functional consequence of this modification is that the G-protein becomes constitutively activated as it is unable to produce the hydrolysis of bound GTP. With the exception of two distinct forms of transducin and the G-like G-protein, Gαi, each of which are expressed in only highly specialized tissues and are as such only found in limited locations (13), cholera toxin is generally believed not to catalyze the ADP-ribosylation of other members of the G-protein family. However, particularly in the neutrophil-like cell line HL60, it has been noted that cholera toxin is able to catalyze ADP-ribosylation of a number of pertussis toxin-sensitive G-proteins when the bacterial chemotactic factor formyl-methionyl-leucyl-phenylalanine (fMLP) is present to activate its receptor (14, 15). This receptor is able to activate a phospholipase C which then generates inositol 1,4,5-trisphosphate from phosphatidylinositol 4,5-bisphosphate.

Relatively little attention has been paid to whether activation of receptors which mediate inhibition of adenylate cyclase will allow equivalent ADP-ribosylation of Gβ by cholera toxin. However, in the neuroblastoma × glioma hybrid cell line, NG108-15, activation of a δ opioid receptor promotes cholera toxin-catalyzed ADP-ribosylation of Gβ (16) and the G-protein which mediates opioid inhibition of adenylate cyclase has been shown to be Gβ2α (11).

In the present study we have examined the ability of the human platelet α2-C10 adrenergic receptor to regulate cholera toxin-catalyzed ADP-ribosylation of pertussis toxin-sensitive G-proteins following transfection of this receptor into Rat 1 fibroblasts. We further examine the requirements which allow such an assay to be used to identify direct interactions between a receptor and a G-protein and demonstrate, in this cell line, the direct interaction of the activated α2-C10 receptor with both Gβ2 and Gδ3.

**EXPERIMENTAL PROCEDURES**

**Materials**

Reagents were obtained from the following sources, [3H]yohimbine (80 Ci/mmol) and [35S]GTP (>10 Ci/mmol) were obtained from Amersham International, [3H]NAD (800 Ci/mmol) from Du Pont-New England Nuclear. Pertussis toxin was from Porton Products, Porton Down, Wiltshire, England, United Kingdom, cholera toxin from Sigma and all materials for tissue culture from Gibco/Bethesda Research Laboratories.

**Methods**

**Expression of Recombinant DNA Encoding the α2-C10 Adrenergic Receptor—** Stable expression of genomic DNA corresponding to the human platelet α2-C10 adrenergic receptor (3) (obtained from ATCC, name of clone, HPAlpha2 GEN) was obtained using the mammalian expression vector pDOL (17). The BamHI fragment of HPAlpha2 GEN, containing the entire coding sequence of the α2-C10 (also called α2A) receptor was cloned into the BamHI site of pDOL. Transcription of the gene is driven by the left viral long terminal repeat, producing a chimeric RNA species that terminates at the right long terminal repeat and includes the neomycin resistance gene downstream of the α2-C10 receptor DNA. Expression of the neomycin (G418) resistance gene is driven by the SV40 early promoter. Rat 1 fibroblasts were transfected by calcium phosphate precipitation (18), and G418 sulfate-resistant clones were selected.

Two clones were selected for further study. Rat 1 α2A 1C (1C) demonstrated the expression of some 3.5 pmol/mg membrane protein of the α2-C10 receptor as assessed by the specific binding of the α2 receptor antagonist [3H]yohimbine. Rat 1 α2A 4D (4D), while resistant to treatment with G418 sulfate did not express detectable levels of the α2-C10 receptor.

**Cell Culture—** Cells of both the 1C and 4D clones were grown in Dulbecco's modified Eagle's medium supplemented with 5% donor calf serum, penicillin (100 units/ml) and streptomycin (100 μg/ml) in 5% CO2 at 37 °C. Cells were grown in 75 cm² tissue culture flasks and were harvested just prior to confluence. In a number of cases, cells were treated with pertussis toxin (25 ng/ml) for 16 h prior to cell harvest or with cholera toxin (500 ng/ml for up to 48 h). Membranes were prepared from the cells by homogenization with a Teflon on glass homogenizer and differential centrifugation as described for a variety of other cells (19).

**Cholera and Pertussis Toxins—** Pertussis toxin (Ptx) and cholera toxin (Ctx) will allow equivalent ADP-ribosylation of Gi by cholera toxin. We further examine the requirements which allow such an assay to be used to identify direct interactions between receptors which mediate inhibition of adenylate cyclase (10, 11) or to uncouple the G-protein from the G-protein signaling system such that the receptor antagonist [3H]yohimbine. Rat 1 α2A 4D (4D), while resistant to treatment with G418 sulfate did not express detectable levels of the α2-C10 receptor.

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screens. We have previously described the use of each of the antisera in immunoprecipitation studies (22).

Binding Experiments—These experiments were performed at 25 °C for 30 min in 10 mM Tris-HCl, 50 mM sucrose, 20 mM MgCl₂, pH 7.5 (buffer B). In saturation experiments using [³H]yohimbine, the concentration of ligand was varied between 0.5 and 20 nM. Nonspecific binding was defined in all cases by parallel assays containing 100 μM noradrenaline. Nonspecific binding increased with [³H]ligand concentration in a linear manner. Binding experiments were terminated by rapid filtration through Whatman GF/C filters followed by three washes of the filter with ice-cold buffer B (6 ml).

High Affinity GTPase Assays—These assays were performed essentially as described in Ref. 11 using [γ-³²P]GTP (0.5 μM, 60,000 cpm) and various concentrations of U.K.14304. Nonspecific GTPase was assayed by parallel assays containing 100 μM GTP.

RESULTS

Stable expression of a construct of the gene encoding the human platelet α₂-C10 receptor linked to the mammalian expression vector pDOL was achieved by transfecting this construct into Rat 1 fibroblasts using calcium phosphate precipitation. Clones which were resistant to G418 sulfate were selected and expanded. Total and nonspecific binding of [³H]yohimbine (10 nM) as defined by the absence or presence of adrenaline (100 μM) in the assay was assessed in membranes of cells of a series of the clones. Membranes from clone 1C cells expressed some 3.5 pmol of the receptor/mg of membrane protein, while specific binding of [³H]yohimbine to membranes of cells of clone 4D was not detectable (Table I). The parental Rat 1 fibroblasts also did not express detectable specific binding sites for [³H]yohimbine (results not shown). Cholera toxin-catalyzed [³²P]ADP-ribosylation of membranes of 1C cells, when performed without the addition of guanine nucleotides to the assay, identified a major polypeptide of 45 kDa, a less prevalent 42-kDa polypeptide and a poorly identified polypeptide of 40 kDa (Fig. 1). When the same experiment was performed but with the addition of the α₂-adrenergic receptor agonist U.K.14304 (10 μM), while similar amounts of radioactivity were incorporated into the 45- and 42-kDa polypeptides, a marked enhancement of the labeling of the 40-kDa polypeptide was evident (Fig. 1). Dose-response curves for UK14304 enhancement of the cholera toxin-catalyzed ADP-ribosylation of the 40-kDa polypeptide indicated that half-maximal stimulation of the effect was observed at some 30 nM (Fig. 2, A and B). Similar concentrations (30 nM) of this agonist were required to produce a half-maximal stimulation of receptor-mediated high affinity GTPase activity in membranes of these cells (Fig. 2C). In both assays, the effect of U.K.14304 was completely reversed by the addition of the α₂ receptor antagonist yohimbine (results not shown). In contrast to membranes from clone 1C, equivalent membranes from cells of clone 4D demonstrated no U.K.14304 stimulated-cholera toxin-catalyzed ADP-ribosylation of a 40-kDa polypeptide (Fig. 2D) or stimulation of high affinity GTPase activity (results not shown).

TABLE I

<table>
<thead>
<tr>
<th>Clone</th>
<th>Specific [³H]yohimbine binding (pmol/mg membrane protein)</th>
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<tbody>
<tr>
<td>1C</td>
<td>3,451 ± 479</td>
</tr>
<tr>
<td>4D</td>
<td>0</td>
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</table>

Fig. 1. Activation of the α₂-C10 receptor in membranes of clone 1C cells with U.K.14304 produces marked enhancement of cholera toxin-labeling of a 40-kDa polypeptide. Membranes (60 μg) of clone 1C cells were treated with thiol-activated cholera toxin and [³²P]NAD in the absence of added guanine nucleotides as described under "Experimental Procedures." U.K.14304 (10 μM) was also present in the sample displayed in 2 but not in 1. Samples were precipitated and resolved by SDS-PAGE (10% (w/v) acrylamide). The gel was stained with Coomassie Blue, dried, and autoradiographed for 48 h. Similar results were observed in membranes isolated from nine separate preparations of clone 1C cells.

We examined whether the presence of guanine nucleotides would regulate agonist promotion of cholera toxin-catalyzed ADP-ribosylation of the 40-kDa polypeptide in membranes of 1C cells. Addition of GDP (100 μM) to the ADP-ribosylation assay completely attenuated the effect of a receptor saturating concentration (10 μM) of U.K.14304 (Fig. 3). As it is impractical to add GTP to such assays such that G-proteins and other nucleotide-dependent proteins present in the membrane will not hydrolyze this nucleotide to GDP, then to assess the effects of GTP on U.K.14304 stimulation of cholera toxin-catalyzed ADP-ribosylation of G-proteins, we performed the assay in the presence of GDP (100 μM) + fluorooraluminate (20 μM) (added as 20 μM aluminum sulfate, 10 mM sodium fluoride). This anion acts in the presence of GDP to mimic the terminal phosphate of GTP at the nucleotide-binding site of G-proteins. This mixture also prevented completely the effect of U.K.14304 on stimulation of cholera toxin-catalyzed ADP-ribosylation of the 40-kDa polypeptide (Fig. 3). By contrast, addition of fluorooraluminate (20 μM) without GDP did not modify the effect of the receptor agonist (Fig. 3). The addition of either GDP or GDP + fluorooraluminate also suppressed the basal incorporation of radioactivity into the 40-kDa polypeptide which was observed in the absence of the agonist (Fig. 3).

The GTP analogue, GTPγS also reduced cholera toxin-catalyzed labeling of the 40-kDa polypeptide at high concentrations. At no concentration of this nucleotide was a stimulation of cholera toxin-labeling of this polypeptide observed (results not shown). This is in contrast to the situation for fMLP stimulation of cholera toxin-catalyzed ADP-ribosylation of a 40-kDa polypeptide in membranes of HL60 cells (15). As such, it appears that cholera toxin-catalyzed ADP-ribosylation of the 40-kDa polypeptide (G_i) can only occur when the G-protein does not have a nucleotide in the binding site. By contrast with its lack of stimulation of cholera toxin-catalyzed ADP-ribosylation of the 40-kDa polypeptide, GTPγS produced a large stimulation of cholera toxin-catalyzed ADP-ribosylation of the forms of Gα at concentrations above 1 μM (data not shown). Inclusion of Mg²⁺ ions in the assay was able to stimulate markedly agonist-independent ADP-ribosylation of the 40-kDa polypeptide by cholera toxin (Fig. 4A). However, agonist-stimulation of labeling was still apparent over and above the agonist-independent labeling in
FIG. 2. Cholera toxin-catalyzed ADP-ribosylation of a 40-kDa polypeptide in membranes of Rat 1α2-C10 cell clones is dependent upon the presence of receptor and displays a similar agonist dependence as stimulation of high affinity GTPase activity. A, agonist promotion of cholera toxin-catalyzed ADP-ribosylation of a 40-kDa polypeptide in membranes of clone 1C cells. Concentration dependence of U.K.14304. Membranes (60 μg) of clone 1C cells were ADP-ribosylated with cholera toxin as detailed in Fig. 1 except that concentrations of U.K.14304 in the assay were 0 (a), 1 nM (b), 10 nM (c), 100 nM (d), 1 μM (e), 10 μM (f), 100 μM (g). Three further experiments produced similar results. B, concentration dependence of U.K.14304 stimulation of cholera toxin-catalyzed ADP-ribosylation in clone 1C cells. Quantitative analysis. The autoradiograph from A was scanned to assess the effectiveness of U.K.14304 the presence of Mg²⁺ (Fig. 4, A and B) and was additive with the effect of Mg²⁺ (Fig. 4B).

Pretreatment of clone 1C cells with pertussis toxin (25 ng/ml, 16 h) prior to cell harvest caused complete ADP-ribosylation of the pool of pertussis toxin-sensitive G-proteins in these cells as subsequent treatment of membranes derived from these cells with dithiothreitol-activated pertussis toxin and [³²P]NAD was unable to produce incorporation of radioactivity into polypeptides of some 40 kDa (Fig. 5). Following such pertussis toxin-pretreatment, cholera toxin was still able to catalyze ADP-ribosylation of the 45- and 42-kDa forms of G₁α (Fig. 5), but U.K.14304 was unable to promote cholera toxin-catalyzed ADP-ribosylation of the 40-kDa polypeptide(s) (Fig. 6A). As such, uncoupling of the α₁C10 adrenergic receptor from the G-protein signaling system by pertussis toxin prevented receptor agonist regulation of the cholera toxin-catalyzed ADP-ribosylation of the 40-kDa polypeptide(s) and thus defined that the 40-kDa polypeptide(s) was the G-protein with which the α₁C10 adrenergic receptor interacts in these cells. By contrast treatment of clone 1C cells with cholera toxin did not prevent subsequent agonist promotion of cholera toxin-catalyzed ADP-ribosylation of the 40-kDa polypeptide(s) but it did prevent cholera toxin-catalyzed ADP-ribosylation of the 45- and 42-kDa forms of G₁α (results not shown). Cholera toxin treatment of clone 1C cells to promote cholera toxin-catalyzed ADP-ribosylation of the 40-kDa polypeptide. C, U.K.14304 stimulation of high affinity GTPase activity in membranes of clone 1C cells. Basal and U.K.14304-stimulated high affinity GTPase activity was assessed in membranes of clone 1C cells. In this experiment the basal high affinity GTPase activity was 63.0 ± 1.3 (mean ± S.D.) pmol/min/mg membrane protein. Parallel experiments with membranes of clone 4D cells produced a basal GTPase activity of 55.3 ± 2.2 (mean ± S.D.) pmol/min/mg membrane protein. This value was not increased by the addition of U.K.14304. D, lack of effect of U.K.14304 on cholera toxin-catalyzed ADP-ribosylation of a 40-kDa polypeptide(s) in membranes of clone 4D cells. Membranes (60 μg) of clone 4D cells were treated with thiol-activated cholera toxin and [³²P]NAD in the absence of added guanine nucleotides as described under “Experimental Procedures.” U.K.14304 (10 μM) were also present in the sample displayed in 2 but not in 1. Samples were precipitated and resolved on SDS-PAGE (10% (w/v) acrylamide) as in Fig. 1. The gel was stained with Coomassie Blue, dried, and autoradiographed for 48 h.
also produced a marked (>90%) reduction of levels of membrane-associated G\(\alpha\) as assessed by immunoblotting with a specific anti-peptide antisera (CS1) against the C-terminal decapeptide of all forms of G\(\alpha\) (results not shown) as has recently been reported for a variety of other cells (23, 24).

Somewhat surprisingly, the effect of Mg\(^{2+}\) to produce agonist-independent cholera toxin-catalyzed ADP-ribosylation of the 40-kDa polypeptide(s) was also attenuated by pertussis toxin-pretreatment of the clone 1C cells (Fig. 6B). The effect of Mg\(^{2+}\) was also evident in membranes from clone 4D cells, which, as noted above, do not express the receptor (results not shown). Furthermore, the effect of Mg\(^{2+}\) in membranes of clone 4D cells was also abolished by pretreatment of the cells with pertussis toxin (results not shown). In membranes from both 1C and 4D cells, as with U.K.14304 stimulation of cholera toxin-catalyzed ADP-ribosylation of the 40-kDa polypeptide, addition of either GDP (100 \(\mu\)M) or GDP (100 \(\mu\)M) + fluoroaluminate (20 \(\mu\)M) completely prevented the Mg\(^{2+}\)-mediated ADP-ribosylation of this polypeptide, although both GDP and GDP + fluoroaluminate produced a very substantial increase in labeling of the 45- and 42-kDa forms of G\(\alpha\). Fluoroaluminate in the absence of GDP had no effect on the labeling of either the forms of G\(\alpha\) or of the 40-kDa polypeptide(s) (Fig. 7).

The 40-kDa polypeptide(s) labeled by cholera toxin in the presence of agonist or of Mg\(^{2+}\) was not a proteolytic fragment of G\(\alpha\) as immunoblots of membranes from clone 1C cells following the cholera toxin-catalyzed ADP-ribosylation did not detect the production of a polypeptide of 40 kDa with G\(\alpha\)-like immunoreactivity (results not shown) and addition of U.K.14304 did not promote the appearance of such a polypeptide (results not shown).

Using a series of anti-peptide antisera directed against sections of the primary amino acid sequence of individual G-proteins (Table II), we examined which G-proteins were expressed by cells of the 1C clone (Fig. 8). Immunoblotting of membranes of these cells with antiserum CS1 which identifies all forms of G\(\alpha\) demonstrated the expression of both 45- and 42-kDa forms of G\(\alpha\). Antiserum SG1, which identifies the \(\alpha\) subunits of both G1 and G2 equally (11), demonstrated that these cells express both G1 and G2 and further experiments in conditions which conveniently resolve these polypeptides (see Ref. 11 for details) demonstrated that G2 was present at markedly higher levels (some 10-fold) than G1 (results not shown). Independent evidence for the expression of G1\(\alpha\) was obtained by immunoblotting membranes from 1C cells with antiserum IM1, which acts as a specific probe for G\(\alpha\), unable to detect expression of this G-protein. Cells of clone 4D expressed the same complement of G-proteins at similar levels as the 1C cells (results not shown).

In order to define the molecular identity of the 40-kDa polypeptide(s) which was [\(^{32}\)P]ADP-ribosylated by cholera toxin in response to activation of the \(\alpha\)2-C10 receptor, we performed a series of such cholera toxin-catalyzed [\(^{32}\)P]ADP-ribosylations in the absence and presence of a receptor saturating concentration of U.K.14304 (10 \(\mu\)M) and subsequently attempted to immunoprecipitate G-protein \(\alpha\) subunits which had been covalently radiolabeled. Immunoprecipitation with antiserum CS1 brought down both the 45- and 42-kDa forms of G\(\alpha\) but did not precipitate a [\(^{32}\)P]ADP-ribosylated 40-kDa polypeptide (Fig. 9, lanes 1 and 2). No difference in the degree of incorporation of [\(^{32}\)P]ADP-ribose into the immunoprecipitated 45- and 42-kDa polypeptides was evident in the samples which had been incubated in the presence or absence of U.K.14304. By contrast, antiserum SG1 did not immunoprecipitate the 45- or 42-kDa polypeptides but did precipitate a 40-kDa polypeptide which had been [\(^{32}\)P]ADP-ribosylated by cholera toxin in a manner which was dependent upon agonist activation of the \(\alpha\)2-C10 adrenergic receptor (Fig. 9, lanes 3 and 4). Immunoprecipitation with antiserum IM3 produced a similar pattern to antiserum SG1. The 45- and 42-kDa polypeptides were not immunoprecipitated by this antiserum but a [\(^{32}\)P]ADP-ribosylated polypeptide of approximately 41 kDa.

![Figure 4](image_url)

**Fig. 4.** A, cholera toxin-catalyzed ADP-ribosylation of a 40-kDa polypeptide in membranes of clone 1C cells. The effect of Mg\(^{2+}\). Membranes (60 \(\mu\)g) of clone 1C cells were ADP-ribosylated with cholera toxin as in Fig. 1 in the absence (a) and presence (b–f) of U.K.14304. Concentrations of U.K.14304 were 1 nM (b), 10 nM (c), 100 nM (d), 1 \(\mu\)M (e), 10 \(\mu\)M (f). Mg\(^{2+}\) was absent from the samples in A but present at 1 mM in the samples in B. Further analysis of the samples was performed as in Fig. 1. B, the effect of Mg\(^{2+}\) on U.K.14304 stimulation of cholera toxin-catalyzed ADP-ribosylation in clone 1C cells. Quantitative analysis. The autoradiograph from A was scanned to assess the effectiveness of U.K.14304 to promote cholera toxin-catalyzed ADP-ribosylation of the 40-kDa polypeptide(s) in the presence (○) and absence ( ●) of Mg\(^{2+}\) (1 mM).
The antisera were generated in New Zealand White Rabbits using a conjugate of the synthetic peptide and keyhole limpet hemocyanin as detailed under "Experimental Procedures." The specificities of the antisera have previously been demonstrated (see Ref. 11 for example). G1α and G2α have identical C-terminal decapetidyls and thus antisera SG1 identifies each of these polypeptides equally. Forms of transducin α (TD1, TD2) are also identified by antisera SG1 but as these G-proteins are restricted in distribution to photoreceptor containing tissues then the antisera can be used as a probe for G1 or G2 in all other locations.

<table>
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<tr>
<th>Antiserum</th>
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<td>SG1</td>
<td>KENLKDCGLF</td>
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**Fig. 7.** The effect of guanine nucleotides on Mg2+-dependent cholera toxin-catalyzed ADP-ribosylation of a 40-kDa polypeptide(s). Cholera toxin-catalyzed ADP-ribosylation of membranes (60 μg) of clone 1C cells was performed as described under "Experimental Procedures" in the presence of 1 mM Mg2+ but in the absence of U.K.14304. In lane b GDP (100 μM) was also included. In lane c GDP (100 μM) + fluoroaluminate (20 μM) (20 μM aluminum sulfate, 10 mM sodium fluoride) was included, and in lane d fluoroaluminate (20 μM) was present. There were no further additions to lane a. Samples were further treated as in Fig. 1.

The very low levels of G1α present in membranes of cells of clone 1C (see above). The immunoprecipitation of both G2α and G3α which have become [32P]ADP-ribosylated by cholera toxin in a fashion which was completely dependent upon agonist activation of the α2-C10 receptor defines that the agonist-activated receptor must be able to interact functionally with both of these G-proteins in membranes of clone 1C cells.

**DISCUSSION**

One of the most useful and universal means of identification of G-proteins has been based on the realization that the majority of identified G-protein α subunits are substrates for mono-ADP-ribosylation catalyzed by either pertussis or cholera toxin. As such, the use of [32P]NAD as substrate for such ADP-ribosylation reactions has allowed the incorporation of a covalent label into G-protein α subunits. This has provided a useful means of identification of the polypeptide(s) either following SDS-PAGE and autoradiography or throughout purification schemes. Further, the fact that ADP-ribosylation of G-protein α subunits by these toxins has important functional consequences has allowed examination of the role(s) of a number of these proteins in particular transmembrane signaling cascades. Pertussis toxin is now known to catalyze the ADP-ribosylation of the α subunits of at least six distinct G-proteins, TD1, TD2, G1, G2, G3, and Gα, and examination of the primary sequence of these polypeptides as predicted from relevant cDNA clones indicates that all pertussis toxin-sensitive G-proteins have a cysteine residue four amino acids from the C-terminal (25). The location of a cysteine residue at such a position is not sufficient, however, to promote pertussis toxin-catalyzed ADP-ribosylation. Construction and
expression of a mutant form of Gα with a cysteine in this position did not cause this polypeptide to become a substrate for pertussis toxin-catalyzed ADP-ribosylation (26). The knowledge of the diversity of G-protein substrates for pertussis toxin-catalyzed ADP-ribosylation has, however, meant that this toxin cannot be used to define a specific function in a cellular signaling process which is obliterated by treatment with the toxin to a specific pertussis toxin-sensitive G-protein (8). More selective approaches, based on either the use of specific antisera (10-12, 27-29) or on agonist-promotion of the binding of photoaffinity labels for the G-proteins (30) are actively being developed.

Cholera toxin is traditionally considered to have a much more limited range of G-protein substrates than pertussis toxin. Indeed, of the G-proteins which are widely expressed, Gα is the only well-characterized G-protein which is ADP-ribosylated by this toxin. The site of cholera toxin-catalyzed ADP-ribosylation in Gα is an arginine residue (Arg-201 in the long form of Gα). This arginine residue appears to be a key site for the GTPase activity of Gα which provides the turn-off reaction for activated Gα. Mutation of this amino acid to any other produces a constitutively active form of the polypeptide (26, 31) and indeed subpopulations of patients with pituitary adenoma, in which the phenotype results from a constitutive activation of adenylate cyclase (32), have been noted to have a somatic mutation of Gα at this amino acid (33).

As all G-protein α subunits act as enzymatic GTPases and an arginine residue is located at the equivalent position in the primary sequence to Arg-201 of Gα (Table III), then it is perhaps surprising that all G-proteins do not appear to be substrates for cholera toxin-catalyzed ADP-ribosylation, particularly as it is well established that transducin (TD1) from rod outer segments can be a substrate for either pertussis or cholera toxin under appropriate conditions (34). In this report we demonstrate that at least some pertussis toxin-sensitive G-proteins can become substrates for cholera toxin-catalyzed ADP-ribosylation and that because of the requirements of the assay to produce such an effect, this approach can be used to identify G-proteins which interact

Table III

<table>
<thead>
<tr>
<th>Gα</th>
<th>C.T.</th>
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<td>G1</td>
<td>172</td>
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<td>G2</td>
<td>173</td>
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<td>G3</td>
<td>172</td>
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<td>Gα</td>
<td>173</td>
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Fig. 8. **G-proteins expressed by clone 1C cells.** Membranes of clone 1C cells, rat glioma C6 cells, and rat brain were resolved on SDS-PAGE (10% (w/v) acrylamide) and transferred to nitrocellulose. The nitrocellulose was blocked as described under “Experimental Procedures” and then incubated with a range of antipeptide antisera which have been demonstrated to identify the α subunits of particular G-proteins (11) (see also Table II). Membranes selected to compare with the 1C cell membranes have previously been demonstrated to express each of the G-proteins in question and hence act as a positive control. A, antiserum 1IC (versus G1α) (1) rat brain membranes, 25 μg. (2) 1C membranes, 50 μg. B, antiserum SG1 (versus G2α) (1) rat glioma C6 membranes, 25 μg. (2) 1C membranes, 50 μg. C, antiserum E3B (versus G3α) (1) rat glioma C6 membranes, 50 μg. (2) 1C membranes, 50 μg. D, antiserum IM1 (versus Gα) rat brain membranes, 10 μg. (2) 1C membranes, 50 μg. E, antiserum CS1 (versus Gα) (1) rat glioma C6 membranes, 25 μg. (2) 1C membranes, 50 μg. Blots were incubated overnight with the primary antiserum, washed extensively, and incubated with an anti-rabbit IgG linked to horseradish peroxidase. Following removal of the secondary antiserum and further washes the immunoblots were developed using o-dianisidine as substrate. Clone 1C membranes have detectable levels of G1, G2, and G3 but not Gα.

Fig. 9. **Immunoprecipitation of pertussis toxin-sensitive G-proteins containing [32P]ADP-ribose introduced by cholera toxin defines that the activated α2-C10 adrenergic receptor interacts directly with both G2 and G3.** Cholera toxin-catalyzed [32P]ADP-ribosylations of membranes of clone 1C cells were performed as in Fig. 1. Samples displayed in lanes 2, 4, and 6 contained labeled pertussis toxin-catalyzed ADP-ribosylations as described under “Experimental Procedures” with antiserum CS1 (lanes 1 and 2), SG1 (lanes 3 and 4), or E3B (lanes 5 and 6). We have previously demonstrated the use and selectivity of these antisera in immunoprecipitation assays (22). Samples were resolved by SDS-PAGE (10% acrylamide) and the resulting gel dried and autoradiographed. Similar results were obtained in three further experiments.
Receptor Activation of Multiple G-proteins

with particular receptors. Cholera toxin has been demonstrated to cause weak ADP-ribosylation of a 49-kDa polypeptide(s) in membranes of a number of tissues, including each of rat adipose tissue (38), RAW 264 macrophages (36), rat glioma C6 cells (37), neuroblastoma x glioma hybrid, NG108-15, cells (16), and HL60 cells (14, 15, 38). The majority of studies which have examined this process in any detail have used membranes from HL60 cells and have noted that the addition of the bacterial chemotactic factor, fMLP markedly promotes cholera toxin-catalyzed [32P]ADP-ribosylation of either one (14, 15) or two (38) polypeptides close to 40 kDa. Based on the known expression of both G2 and G3 by HL60 cells (38) it is likely, although it has not been demonstrated directly, that the two polypeptides identified by Gierschik et al. (38) are indeed the α subunits of these G-proteins. fMLP causes activation in these cells of, at least, a phospholipase A2 and a phospholipase C which promotes the production of inositol 1,4,5-trisphosphate. However, no evidence is available to define if the various effects of fMLP are produced by a single receptor or, as has been demonstrated recently for a number of pharmacologically similar receptors, that the various effects may be produced by distinct receptors produced either from highly homologous genes or by differential splicing of pre-mRNA transcribed from a single gene.

To circumvent problems associated with a lack of detailed knowledge of the receptor complement of a cell type, in these experiments, we have taken advantage of the availability of DNA corresponding to a single molecularly defined receptor to address the question of whether, in a native membrane a single receptor can interact functionally with more than a single species of pertussis toxin-sensitive G-protein. We have demonstrated clearly that the agonist occupied form of the α2-C10 receptor has the intrinsic ability to interact with both G2 and G3, and as far as we are aware this is the first report to demonstrate the interaction of what is clearly a single receptor subtype with multiple pertussis toxin-sensitive G-proteins. We have unambiguously defined the identity of these G-proteins by immunoprecipitation of the polypeptides with selective antisera (Fig. 9). It is of vital importance to the conclusions produced by these data that it be well established that the antisera used show no mutual cross-reactivity. We have demonstrated clearly that the agonist occupied form of the α2-C10 receptor has the intrinsic ability to interact with both G2 and G3, and as far as we are aware this is the first report to demonstrate the interaction of what is clearly a single receptor subtype with multiple pertussis toxin-sensitive G-proteins. We have unambiguously defined the identity of these G-proteins by immunoprecipitation of the polypeptides with selective antisera (Fig. 9). It is of vital importance to the conclusions produced by these data that it be well established that the antisera used show no mutual cross-reactivity.

Receptor  Activation  of  Multiple  G-proteins

Multiple  G-proteins

before it could act as a substrate for cholera toxin-catalyzed [32P]ADP-ribosylation, we performed a series of experiments to assess the role of guanine nucleotides in the effect. Addition of GDP (100 μM) prevented completely the ability of U.K.14304 to stimulate cholera toxin-catalyzed ADP-ribosylation of the 40-kDa polypeptides. Because 1) GTP added to such an assay would be likely to be converted rapidly to GDP, 2) inconsistencies in the effects of GTPγS and Gpp(NH)p as analogues of GTP have been noted in similar experiments with HL60 cells (14) and, 3) we wished to mimic the effects of the natural guanine nucleotides which regulate G-protein activation/deactivation in the cell as closely as possible, then as a mimic of GTP we added GDP + fluoroaluminate. This anion can interact with GDP bound to G-proteins and imitate the terminal phosphate of GTP, hence leading to quasipersistent activation of the G-protein. This mixture also completely abolished the effect of U.K.14304. However, addition of fluoroaluminate without GDP did not alter the ability of U.K.14304 to promote cholera toxin-catalyzed ADP-ribosylation of the 40-kDa polypeptide demonstrating that the effect was not produced directly by the fluoroaluminate anion.

The addition of Mg2+ to such assays promoted agonist-independent cholera toxin-catalyzed [32P]ADP-ribosylation of the 40-kDa polypeptides when membranes of either clone 1C or 4D cells were used as the acceptor. This fact defines that the effect of Mg2+ is not produced at the level of the α-C10 receptor. Further, addition of U.K.14304 in the presence of Mg2+ to assays using membranes from clone 1C produced an additive effect of these two agents. As with the effect of U.K.14304, the effect of Mg2+ to stimulate cholera toxin-catalyzed ADP-ribosylation of the 40-kDa polypeptide(s) in 1C membranes was attenuated by pretreatment of the cells with pertussis toxin. This was unexpected as the effect of pertussis toxin is generally believed to be restricted to preventing contact between receptors and pertussis toxin-sensitive G-proteins. It was perhaps even more surprising that pertussis toxin-pretreatment of cells of clone 4D prevented subsequent Mg2+ stimulation of cholera toxin-catalyzed ADP-ribosylation of the 40-kDa polypeptide in membranes of these cells. We cannot provide a ready explanation of these results at this stage, but it is true that Mg2+ and other divalent cations promote the formation of high affinity states for agonists of G-protein-coupled receptors (11) which are believed to represent the complex of receptor and G-protein. It may be that Rat 1 cells express an as yet undefined receptor which can interact with the G-protein signaling system to mediate inhibition of adenylate cyclase. A receptor for lysophosphatidic acid may be a potential candidate (40). It appears that agonist-unoccupied receptors can stimulate G-protein GTPase activity, at least in certain circumstances (41, 42). This implies that GDP must be released from the G-protein by the empty receptor, which in the conditions of the cholera toxin-catalyzed ADP-ribosylation assays performed here would be anticipated to provide a form of the G-protein which could act as a substrate for cholera toxin-catalyzed ADP-ribosylation.

The approach which we have defined in these studies, in which incorporation of a covalent label into G-proteins in a manner which is strictly dependent upon the interaction of that G-protein with an agonist-activated receptor, is likely to be useful for the identification of functional interactions between receptors and pertussis toxin-sensitive G-proteins. The further ability to identify these G-proteins unambiguously by immunoprecipitation of the labeled polypeptide(s) with selective antisera provides a novel approach, distinct from reconstitutive assays, which will allow detailed assess-
ments of the specificity (or otherwise) of such interactions in
the native membrane.

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