Coupling of the α_2 -adrenergic receptor to the inhibitory G-protein G_i and adenylate cyclase in HT29 cells

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Previous studies have established that the human colon carcinoma cell line HT29 expresses an α_2 -adrenergic receptor of the α_2A subtype, which is negatively coupled to adenylate cyclase. The purpose of the present study was to examine the mechanisms of α_2 -adrenergic signal transduction in these cells. [32 P]ADP-ribosylation with pertussis toxin and immunoblots using antibodies specific for the G_i α -subunits indicated that two distinct G_i -proteins (G_{i2} and G_{i3}) were present in HT29-cell membranes. Treatment of intact cells with pertussis toxin resulted in a time-dependent decrease in the amount of [32 P]ADP-ribosylatable G_{i2} and G_{i3} , which coincided with a diminution in the number of α_2 -adrenergic receptors in high-affinity state for agonists and with a progressive loss of ability of UK14304 to inhibit forskolin-stimulated accumulation of cyclic AMP. When membranes were [32 P]ADP-ribosylated with cholera toxin in the absence of

exogenous added guanine nucleotides, radioactivity was incorporated into a 45 kDa polypeptide representing G_s , as well as into 40–41 kDa polypeptides corresponding to G_{i3} and G_{i2} . The amount of radioactivity incorporated into the two G_i s under basal conditions was decreased by addition of the α_2 -antagonist RX821002. It was not significantly affected by addition of clonidine (partial α_2 -agonist), but was doubled by the addition of UK14304 (full α_2 -agonist). This effect was blocked by RX821002. Study of adenylate cyclase activity indicated that preincubation of HT29 membranes with the antibody AS/7 (anti- α_1/α_2), but not with the antibody EC/2 (anti- α_3), attenuated the inhibitory effect of UK14304 on forskolin-stimulated adenylate cyclase. These data demonstrate that the α_2A -adrenergic receptor is coupled to both G_{i2} and G_{i3} , and identify G_{i2} as the major mediator of inhibition of adenylate cyclase in HT29 cells.

INTRODUCTION

The α_2 -adrenergic receptors have a wide distribution in the organism and participate in the regulation of important physiological functions such as lipolysis in adipose tissue, insulin release from pancreatic- β cells, electrolyte secretion in the intestine and aggregation of platelets [1]. Lowering of intracellular cyclic AMP via inhibition of adenylate cyclase is a general pathway of α_2 -adrenergic signal transduction. This mechanism of coupling is, however, not unique, since α_2 -adrenergic receptors were demonstrated to couple with other effectors, including K^+ channels, voltage-operated Ca^{2+} channels, phospholipase C and phospholipase D [2]. In all tissues or cells examined so far, the response to α_2 -agonists was abolished by prior treatment with pertussis toxin, demonstrating the crucial role of G-proteins as mediators of signal progression across the membrane [3].

Cloning techniques have led to the discovery of multiple forms of pertussis-toxin-sensitive G-proteins. These include three forms of G_i proteins (G_{i1} , G_{i2} and G_{i3}) and G_o [4,5]. All these G-proteins are heterotrimers ($\alpha\beta\gamma$) and differ each other by their α -subunit (α_1 , α_2 , α_3 and α_o). Their activation by the agonist-occupied receptor provokes an exchange of the α -subunit-bound GDP for GTP and results in their dissociation into α and $\beta\gamma$ subunits. The molecular mechanisms whereby dissociation of G_i leads to inhibition of the catalytic unit of adenylate cyclase have been a matter of extensive discussion. It is generally accepted that both α_i subunit and $\beta\gamma$ complex participate in the decrease in the enzyme activity [6].

Quantification of the mRNA levels of the different forms of α_i and of α_o in various tissues demonstrated that their expression is

tissue-specific. However, the biological significance of the G_i diversity and the functional specificity of each G_i remain poorly understood. Reconstitution experiments demonstrated that the α_2 -adrenergic receptor can interact with inhibitory G-proteins with the order of potency $G_{i3} > G_{i1} \geq G_{i2} > G_o$ [7]. Besides this approach, which is only indicative of the potential interactions between the receptor and G-proteins in an artificial environment, several techniques have recently been developed which allow examination of the physical interactions between a given type of receptor, individual G_i -proteins and their effectors in native membranes. These techniques, which are based on the use of specific antibodies directed against the C-terminal decapeptide of the different α_i s [8] and on the property of cholera toxin to ADP-ribosylate α_i s under certain conditions [9], provided valuable information on the specificity of receptor- G_i -effector interactions. However, their use was so far limited to a small number of cell models.

The human adenocarcinoma cell line HT29 has been widely used to study intestinal cell differentiation and function, because it has kept the potency to express a large spectrum of receptors which are present in normal intestinal mucosa. In this respect, the HT29 model has been used by several groups to study α_2 -adrenergic receptivity. Previous results have demonstrated that the receptor is of the α_2A subtype [10] and that it is negatively coupled to adenylate cyclase [11]. The present study was carried out to determine more precisely the pathways of α_2 -adrenergic signal transduction in HT29 cells. We demonstrated that the receptor is coupled to both G_{i2} and G_{i3} . However, it is the activation of G_{i2} which is predominantly responsible for the inhibition of adenylate cyclase by α_2 -agonists.

Abbreviations used: RX821002, 2-(2-methoxy-1,4-benzodioxan-2-yl)-2-imidazoline; UK14304, 5-bromo-6-(2-imidazolin-2-ylamino)-quinoxaline; IBMX, 3-isobutyl-1-methylxanthine; DTT, dithiothreitol; B_{max} , maximal number of binding sites; K_d , equilibrium dissociation constant.

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MATERIALS AND METHODS

Drugs and reagents

[³H]RX821002 (sp. radioactivity 55 Ci/mmol) and ¹²⁵I-Protein A (sp. radioactivity 30 mCi/mg) were from Amersham International (Les Ulis, France). [³H]UK14304 (sp. radioactivity 63 Ci/mmol), [³²P]NAD⁺ (sp. radioactivity 800 Ci/mmol) and [α -³²P]ATP were from New England Nuclear (Boston, MA, U.S.A.). RX821002 was a gift from Reckitt and Colman Laboratories (Kingston-upon-Hull, U.K.). Other donated drugs came from the following sources: UK14304 tartrate (Pfizer, Sandwich, Kent, U.K.); clonidine hydrochloride (Boehringer, Ingelheim, Germany). Fetal-calf serum was purchased from IBF (Villeneuve-la-Garenne, France). Dulbecco's modified Eagle's medium (DMEM) was from Gibco BRL (Cergy-Pontoise, France). Radioimmunoassay kits for cyclic AMP determination were from Immunotech (Luminy, France). NAD⁺, creatine kinase, phosphocreatine, ATP and cyclic AMP were from Boehringer (Mannheim, Germany). Yohimbine, dithiothreitol (DTT), EDTA, thymidine, arginine, BSA, alumina, pertussis toxin and cholera toxin, forskolin, 3-isobutyl-1-methylxanthine (IBMX) and all other chemicals were from Sigma (St. Louis, MO, U.S.A.). The anti-G α antibodies (anti- α_o and anti- α_1/α_2) used for identification of G-proteins were prepared as described by Homburger et al. [12] and Rouot et al. [13]. When specified, we also used the commercial antibodies AS/7 and EC/2 from New England Nuclear (Boston, MA, U.S.A.).

Cell culture

The HT29 cell line was cultured in DMEM containing 25 mM glucose supplemented with 5% (v/v) heat-inactivated fetal-calf serum, 100 μ g/ml streptomycin and 100 i.u./ml penicillin under a humidified atmosphere (air/CO₂, 19:1). The cells were routinely passaged weekly by seeding at a density of 2×10^4 cells/cm² in plastic Petri dishes, and the medium was changed every 2 days. All experiments were performed on post-confluent attached cells (day 13 of culture) previously deprived of fetal-calf serum for 48 h.

Treatment of intact cells with pertussis toxin

Pertussis toxin (100 μ g/ml in 100 mM Tris/0.1% BSA/2 M urea, pH 7.4) was preactivated by treatment with 100 mM DTT for 30 min at 37 °C and then added to the culture medium at 250 ng/ml final concentration. Control cells were treated with pertussis-toxin adjuvant containing DTT. At the indicated time, medium was removed and the cell layers were rinsed with PBS. According to their further use, cells were isolated or quickly frozen at -80 °C until analysis.

Crude membrane preparation

The frozen cell layers were scraped in 5 ml of ice-cold TE buffer (50 mM Tris/HCl/5 mM EDTA, pH 7.5) and were further disrupted with a glass pestle homogenizer. The lysate was centrifuged for 10 min at 39000 g (4 °C). The particulate fraction obtained was washed once in TM buffer (50 mM Tris/HCl, 0.5 mM MgCl₂, pH 7.5). The final crude membrane pellet was resuspended in the required volume of TM buffer for immediate use.

[³²P]ADP-ribosylation of HT29-cell membrane by pertussis toxin

ADP-ribosylation was carried out essentially as described by Ribeiro-Neto [14]. Membranes (50–75 μ g of protein) suspended

in Tris/HCl buffer containing 1 mM EDTA, 1 mM DTT and 0.05% (v/v) Lubrol were incubated for 60 min at 30 °C in the presence of 1 μ Ci of [³²P]NAD⁺ and 100 ng of preactivated pertussis toxin in a final volume of 60 μ l. The reactional mixture contained 70 mM Tris/HCl (pH 8.0), 0.5 μ M NAD⁺, 1 mM ATP, 10 mM thymidine, 1 mM EDTA, 0.1 mM MgCl₂, 1 mg/ml L-myristoyl phosphatidylcholine, 10 mM nicotinamide and 25 mM DTT. The reaction was stopped by addition of 2 μ g BSA in 0.04% SDS, and the proteins were precipitated with 70 μ l of 10% trichloroacetic acid. After centrifugation, the pellets were washed twice with diethyl ether and finally dissolved in Laemmli's [14a] sample buffer by heating at 100 °C for 3 min. The samples were subjected to SDS/PAGE in 10% gels, stained with Coomassie Brilliant Blue R-250, destained, dried and exposed to Amersham Hyperfilm for 48 h at -80 °C. The amounts of G_i were determined by measuring the density of the specific band with a Biocom analyser.

Immunoblotting of α_o and α_1 subunits of G-proteins

Immunoblotting was carried out as described by Homburger et al. [12]. After SDS/PAGE on 10% gels, proteins were blotted on a nitrocellulose membrane (0.45 μ m) during 16 h at 150 mA. To minimize non-specific protein binding, the nitrocellulose sheet was treated with Tris/saline buffer (10 mM Tris/HCl, 500 mM NaCl, pH 7.5) containing 2% gelatin. The blots were incubated overnight at room temperature in the same buffer supplemented with 1% gelatin and containing 10 μ g/ml antibody (anti- α_1/α_2 or anti- α_o). After a series of washing in 50 mM Tris/HCl/500 mM NaCl/0.05% Tween 20, the blots were then incubated for 1 h with ¹²⁵I-Protein A (0.14 μ Ci/ml) in 50 mM Tris/HCl/500 mM NaCl/0.02% NaN₃. After extensive washing in Tris/HCl buffer containing 500 mM NaCl and 0.05% Tween 20, blots were dried and autoradiographed as indicated above.

Binding studies

Quantification of α_2 -adrenergic receptors was carried out as described elsewhere [15]. Total binding was measured by incubating membranes with [³H]RX821002 (0.2–10 nM) or [³H]UK14304 (0.05–3 nM) in TM buffer. After 60 min at 25 °C, the reaction was stopped by adding 4 ml of ice-cold washing buffer (10 mM Tris/HCl, 0.5 mM MgCl₂, pH 7.5). Bound radioligand was separated from free by filtration through GF/C Whatman filters under vacuum. The filters were immediately washed with 2×10 ml of washing buffer, placed in scintillation vials and counted for radioactivity by liquid-scintillation spectrometry. Specific binding was defined as the difference between total binding and non-specific binding measured in the presence of 10 μ M yohimbine. The binding parameters (B_{max} , K_d) were determined from equilibrium data by using a computer-assisted linear transformation of saturation isotherms [16]. Protein concentration was measured as described by Bradford [17], with BSA as standard.

Determination of cyclic AMP content

Cells were isolated by treating the culture dishes with 2.5 mg/ml trypsin and 0.6 mM EDTA in PBS and centrifuged (400 g, 5 min, 37 °C). The cellular pellet was resuspended in DMEM medium buffered with 25 mM Hepes. A 100 μ l portion of cell suspension (4 mg of protein/ml) was incubated in the presence of 0.2 mM IBMX and the indicated concentration of drug to be tested. After 20 min at 37 °C, the reaction was stopped by adding 2 ml of methanol/formic acid (19:1 v/v). The cell lysate was centri-

fused (3000 g, 10 min, 4 °C) and a portion of the supernatant was evaporated. The dry samples were resuspended in acetate buffer containing 0.1 % NaN_3 and their cyclic AMP content was determined by radioimmunological assay [18].

[^{32}P]ADP-ribosylation of HT29-cell membrane by cholera toxin

ADP-ribosylation with cholera toxin was performed in absence of added guanine nucleotide as described by Milligan [19]. Cholera toxin (50 $\mu\text{g}/\text{ml}$) was preactivated with 10 mM DTT in 100 mM KH_2PO_4 , pH 7.4, for 30 min at 37 °C. Membranes from HT29 cells were incubated for 90 min at 37 °C in 100 mM KH_2PO_4 buffer containing 0.5 μM NAD^+ , 1 mM ATP, 10 mM thymidine, 10 mM arginine, 0.01 % BSA, pH 7.4, 2.5 μCi of [^{32}P]NAD $^+$ and preactivated cholera toxin. The samples were then treated as indicated above for ADP-ribosylation experiments with pertussis toxin.

Measurement of adenylate cyclase activity

Adenylate cyclase activity was measured as described by Alvarez and Daniels [20]. Membranes (20 μg of protein) were incubated with 0.2 mM [α - ^{32}P]ATP in 100 μl of Tris/HCl buffer (40 mM, pH 7.4) containing 2 mM MgCl_2 , 5 mM phosphocreatine 50 units/ml creatine kinase, 1 mM cyclic AMP, 0.5 mM IBMX, 0.1 mM EGTA, 0.1 μM GTP, 0.2 mM ATP and 0.2 % BSA. After 30 min at 30 °C the reaction was stopped by addition of 20 μl of 2.2 M HCl containing 12 nCi of cyclic [^3H]AMP as an internal standard. Cyclic AMP was separated from ATP by chromatography on an alumina column. Results from measurement of adenylate cyclase are expressed as percentage inhibition of forskolin-stimulated activity.

RESULTS

Identity of the G_i proteins present in HT29 cells

A previous study on the HT29 cell line has demonstrated the existence of a 41 kDa protein which is [^{32}P]ADP-ribosylated by pertussis toxin [21]. This protein was referred to as G_i , but its subtype was not determined. The first aim of our study was therefore to identify the species of G-proteins expressed in these cells. SDS/PAGE analysis of the proteins [^{32}P]ADP-ribosylated by pertussis toxin indicated the presence of approximately equal amounts of two polypeptides in membrane from HT29 cells (Figure 1). Comparison of the mobility of these two proteins with that of pertussis-toxin substrates in bovine brain membrane suggested that the 40 kDa band corresponded to α_2 , whereas that of 41 kDa could be either α_1 or α_3 . Western-blot analyses were thus performed in order to identify these polypeptides definitively. Two antibodies generated against the C-terminal decapeptide of either α_1/α_2 or α_o subunit were used (Figure 2). The anti- α_1/α_2 antibody reacted with a single polypeptide in HT29 membranes which co-migrated with an equivalent polypeptide in human platelets, a cell type expressing α_2 but not α_1 [8]. As expected, this antibody recognized two polypeptides corresponding to α_1 and α_2 in bovine brain. On the other hand, the anti- α_o antibody, which is known to cross-react with α_3 , recognized a single band in HT29 membranes. This protein, which was also present in human platelets, corresponded to α_3 [8]. In membranes from bovine brain, besides α_3 , this antibody revealed a major 39 kDa protein corresponding to α_o . Strictly similar observations were made when the commercial antibodies

(AS/7 and EC/2) were used. Thus HT29 cells express two forms of G_i -proteins corresponding to G_2 and G_3 . According to densitometric analysis of the autoradiograms from a series of [^{32}P]ADP-ribosylation experiments ($n = 5$), the relative amounts of these two G_i -proteins were $38 \pm 1\%$ for G_2 and $62 \pm 3\%$ for G_3 .

Effect of pertussis toxin on α_2 -adrenergic receptivity

The coupling of α_2 -adrenergic receptors to G_i -proteins was investigated by treating HT29-cell layers with pertussis toxin during periods of time ranging from 0 to 24 h. The kinetics of ribosylation of the two G_i s were estimated by performing a second pertussis-toxin-catalysed ADP-ribosylation, but in the presence of [^{32}P]NAD $^+$. The consequences of G_i -protein inactivation were examined by studying the binding of selective radioligands to the α_2 -adrenergic receptors and by measuring the inhibitory effect of UK14304 on cyclic AMP production.

As depicted in Figure 3(a), where the amounts of [^{32}P]ADP-

Figure 1 [^{32}P]ADP-ribosylation of HT29-cell membranes by pertussis toxin

Membranes from HT29 cells were incubated for 60 min at 30 °C with thiol-preactivated pertussis toxin and [^{32}P]NAD $^+$. At the end of the incubation, the [^{32}P]ADP-ribosylated proteins were precipitated with trichloroacetic acid, resolved by SDS/PAGE and autoradiographed. The use of molecular-mass markers indicated that the only bands labelled were of apparent molecular masses 40 and 41 kDa. The respective amounts of radioactivity incorporated in the two proteins were determined by densitometric analysis of the autoradiogram with a Biocom analyser.

Figure 2 Identification of G-protein α -subunits with specific antisera

Membrane proteins from HT29 cells, human platelets or bovine brain were separated by SDS/PAGE and then transferred on to nitrocellulose sheets. Immunoblotting was performed with either anti- α_1/α_2 (left panel) or anti- α_3/α_o (right panel) antisera at a 1:250 dilution. Immunoblots were revealed by using ^{125}I -Protein A as indicated in the Materials and methods section.

Figure 3 Evaluation of the amounts of [32 P]ADP-ribosylatable G_i-proteins and of the number of binding sites for α_2 -agonist and α_2 -antagonist in membranes of HT29 cells treated with pertussis toxin

HT29 cells were treated with pertussis toxin, collected at the indicated time and used for the determination of their content of pertussis-toxin [32 P]ADP-ribosylatable G-proteins (a) and for the quantification of α_2 -adrenoceptors by using the specific agonist [3 H]UK14304 (■) or the specific antagonist [3 H]RX821002 (□) (b). In (a) the amounts of G_{i3} (■) and G_{i2} (▨) are expressed as percentages of that in controls. The maximum number of binding sites (B_{max}) is expressed in fmol/mg of membrane proteins. Reported values represent the means $\pm$ S.E.M. of three experiments.

ribosylatable G_i-proteins are plotted as a function of the time of treatment, a 18 h period of exposure of the cells to the toxin led to complete abolition of incorporation of 32 P into both G_{i2} and G_{i3}. The disappearance of G_{i2} and G_{i3} was progressive and occurred with similar kinetics.

In the range of concentration used (0.05–3 nM), [3 H]UK14304 only labelled the high-affinity-coupled receptors [22]. The study of [3 H]UK14304 binding (Figure 3b) revealed a time-dependent diminution of the number of high-affinity state receptors, with a total disappearance of [3 H]agonist-binding sites after 24 h of treatment. No change in the K_d value of [3 H]UK14304 (0.32 nM) was observed during the period of treatment. On the other hand, treatment with pertussis toxin had no effect on the number of receptors labelled by the antagonist radioligand [3 H]RX821002, indicating that the whole receptor population did not change during the experiment.

It has been previously shown that HT29-cell α_2 -adrenergic receptors are negatively coupled to adenylate cyclase and that their stimulation leads to a strong attenuation of the accumulation of intracellular cyclic AMP induced by either forskolin or vasoactive intestinal peptide [23,24]. The functional consequence

Figure 4 Evaluation of the inhibitory effect of UK14304 on the accumulation of cyclic AMP induced by VIP in HT29 cells treated with pertussis toxin

HT29 cells treated with pertussis toxin for periods ranging from 0 to 18 h were isolated by trypsin treatment as described in the Materials and methods section. Samples of the cell suspension were incubated in presence of 0.2 mM IBMX (■), IBMX + 10 nM VIP (□) or IBMX + VIP + 1 μ M UK14304 (▨). Intracellular cyclic AMP content was determined by radioimmunoassay and is expressed as pmol/mg of cell protein. Triplicate determinations were performed for each experimental point. Reported values are means $\pm$ S.E.M. of four independent experiments.

of the decrease in the number of high-affinity-state receptors was examined by measuring the inhibitory effect of UK14304 on forskolin-stimulated accumulation of cyclic AMP. Pertussis-toxin treatment of the cells had no effect on the basal level of cyclic AMP or on its accumulation provoked by forskolin (Figure 4). By contrast, the inhibitory effect of UK14304 was diminished by the treatment and was completely abolished after 12 h of culture in presence of toxin.

Identification of G_i-proteins coupled to the α_2 -adrenergic receptor

Recent reports have demonstrated that cholera toxin can catalyse the ribosylation of pertussis-toxin substrates. This reaction occurs in the absence of added guanine nucleotides and is enhanced when the G-protein-coupled receptor is stimulated by its specific agonist [25].

As shown in Figure 5, in the absence of exogenously added nucleotides, cholera toxin catalysed the labelling of a prevalent polypeptide species with an apparent molecular mass of 45 kDa. Besides this major substrate, which corresponds to the stimulatory G-protein G_s, cholera toxin also catalysed the incorporation of radioactivity into certain other substrates (lane 2). Among these substrates was a doublet (apparent molecular masses 40–41 kDa) which co-migrated with the α -subunits of the G_i-proteins (lane 6). This doublet corresponded to α_2 and α_3 , since, when a similar experiment was carried out with membranes prepared from HT29 cells previously treated with pertussis toxin for 12 h, cholera toxin could no longer label the 40–41 kDa bands (results not shown).

Addition of RX821002 into the reaction mixture (lane 1) provoked a significant decrease (68%) in the labelling of the 40–41 kDa polypeptides observed under basal conditions. This effect was specific, since the α_2 -adrenergic antagonist did not affect the incorporation of radioactivity into the other bands. In contrast, the addition of the full α_2 -adrenergic agonist UK14304 provoked a 2-fold increase in the labelling of the 40–41 kDa substrates (lane 3). Such an effect was not observed in the

Figure 5 Effect of stimulation of the α_2 -adrenoceptors on cholera-toxin-catalysed ADP-ribosylation of G_i -proteins

Membranes from HT29 cells were incubated with [32 P]NAD $^{+}$ and cholera toxin in the absence of added nucleotides. The reaction was performed in the presence of 10 μ M RX821002 (lane 1), in the absence of drug (lane 2), in the presence of 10 μ M UK14304 (lane 3), in the presence of 10 μ M clonidine (lane 4) and in the presence of 10 μ M UK14304 plus 10 μ M RX821002 (lane 5). At the end of the incubation, the labelled proteins were precipitated with trichloroacetic acid, separated by SDS/PAGE and autoradiographed for 48 h at -80°C . The only bands labelled in a toxin-dependent manner were of apparent molecular masses 40, 41 and 45 kDa. The amount of radioactivity incorporated in the 40 and 41 kDa bands was determined by densitometry. The results of this analysis are expressed in Table 1. Lane 6 corresponds to HT29-cell membranes incubated with [32 P]NAD $^{+}$ and pertussis toxin.

Table 1 Densitometric analysis of the amount of radioactivity incorporated in G_i -proteins [32 P]ADP-ribosylated by cholera toxin

HT29 cell membranes were [32 P]ADP-ribosylated by cholera toxin in the presence of different α_2 -adrenergic compounds as described in the legend of Figure 5. The amount of the 40–41 kDa labelled bands was quantified by densitometric analysis of the autoradiogram with a Biocom analyser. The results are expressed as percentages of control and represent means $\pm$ S.E.M. of four experiments.

α_2 -Adrenergic drug added (10 μ M)	Amount of cholera-toxin labelled G_i -proteins (% of control)
None	100
RX821002	32 $\pm$ 5
UK14304	181 $\pm$ 10
Clonidine	105 $\pm$ 3
UK14304 + RX821002	30 $\pm$ 2

Table 2 Effect of anti- α_i -subunit antibodies on the inhibition of adenylate cyclase activity by UK14304

Membranes from HT29 cells were incubated for 60 min at 37 $^{\circ}\text{C}$ with normal rabbit serum (control), with anti- α_i1/α_i2 antiserum (AS/7), with anti- α_i/α_i3 antiserum (EC/2) or with anti- α_i1/α_i2 plus anti- α_i/α_i3 antiserum (AS/7 + EC/2). All antisera were used at a 1:50 dilution. The activity of adenylate cyclase was determined in the presence of 10 μ M forskolin alone or plus 10 μ M UK14304. The inhibitory effect of the α_2 -agonist is expressed as a percentage. Reported values are means $\pm$ S.E.M. of three experiments. Quintuplicate determinations were performed for each experiment point. * indicates a significant difference from control ($P < 0.05$) by Student's t test (NS, no significant difference).

	Inhibition (%)
Control	29 $\pm$ 3.5
AS/7	12 $\pm$ 2*
EC/2	22.5 $\pm$ 6 (NS)
AS/7 + EC/2	13 $\pm$ 1.5*

presence of the partial agonist, clonidine (lane 4). Furthermore, the enhancement of the cholera-toxin-catalysed ribosylation of the 40–41 kDa polypeptides by UK14304 was antagonized by

RX821002 (lane 5). Again, none of these additions affected the labelling of the 45 kDa protein. The results of densitometric analysis of the amount of radioactivity incorporated in G_i -proteins are summarized in Table 1.

Identification of $G_i\alpha$ -subunit(s) involved in the inhibition of adenylate cyclase

Methods involving preincubation of membrane with antibodies raised against the C-terminal decapeptide of α_i -subunits have been used successfully in several cell types to determine which of the G_i -subtypes triggers inhibition of adenylate cyclase [8,26,27]. We therefore questioned whether such techniques could also be used in the HT29 model.

Measurement of adenylate cyclase on HT29 membranes indicated that UK14304 produced a $29 \pm 6\%$ inhibition of forskolin-stimulated activity (Table 2). Such a degree of inhibition is in agreement with that previously reported in these cells [24]. The inhibitory effect of UK14304 could be blocked by the α_2 -adrenergic antagonist RX821002 and was not affected by preincubation of the membranes with normal rabbit serum. Preincubation of membranes with the antiserum AS/7, which is specific for the α_i1/α_i2 C-terminus, strongly diminished the α_2 -adrenergic-receptor-mediated inhibition of adenylate cyclase (59% decrease of UK14304-induced effect). Under the same conditions, the antiserum EC/2 against the C-terminal decapeptide of α_i3 had no significant effect. Moreover, co-preincubation with both antisera did not produce a statistically greater attenuation of the UK14304-elicited effect than did AS/7 alone.

DISCUSSION

This work was carried out to investigate the coupling of the α_2 -adrenergic receptor to G_i -proteins and to adenylate cyclase in the colon carcinoma cell line HT29. [32 P]ADP-ribosylation experiments and Western-blot analyses demonstrated that HT29 cell membranes contained both G_i2 - and G_i3 -protein. Two reasons make the presence of G_i2 and G_i3 not surprising in HT29 cells. First, these two G_i -subtypes were found in all cells examined so far [28]. Second, both G_i2 and G_i3 , but not G_i1 and G_{i0} , were expressed by epithelial cells from rat jejunum mucosa [29,30] or from human duodenum and colon mucosa [31a]. As expected, pertussis toxin abolished the binding of [^3H]UK14304 and impaired the inhibitory effect of this α_2 -agonist, indicating that the toxin-modified G_i -proteins are involved in the maintenance of the high-affinity agonist-binding state of the receptor and in the inhibition of adenylate cyclase. However, from this sole experiment it was impossible to determine which of the two toxin substrates is coupled to the receptor and ensures inhibition of adenylate cyclase.

Milligan and colleagues have recently developed a technique which can be used to investigate the coupling between an inhibitory receptor and G_i -proteins in native membranes [9,25]. This technique, which is based on the ability of cholera toxin to ribosylate G_i -proteins in the absence of added nucleotide and on the property of receptor agonists to promote this reaction, was applied to HT29 cells. When cholera-toxin-catalysed [32 P]ADP-ribosylation was performed in the absence of adrenergic compound (basal condition), radioactivity was incorporated into the 45 kDa form of G_s as well as into 40–41 kDa polypeptides corresponding to G_i2 and G_i3 . Thus, as previously observed in membranes from other cell types, G_i -proteins are weak substrates for cholera toxin in HT29 cells. The amount of radioactivity in G_i2 and G_i3 , but not that in G_s , was increased by addition of

the α_2 -agonist UK14304. This effect was antagonized by the α_2 -antagonist RX821002, indicating that both G_{i2} and G_{i3} are coupled to the α_2 A-adrenergic receptor in HT29 cells. Such results are in agreement with those obtained in Rat-1 fibroblasts [25,31] or CHO-K1 cells [27] transfected with the α_2 -C10 gene coding for the α_2 A-adrenergic receptor. Contrarily to UK14304, clonidine was unable to promote cholera-toxin-catalysed ADP-ribosylation of G_i -proteins. The lack of effect of clonidine may be related to its partial agonist properties [22] and to the fact that we were unable to obtain any significant inhibition of adenylate cyclase by clonidine when activity was measured in membranes prepared from HT29 cells. Much more surprising was the finding that RX821002 by itself decreased the labelling of G_{i2} and G_{i3} observed under basal conditions. Such inhibition was unexpected, as antagonists are generally believed to be devoid of any effect. We do not have a ready explanation of this observation, but it is worth mentioning that the α_2 -adrenergic receptor was demonstrated to exist in a precoupled state in HT29 cells [22] as well as in human platelets [32]. The effect of RX821002 could therefore be interpreted as a blockade of G_i -protein activation by unoccupied receptors. Further experiments will be necessary to verify this hypothesis. Interestingly, Milligan et al. [25] found that Mg^{2+} increased the agonist-independent cholera-toxin ADP-ribosylation of G_{i2} and G_{i3} in Rat-1 fibroblasts, and this bivalent cation is known to be indispensable in the formation of the α_2 -adrenergic-receptor/ G_i -protein complex [22]. The inhibition of cholera-toxin-catalysed G_i -ribosylation provoked by RX821002 alone was not total. This may be due to the fact that HT29 cells express other inhibitory receptors such as somatostatin, which could be also precoupled with G_{i2} and G_{i3} .

Measurement of the inhibition of adenylate cyclase by UK14304 in the presence of anti- G_i antisera demonstrated that the anti- G_{i2} antiserum AS/7 blocked 59% of the α_2 -adrenergic-receptor-mediated inhibition of adenylate cyclase. On the other hand, addition of the anti- G_{i3} antiserum EC/2 had no effect. According to these results, it can be concluded that it is G_{i2} which is responsible for inhibition of adenylate cyclase. In this respect the situation in HT29 cells resembles that in human platelets [8] and transfected Rat-1 fibroblasts [25,31]. It is by contrast different from that in transfected CHO cells, where both G_{i2} and G_{i3} were shown to participate in inhibition [27]. The role of G_{i3} in HT29 cells remains to be clarified. The α_2 -adrenergic receptor is known to regulate chloride secretion in intestinal cells, and this effect is in some way independent of the cyclic AMP pathway [33]. Interestingly, patch-clamp experiments on apical membranes from the HT29-19A clone revealed the existence of a Cl^- channel which can be activated by GTP analogues [34]. The identity of the G-protein responsible for the modulation of this Cl^- channel is not established yet, but G_{i3} may be a good candidate for such a role.

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