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G_i3 does not contribute to the inhibition of adenylate cyclase when stimulation of an α_2-adrenergic receptor causes activation of both G_i2 and G_i3

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Agonist occupancy of the α_2-C10 adrenergic receptor in a stable clone (1C) of Rat 1 fibroblasts produced by transfection of cells with genomic DNA encoding this receptor causes the activation of both of the pertussis-toxin-sensitive G-proteins G_i2 and G_i3 [Milligan, Carr, Gould, Mullaney & Lavan (1991) J. Biol. Chem. 266, 6447–6455]. An IgG fraction from an antiserum (I3B) which identifies the *C*-terminal decapeptide of $G_i3\alpha$ only was able to inhibit partially receptor stimulation of high-affinity GTPase activity. An equivalent fraction from an antiserum (AS7) able to identify the *C*-terminal decapeptide of $G_i1\alpha + G_i2\alpha$, but not $G_i3\alpha$, was also able to inhibit partially receptor stimulation of GTPase activity, and the effects of the two antisera were additive. By contrast, agonist-mediated inhibition of forskolin-amplified adenylate cyclase activity was abolished completely by the IgG fraction of antiserum AS7, but was not decreased by treatment with antiserum I3B. Based on the proportion of agonist-stimulated high-affinity GTPase which was prevented by each antiserum and on the measured membrane levels of G_i2 and G_i3, calculations indicated that essentially all of the cellular G_i3, but only 15% of the available G_i2, can be activated by the α_2-C10 adrenergic receptor in these cells. These results demonstrate that, although G_i3 is activated by α_2-adrenergic agonists in membranes of clone 1C cells, it does not contribute to the transduction of receptor-mediated inhibition of adenylate cyclase.

INTRODUCTION

Receptor-mediated inhibition of adenylate cyclase is transduced by a pertussis-toxin-sensitive heterotrimeric guanine nucleotide-binding protein (G-protein) called 'G_i' [1]. Three of the heterotrimeric G-proteins which are substrates for pertussis-toxin-catalysed ADP-ribosylation are named G_i1, G_i2 and G_i3 [1]. The term 'G_i' was used initially to describe the pertussis-toxin substrate which was responsible for transducing receptor-mediated inhibition of adenylate cyclase, and, as it appeared that there was only a single polypeptide which was modified by the toxin, this definition was adequate. It is now clear that the α subunits of at least six G-proteins can be modified by pertussis-toxin-catalysed ADP-ribosylation [1]. Although the limited tissue distribution of four of these G-proteins, G_i1, G_o, TD1 and TD2 [2], defines that they could not function universally as 'G_i', both G_i2 and G_i3 appear to be expressed by all cells [2]. In studies on both neuroblastoma × glioma hybrid NG108-15 cells [3] and human platelets [4], it has been noted that inhibition of adenylate cyclase is transduced by G_i2. However, in the studies to date it has appeared that G_i2 has been the only G-protein to have been activated by the receptor, and hence it has been unclear if receptor-activated G_i3 would be physically capable of regulating adenylate cyclase.

We have recently generated a clonal cell line (1C) by transfection of Rat 1 fibroblasts with genomic DNA encoding the α_2-C10 adrenergic receptor [5]. Agonist activation of the receptor in membranes of these cells causes the activation of both G_i2 and G_i3 [5]. In this study we demonstrate that activated G_i3 does not contribute to α_2-C10 receptor-mediated inhibition of adenylate cyclase in these cells and that this effect can be accounted for by the action of activated G_i2.

MATERIALS AND METHODS

Materials

[γ-^{32}P]GTP, cyclic [^{32}P]AMP and [α-^{32}P]ATP were obtained from Amersham International. All materials for tissue culture were from GIBCO/BRL. Recombinant G-protein α subunits were expressed in *Escherichia coli* and subsequently purified as described previously [6].

Methods

Cells. Clone 1C was generated as previously described in detail [5] by transfection by calcium phosphate precipitation of Rat 1 fibroblasts with genomic DNA encoding the human α_2-C10 adrenergic receptor [7] linked to the mammalian expression vector pDOL. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) donor calf serum, penicillin (100 units/ml) and streptomycin (100 μg/ml) in 5% CO_2 in air at 37 °C. Membranes from the cells were prepared by homogenization with a Teflon/glass homogenizer and differential centrifugation as described for a variety of cells [8].

Adenylate cyclase assays. Forskolin (10 μM)-amplified adenylate cyclase activities and α_2-C10 receptor modulation of this activity were measured as in [9]. Resolution of [α-^{32}P]ATP and cyclic [^{32}P]AMP was performed by the method of Salomon *et al.* [10].

High-affinity GTPase assays. These were performed as in [3] with [γ-^{32}P]GTP (0.5 μM, 60000 c.p.m.). Low-affinity GTPase activity was assessed by parallel assays containing 100 μM-GTP. The effect of agonist was assessed by the addition of the selective α_2-adrenergic agonist UK14304. This agent had no effect on low-affinity GTPase.

Immunological studies. The generation of antisera AS7 [11],

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Table 1. Antisera against the *C*-terminal decapeptide of $G_i1\alpha + G_i2\alpha$ and against the same region of $G_i3\alpha$ both partially prevent α_2-C10-adrenergic stimulation of high-affinity GTPase activity in membranes of clone 1C cells

Membranes (5 μg) from 1C cells were preincubated with Protein A–Sepharose-purified IgG fractions (10 μg) from normal rabbit sera (NRS) and from the anti-G-protein antisera AS7 and I3B for 60 min at 37 °C, and subsequently the ability of UK14304 to stimulate high-affinity GTPase activity was assessed as in [3]. Both AS7 and I3B in isolation produced a statistically significant decrease in the effect of the agonist compared with NRS ($P < 0.001$ in each case, Student's *t* test) and the combination of AS7 and I3B produced a statistically significant decrease compared with either antibody alone ($P < 0.001$ in each case) (see the Results and discussion section). High-affinity GTPase activity in the absence of UK14304 (basal activity) after preincubation with the IgG fraction of NRS was 22.3 ± 1.4 pmol/min per mg of membrane protein. Results are presented as means ± S.E.M. ($n = 3$).

Antiserum	High-affinity GTPase activity stimulated by UK14304 (10 μM) (pmol/min per mg of membrane protein)	(% decrease)
NRS	42.0 ± 2.3	–
AS7	24.7 ± 1.6	41
I3B	31.6 ± 1.6	25
AS7 + I3B	15.5 ± 0.8	65

I3B [12] and CS1 [13] has been described previously. IgG fractions were prepared from these antisera and from normal rabbit serum by chromatography on a column of Protein A–Sepharose 4B (Pharmacia) as previously described [3]. Preincubation of membranes (5 μg) of clone 1C cells with purified IgG fractions was for 60 min at either 30 °C (adenylate cyclase) or 37 °C (GTPase). Subsequently forskolin-amplified adenylate cyclase activity and its modulation by UK14304 and agonist regulation of high-affinity GTPase activity were measured as above.

Immunoblotting studies. Membrane samples were resolved by SDS/PAGE (10% acrylamide) in 14 cm × 16 cm slab gels overnight at 60 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, 500 mM-NaCl). Primary antiserum (1:200 dilution unless otherwise noted) 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 0.1% Tween 20 and then TBS. The antigen–antibody complex was detected with ^{125}I-Protein A.

RESULTS AND DISCUSSION

After a 60 min preincubation of membranes of clone 1C cells at 37 °C in the presence of an IgG fraction (10 μg) of normal rabbit serum, a receptor-saturating concentration (10 μM) of the α_2-adrenergic agonist UK14304 stimulated high-affinity GTPase activity by some 42.0 ± 1.2 pmol/min per mg of membrane protein (mean ± S.E.M., $n = 4$) (Table 1). Preincubation of membranes of clone 1C cells with an IgG fraction from antiserum AS7, which identifies $G_i1\alpha$ and $G_i2\alpha$, but not $G_i3\alpha$ (Fig. 1), caused a statistically significant decrease (42%; $P < 0.001$, Student's *t* test) in the ability of UK14304 to stimulate high-affinity GTPase activity (Table 1). Preincubation of clone 1C membranes with an IgG fraction from antiserum I3B, which identifies $G_i3\alpha$, but not $G_i1\alpha$ or $G_i2\alpha$ (Fig. 1), also caused a statistically significant decrease (25%; $P < 0.001$, Student's *t* test) in UK14304 stimulation of high-affinity GTPase activity (Table 1). Preincubation of membranes with a combination of the IgG fractions from antiserum AS7 and I3B produced an additive effect (67%) (Table 1). These results indicate that both G_i3 and $G_i1 + G_i2$ are activated by agonist occupation of the α_2-C10 adrenergic receptor expressed in the membranes of 1C cells.

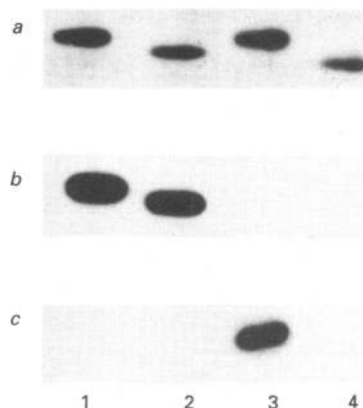

Fig. 1. Specificity of antiserum AS7 for $G_i1\alpha$ and $G_i2\alpha$ and of antiserum I3B for $G_i3\alpha$

(*a*) Recombinant $G_i1\alpha$ (1), $G_i2\alpha$ (2), $G_i3\alpha$ (3) and $G_o\alpha$ (4) (50 ng/lane) were resolved by SDS/PAGE (10% acrylamide/0.13% bisacrylamide) and silver-stained. Such gels were subsequently immunoblotted with either antiserum AS7 (*b*) or I3B (*c*) to define the specificity of these antisera.

After such preincubations, UK14304 (10 μM) was able to produce inhibition, by some 33%, of forskolin-amplified adenylate cyclase activity in membranes of 1C cells which had been preincubated with the IgG fractions from normal rabbit serum. This effect of UK14304 was not decreased by preincubation of the membranes with the IgG fraction from antiserum I3B (38%), but was essentially abolished by preincubation with the IgG fraction of antiserum AS7 (5%) (Table 2). Preincubation of 1C cell membranes with an equivalent IgG fraction from an antiserum directed against the *C*-terminal decapeptide of forms of $G_s\alpha$, the stimulatory G-protein of the adenylate cyclase cascade, also did not decrease UK14304-mediated inhibition of adenylate cyclase (39%) (Table 2), although this antiserum has previously been shown to be capable of preventing agonist stimulation of adenylate cyclase [4,14].

We quantified levels of the α subunits of G_i1, G_i2 and G_i3 present in membranes of 1C cells by immunoblotting these membranes alongside different amounts of *E. coli*-expressed recombinant G-proteins (Table 3). G_i2 was the most prevalent of the 'G_i-like' G-proteins, being present at some 50 pmol/mg of membrane protein. Both G_i3 and G_i1 were present at some 10-fold lower levels than G_i2. It was thus of interest to know if the lack of effect of the anti-G_i3 antiserum on receptor-mediated inhibition of adenylate cyclase was simply a reflection that only a very small amount of G_i3 in comparison with G_i2 was activated by agonist occupancy of the α_2-C10 receptor. This is not the case: $G_i3\alpha$ provides some 37% of the total pertussis-toxin-sensitive G-protein pool activated by the agonist-occupied receptor (Table 3), and as such, if this G-protein were to contribute stoichiometrically to receptor-mediated inhibition of adenylate cyclase, we would have been able to measure this effect easily. Calculations employing the fraction of the agonist-stimulated GTPase that was inhibited by the anti-$G_i3\alpha$ antibody (I3B) (Table 1) and the level of G_i3 determined by immunoblotting (Table 3) indicated a molar turnover of about 2 min^{-1} for $G_i3\alpha$, which corresponds to

Table 2. An antiserum against the *C*-terminal decapeptide of $G_i1\alpha + G_i2\alpha$ abolishes α_2-C10-adrenergic-receptor-mediated inhibition of adenylate cyclase in membranes of clone 1C cells: lack of effect of antisera against the equivalent regions of $G_i3\alpha$ and $G_s\alpha$

Forskolin (10 μM)-amplified adenylate cyclase activity and its modulation by UK14304 (10 μM) were assessed in membranes of clone 1C cells after preincubation for 60 min at 37 °C in the presence of 10 μg of IgG fractions produced as in the Materials and methods section from normal rabbit serum (NRS), antiserum AS7 [anti-($G_i1 + G_i2$)], antiserum I3B (anti-G_i3) or antiserum CS1 (anti-G_s). Results are presented as means ± S.E.M. ($n = 6$ for NRS and AS7, 5 for I3B and 4 for CS1) from individual experiments. Statistical analysis (Student's t test) of the percentage decrease in adenylate cyclase activity produced by UK14304 demonstrated that AS7 produced a significant decrease ($P < 0.001$) compared with NRS. The effects of both I3B and CS1 were non-significant. Analysis of the adenylate cyclase activity data indicated statistically significant decreases by UK14304 for NRS ($P = 0.0007$), I3B ($P = 0.02$) and CS1 ($P = 0.02$). In the presence of AS7 the decrease in adenylate cyclase activity by UK14304 was non-significant. Basal adenylate cyclase activity was not altered by addition of NRS.

IgG fraction	Adenylate cyclase activity (pmol/min per mg of membrane protein) Basal	Forskolin	Forskolin + UK14304	Decrease in forskolin stimulation by UK14304 (%)
NRS	116 ± 9	285 ± 26	229 ± 24	33
AS7	120 ± 11	271 ± 24	264 ± 26	5
I3B	114 ± 9	252 ± 21	199 ± 8	38
CS1	115 ± 8	256 ± 22	201 ± 11	39

the $k_{cat.}$ determined for purified $G_i3\alpha$ [20]. This suggests that essentially all of the $G_i3\alpha$ molecules in the membrane have been activated by the receptor. Similar calculations yield a turnover number of about 0.3 min^{-1} for $G_i2\alpha$, which translates into the activation of roughly 15% of the theoretically available molecules of $G_i2\alpha$. We have noted that in a series of separate clones isolated from the transfection which generated clone 1C a high correlation is obtained between maximal receptor-stimulated GTPase activity and α_2-C10 adrenergic-receptor number [19], an observation which would be consistent with the observation that the G-protein signalling capacity of the membrane has not been saturated by full activation of the α_2-C10 receptor population in clone 1C cells.

It is unclear if, *in vivo*, all of the 'G_i-like' G-proteins [15] can interact with and be activated by receptors known to produce inhibition of adenylate cyclase and whether, when activated, each of these G-proteins will produce inhibition of adenylate cyclase. In human platelets G_i2 was shown to mediate α_2-adrenergic inhibition of adenylate cyclase activity, on the basis of the ability of an anti-peptide antiserum which identifies the *C*-terminal decapeptide of this G-protein to uncouple this effect of a receptor agonist [4]. As an equivalent antiserum directed against the *C*-terminal decapeptide of G_i3 was unable to mimic this effect [4], it appeared that G_i3 was unlikely to provide a substantial contribution to this effect. However, as noted by the authors [4], they were unable to assess if G_i3 had been activated in these experiments by agonist occupation of the receptor. In neuroblastoma × glioma hybrid NG108-15 cells, by a similar antibody-based approach, opioid inhibition of adenylate cyclase was also shown to be mediated by G_i2 [3]. However, in this case it was clear that the receptor had caused activation of substantial amounts of G_i2, but not of G_i3, because opioid activation of high-affinity GTPase activity was attenuated by pre-treatment

Table 3. Determination of levels of the α subunits of G_i1, G_i2 and G_i3 in 1C cell membranes and their activation by agonist activation of the α_2-C10 adrenergic receptor

Membrane-associated levels of these G-proteins were assessed by immunoblotting membranes of 1C cells in conjunction with recombinant G-proteins purified after expression in *E. coli* [6,20]. $G_i1\alpha$ was identified by using antiserum I1C (raised against amino acids 159–168 of $C_i1\alpha$) [26]. $G_i2\alpha$ and $G_i3\alpha$ were identified by using antisera AS7 and I3B respectively (see Fig. 1 and [11,12]). $G_o\alpha$ was identified by using antiserum IM1, which was raised against amino acids 22–35 of $G_o\alpha$ [27]. In immunoblots using antiserum I1C, 25 and 75 μg of 1C membranes were compared with 5, 10, 20 and 40 ng of recombinant $G_i1\alpha$. For G_i2, 12.5 and 25 μg of 1C membranes were compared with 5, 10, 20 and 40 ng of recombinant $G_i2\alpha$. For $G_i3\alpha$, 25 and 75 μg of 1C membranes were compared with 1, 5, 15 and 30 ng of recombinant $G_i3\alpha$. Levels of $G_o\alpha$ in 1C membranes were below the detection limit of antiserum IM1. Results are presented as the arithmetic means from two separate experiments. Antiserum AS7 identifies both $G_i1\alpha$ and $G_i2\alpha$ in membranes of 1C cells [23], but densitometric analysis was conducted against the recombinant protein only for the polypeptide identified which corresponds to $G_i2\alpha$. From calculations of the amount of receptor-stimulated GTPase activity which was prevented by antisera AS7 and I3B, the previously determined $k_{cat.}$ for the GTPase activity of G_i2 and G_i3 and the measured levels of these polypeptides (see the Results and discussion section), we estimated the percentage of the theoretically available G-protein activated by full agonist occupancy of the expressed α_2-C10 adrenergic receptor. These calculations indicate that $G_i3\alpha$ contributes 37% of the total activated pertussis-toxin-sensitive G-proteins. The contribution of $G_i1\alpha$ was too small to be assessed accurately.

G-protein	(pmol/mg of membrane protein)	Percentage activated by receptor	(pmol activated/mg of membrane protein)
$G_i1\alpha$	5.1	Unknown	
$G_i2\alpha$	53.8	15	8.1
$G_i3\alpha$	4.8	100	4.8
G_o	Not detected		

with the antibody which identified G_i2 but was completely unaffected by the anti-G_i3 antibody [3].

Reconstitution experiments using purified receptors and defined G-protein α subunits belonging to the G_i/G_o group have been used to test the ability of receptors to discriminate between these highly related polypeptides [16–18]. Compared with $G_i1\alpha$, $G_i2\alpha$ and $G_o1\alpha$, $G_i3\alpha$ displays the highest relative affinity for α_2-adrenergic receptors [17] as well as the bovine brain A_1 adenosine receptor [18], and selective contact between the dopamine D2 receptor and G_i2 has been recorded [16]. Although such experiments provide evidence for specificity in the interaction between receptors and G-proteins, it is unclear whether the observed selectivities control signal-transduction processes *in vivo*.

We have recently shown that in clone 1C the human α_2-C10 adrenergic receptor causes both inhibition of adenylate cyclase and activation of phosphatidylcholine hydrolysis by a phospholipase D activity [19]. Both of these effects are blocked by pretreatment of the cells with pertussis toxin [19], defining that they are mediated by one or more pertussis-toxin-sensitive G-proteins. Furthermore, these two actions of the receptor appear to be independent. We have also noted previously that α_2-receptor agonists cause the activation of both G_i2 and G_i3 in membranes of these cells [5]. This conclusion was based on the ability of cholera toxin to catalyse the [^{32}P]ADP-ribosylation of pertussis-toxin-sensitive G-proteins only if they had been activated by a receptor.

Our observations herein complement and extend previous

results inasmuch as the coupling between the α_2-C10 adrenergic receptor and $G_i3\alpha$ appears to be very efficient. Even though we note that levels of $G_i3\alpha$ in these membranes are only some 10% of those of $G_i2\alpha$ (Table 3), this G-protein provides more than half as much of the α_2C10-receptor-stimulated high-affinity GTPase activity as does $G_i2\alpha$ (see Table 1). The resulting higher turnover number of $G_i3\alpha$ cannot be attributed to intrinsic differences in the ability of $G_i2\alpha$ and $G_i3\alpha$ to hydrolyse GTP, since the catalytic rate ($k_{cat.}$) is similar for both proteins. In addition, the steady-state rate of hydrolysis, which is limited by the GDP-dissociation rate, is actually slower for $G_i3\alpha$ [20]. Unless physical constraints on mobility limit the potential interactions of the α_2-C10 adrenergic receptor and these two G-proteins, it seems that $G_i3\alpha$ competes efficiently with G_i2 for activation by the α_2-C10 adrenergic receptor in this cell line. Although we have been unable to detect receptor activation of $G_i1\alpha$ in these membranes [5], it should be noted that $G_i1\alpha$ and $G_i2\alpha$ share a common *C*-terminal region. As the *C*-terminal region of G-proteins has been implicated as the site of G-protein/receptor contact [21], it might be expected that the α_2-C10 receptor would be able to interact with these polypeptides equally, unless compartmentalization or other physical constraints apply. In this context, it has been noted that G_i1, but not G_i2, is able to interact in a selective manner with tubulin [22].

In spite of the efficient coupling of G_i3 to the α_2-C10 receptor, which leads to the accumulation of similar amounts of activated G_i2 and G_i3 molecules in the membrane, adenylate cyclase inhibition arises only from receptor-activated G_i2. This is demonstrated by the fact that the anti-G_i3 antibody has no ability to uncouple agonist-mediated inhibition of adenylyl cyclase (Tables 1 and 2). In contrast, the anti-(G_i1+G_i2) antibody is able to attenuate fully α_2-C10-receptor-mediated inhibition of adenylate cyclase (Tables 1 and 2). We have noted previously that, even at antisera concentrations up to 100 μg/ml, combinations of antisera AS7 and I3B are unable to eliminate entirely α_2-C10-adrenergic-receptor stimulation of high-affinity GTPase activity in membranes of 1C cells [23], although all of this stimulated activity is attenuated by pretreatment of the cells with pertussis toxin [5,23]. The presence of G_o is undetectable in these cells [5,23], and it remains unclear whether a further pertussis-toxin-sensitive G-protein(s), which is not identified by any of our available antisera, is expressed by these cells. If so, this would have to be an entirely new G-protein, as we have antisera which detect all of the mammalian pertussis-toxin-sensitive G-proteins which have been characterized at a molecular level.

A recent report [24] has indicated that, after reconstitution of pertussis-toxin-treated hepatocyte membranes with recombinant $G_i3\alpha$, it is possible to restore angiotensin II-mediated inhibition of adenylate cyclase. This study did not report equivalent experiments with recombinant G_i2, and it is worth noting that treatment of intact hepatocytes with angiotensin II, and a variety of other agents which activate protein kinase C via production of diacylglycerol, cause the phosphorylation of $G_i2\alpha$, but not $G_i3\alpha$, and that under such conditions guanine-nucleotide-mediated inhibition of adenylate cyclase is abolished [25]. Such observations argue strongly that G_i2 and not G_i3 is the true inhibitory regulator of adenylate cyclase in native hepatocytes.

On the basis of the complete blockade of agonist-mediated-inhibition of adenylate cyclase by antiserum AS7 and the lack of effect of antiserum I3B in parallel assays, the results from this study provide the first definitive demonstration, in a native membrane system, that receptor-activated G_i3 does not contribute to receptor-mediated inhibition of adenylate cyclase.

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