Inhibition of adenylate cyclase activity by galanin in rat insulinoma cells is mediated by the G-protein G_{i3}

Philippe DE MAZANCOURT,* Paul K. GOLDSMITH and Lee S. WEINSTEIN†
Metabolic Diseases Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892, U.S.A.

Galanin inhibits adenylate cyclase activity and insulin secretion and modulates ion channels in pancreatic β-cells through pertussis-toxin-sensitive G-protein(s). Antibodies directed against the C-terminal region of specific G-protein α-subunits were used to determine which G-protein(s) couple galanin receptors to inhibition of adenylate cyclase in the rat insulinoma cell line RINm5F. Preincubation of membranes with EC antibody (anti-α_{i3}) decreased the inhibition of forskolin-stimulated adenylate cyclase activity by galanin (100 nM) by 45% compared with control IgG ($P < 0.05$) whereas preincubation with AS (anti-α_{i1}, α_{i2}) or GO (anti-α_o) antibodies had no significant effect. To confirm these results, RINm5F cells were exposed intermittently over a 4-day period to phosphorothioate oligodeoxynucleotides that were either sense or antisense to α_{i1}, α_{i2}, α_{i3} or α_o. Oligodeoxynucleotides antisense to α_{i2}, α_{i3} and α_o specifically decreased the levels of the targeted α-subunit in membranes. α_{i1} was undetectable in these cells. Inhibition of adenylate cyclase activity by galanin was largely abolished in membranes from cells exposed to the oligodeoxynucleotide antisense to α_{i3}, whereas all other oligodeoxynucleotides had no significant effect on this pathway. Indirect immunofluorescence and immunoblotting of specific membrane fractions with EC antibody show significant localization of α_{i3} to intracellular membrane compartments. These results suggest that G_{i3} is the G protein that couples galanin receptors to inhibition of adenylate cyclase activity in RINm5F cells.

INTRODUCTION

Heterotrimeric GTP-binding proteins (G-proteins) couple the activation of membrane receptors to the modulation of intracellular effectors, such as adenylate cyclase, phospholipases C and A_2, and ion channels (Bourne et al., 1990, 1991; Simon et al., 1991; Spiegel et al., 1992; Wilkie et al., 1992). G-proteins consist of three subunits, α, β and γ. On interaction of a G-protein with an activated receptor, GTP is exchanged for GDP at the guanine nucleotide-binding site on the α-subunit, and the α-subunit is released from the $\beta\gamma$ complex. Although α-subunits are believed to be the major determinant of coupling specificity to receptors and effectors, recent evidence suggests that β- and γ-subunits may also play a role in coupling specificity (Kleuss et al., 1992, 1993). For many signal-transduction pathways it is not known which specific G-protein is involved.

A physiologically important role for pertussis toxin (PTX)-sensitive G-proteins in the inhibition of insulin secretion was first demonstrated *in vivo* in rats exposed to *Bordetella pertussis* (Katada and Ui, 1977). A variety of hormones and neurotransmitters, including galanin, somatostatin and α_2-adrenergic agents, modulate several intracellular effectors in the pancreatic β-cell via PTX-sensitive signal-transduction pathways (Robertson et al., 1991). These effectors include adenylate cyclase (inhibition), ATP-sensitive K^+ channels (stimulation), voltage-sensitive Ca^{2+} channels (inhibition) and Ca^{2+}-stimulated insulin release (inhibition). The α-subunits of all known PTX-sensitive G-proteins, namely G_{i1}, G_{i2}, G_{i3}, G_{o1} and G_{o2}, have been shown to be present in pancreatic islet cells (Cormont et al., 1991). The α_is are products of separate genes (Itoh et al., 1988), while α_{o1} and α_{o2} are produced by alternative splicing of a single gene (Tsukamoto et al., 1991). Little is known about the specificity of coupling of these G-proteins to receptors or effectors in the β-cell.

Galanin is a 29-amino acid peptide originally isolated from porcine intestine (Tatemoto et al., 1983). Among its many potential biological functions, galanin has been shown to be present in high abundance in nerve endings within the pancreas and to suppress insulin release (Ahrén et al., 1988). In the rat insulin-secreting cell line RINm5F (Gazdar et al., 1980), galanin binds to what is likely to be a single-cell surface receptor (Amiranoff et al., 1989; Lagny-Pourmir et al., 1989) and is coupled through PTX-sensitive G-proteins to adenylate cyclase, Ca^{2+} and K^+ channels and insulin secretion (Amiranoff et al., 1988; de Weille et al., 1988; Dunne et al., 1989; Sharp et al., 1989; Ullrich and Wollheim, 1989; Homaidan et al., 1991). Defining the specificity of coupling of G-proteins to receptors and effectors in insulin-secreting cells remains an important task.

Reconstitution experiments with purified components can identify potential receptor and effector interactions, but these may not necessarily reflect which interactions are occurring *in situ* (Spiegel et al., 1992). Antibodies directed towards the C-terminus of different α-subunits, a region known to be crucial for receptor coupling, are capable of disrupting G-protein–receptor coupling *in situ* and have proven useful in defining receptor and effector coupling specificity within membranes from several cell types (McFadzean et al., 1989; Spiegel et al., 1992; Simonds et al., 1989a; Shenker et al., 1991). A complementary approach is to lower the expression of specific G-protein subunits in cells with antisense oligodeoxynucleotides. This has been accomplished by microinjection (Kleuss et al., 1991, 1992, 1993) or preincubation (Wang et al., 1992) of cells with antisense oligonucleotides, or expression of antisense cDNAs in cultured cells

Abbreviations used: G-protein, heterotrimeric guanine nucleotide regulatory protein; PTX, pertussis toxin; WGA–TRITC, wheat germ agglutinin conjugated to tetramethylrhodamine isothiocyanate; FITC, goat anti-rabbit IgG conjugated to fluorescein isothiocyanate; AEBSF, 4-(2-aminoethyl)benzenesulphonyl fluoride hydrochloride; S-ODN, phosphorothioate oligodeoxynucleotide; IBMX, isobutyl methylxanthine.

* Present address: Hôpital de Poissy, Laboratoire de Biochimie, F78303 Poissy Cedex, France.
† To whom correspondence should be addressed, at Bldg. 10, Rm. 8C101, NIDDK/NIH, Bethesda, MD 20892, U.S.A.

(Watkins et al., 1992) or transgenic animals (Moxham et al., 1993). By two different experimental approaches, preincubation of membranes with specific C-terminal antibodies and preincubation of cells with specific antisense phosphorothioate oligodeoxynucleotides (S-ODNs), we show that G_{i3} is the G-protein that specifically couples galanin receptors to inhibition of adenylate cyclase activity in RINm5F cells.

EXPERIMENTAL

Materials

Galanin, ATP, isobutyl methylxanthine (IBMX), phosphocreatine, creatine kinase, *N*-ethylmaleimide and wheat germ agglutinin conjugated to tetramethylrhodamine isothiocyanate (WGA–TRITC) were purchased from Sigma. GTP, leupeptin and aprotinin were from Boehringer-Mannheim. BSA (fraction V) was from Intergen and forskolin from Calbiochem. Goat anti-rabbit IgG conjugated to fluorescein isothiocyanate (FITC) was from Kierkegaard and Perry (Gaithersburg, MD, U.S.A.). 4-(2-Aminoethyl)benzenesulphonyl fluoride hydrochloride (AEBSF) was from ICN Biochemical.

Cell culture

RINm5F cells were obtained from G. Sharp and cultured in RPMI 1640 (Gibco–BRL) supplemented with 10% fetal calf serum (Gibco–BRL), 100 units/ml penicillin and 100 μg/ml streptomycin at 37 °C in a 95% air/5% CO_2 atmosphere. Cells were studied at passages 56–65 and were passaged every 7–10 days. Media were changed three times a week.

S-ODN exposure

Cells were split at a density of 8×10^4 cells/cm^2 and 24 h later the media were replaced with serum-free RPMI 1640 containing penicillin, streptomycin and various concentrations of specific S-ODN. After 12 h, 1 vol. of RPMI 1640 with 20% fetal calf serum, penicillin and streptomycin was added. This protocol was repeated for 4 days at which point the cells were usually confluent. S-ODNs were synthesized on an Applied Biosystems 380B DNA synthesizer using the sulphurizing reagent 3*H*-1,2-benzodithiol-3-one-1,1-dioxide (thiosulphonate; Glen Research, Sterling, VA, U.S.A.) according to the manufacturer's specifications (Iyer et al., 1990). S-ODNs were cleaned by ethanol precipitation and resolved on 15% polyacrylamide/6 M urea gels to verify that the synthesis was satisfactory. The sequences chosen were 22-mers encoding either the sense or antisense strand of rat G-protein α-subunit cDNAs including the last 16 bases of the 5′ untranslated region and the first two codons of the coding region (Jones and Reed, 1987). The specific sequences were: sense α_o, 5′-CAGGGAAGGGGCCACCATGGGA-3′; antisense α_o, 5′-TCCCATGGTGGCCCCTTCCCTG-3′; sense α_{i1}, 5′-GCGACGCTCGGCCACCATGGGA-3′; antisense α_{i1}, 5′-TCCCATGGTGGCCGAGCGTCGC-3′; sense α_{i2}, 5′-GCCGGCGGACGGCAGGATGGGC-3′; antisense α_{i2}, 5′-GCCCATCCTGCCGTCCGCCGGC-3′; sense α_{i3}, 5′-CTCTCCGGCCGCCGTCATGGGC-3′; antisense α_{i3}, 5′-GCCCATGACGGCGGCCGGAGAG-3′.

Table 1 Specificity of antibodies to G-protein α-subunits

Antibody	Domain	Sequence	Proteins	Cross-reactivity
AS7	C-Terminal	KENLKDCGLF	α_{i1}, α_{i2}	α_{i3} (weak)
EC	C-Terminal	KNNLKECGLY	α_{i3}	α_o
GO	C-Terminal	ANNLRGCGLY	α_o	α_{i3}
LD	Internal	LDRIAQPNYI	α_{i1}	

Membrane preparation

Cells were scraped and washed in PBS, then resuspended in 5 mM Hepes/NaOH, pH 7.5, containing 300 kallikrein inhibitor units/ml aprotinin and 0.1 mg/ml leupeptin at a concentration of 3×10^6 cells/ml and agitated for 15 min at 4 °C. The preparation was then centrifuged at 750 ***g*** for 4 min at 4 °C to remove unbroken cells and nuclei, and the resulting supernatant was centrifuged at 16000 ***g*** for 15 min at 4 °C. The pellet was resuspended once in an equal volume of 20 mM Hepes/NaOH, pH 7.5, containing aprotinin and leupeptin at the above concentrations, re-centrifuged at 16000 ***g*** for 15 min at 4 °C, and resuspended in the same buffer. Protein concentration was determined by the Bradford (1976) method (Bio-Rad).

Adenylate cyclase assay

Freshly prepared membranes (10 μl; 1 mg/ml) from confluent cells were incubated for 15 min at 30 °C in a 95 μl reaction volume containing 20 mM Hepes/NaOH, pH 7.5, 2.5 mM $MgCl_2$, 80 mM NaCl, 0.1 mM IBMX, 0.4 mM ATP, 5 mM phosphocreatine, 5 units/ml creatine kinase, 0.1% BSA (fraction V), 10 μM forskolin, 1 μM GTP, 300 kallikrein inhibitor units/ml aprotinin, 0.1 mg/ml leupeptin and galanin at the indicated concentration. The reactions were stopped by adding 1 vol. of 0.1 M HCl/0.1 mM $CaCl_2$. The samples were then immediately frozen. Samples (200 μl) were acetylated by adding 25 μl of acetic anhydride/triethylamine (1:2.5, v/v) and cyclic AMP was measured using a radioimmunoassay (Harper and Brooker, 1975). Assays were performed in triplicate. The results are the means ± S.E.M. of the number of experiments indicated.

Antibodies

C-Terminal decapeptides of transducin (α_t), α_{i3} and α_{o1} were synthesized, conjugated to keyhole limpet haemocyanin with glutaraldehyde, and injected into rabbits (Goldsmith et al., 1987). The resulting antisera were designated AS7, EC and GO respectively [see Table 1 and Jones and Reed (1987) and Tsukamoto et al. (1991)]. AS7 cross-reacts with the common C-terminal decapeptide of α_{i1} and α_{i2}. Antisera were affinity-purified on Affi-Gel 15 columns (Bio-Rad) containing the immobilized corresponding peptide (Simonds et al., 1989a,b). Affinity-purified antibodies were applied to PD-10 columns (Pharmacia LKB) equilibrated with 100 mM Hepes/NaOH, pH 7.9, containing 500 mM NaCl to exchange the buffer and then stored at 4 °C at a concentration of 2.5 mg/ml. Control rabbit IgG (Pel-Freez) was suspended in the same buffer and the same concentrations as affinity-purified antibodies. For functional studies, 150 μl of membranes (1.1 mg/ml) were preincubated with 6 μl of affinity-purified antibodies or IgG and 3 μl of dithiothreitol at 4 °C for various lengths of time. After preincubation, membranes (10 μl) were added to adenylate cylase reactions.

Immunoblotting

Membranes were treated with *N*-ethylmaleimide as previously described (Amatruda et al., 1988), resolved on an SDS/

polyacrylamide gel (11% acrylamide, 0.08% bisacrylamide, 75 μg of protein/lane) and transferred to poly(vinylidene difluoride) filters (Millipore). Specific G-protein α-subunits were detected by incubation of filters with affinity-purified antisera (2–10 μg/ml) for 20 h at room temperature followed by incubation with [^{125}I]Protein A (Amersham). Radioactivity was quantified with a phosphor imager (Molecular Dynamics).

Indirect immunofluorescence

RINm5F cells were grown overnight on glass coverslips, fixed and permeabilized in PBS containing 2.5% paraformaldehyde and 0.1% Triton X-100 for 30 min at room temperature. The coverslips were then rinsed three times with PBS and incubated for 20 min in PBS containing 3% BSA. They were then incubated in PBS containing 3% BSA, affinity-purified EC antibody (5 μg/ml) and WGA–TRITC (1:40 dilution) for 2 h at 37 °C. After being rinsed with PBS, the coverslips were incubated with PBS containing 3% BSA and FITC (1:40 dilution) for 1 h at 37 °C. They were then rinsed with PBS, mounted on glass slides with Citifluor and visualized by conventional fluorescence microscopy.

Cell fractionation

RINm5F cells were washed twice with PBS and then resuspended in lysis buffer containing 250 mM sucrose, 25 mM Tris/HCl, pH 7.4, 1 mM EDTA, 3 mM ATP, 0.1 mg/ml leupeptin and 100 μg/ml AEBSF. Cells were broken in a Polytron for 20 s at 4 °C and centrifuged at 2000 ***g***. The supernatant was centrifuged at 16000 ***g*** for 15 min at 4 °C. The resulting supernatant was saved and the pellet resuspended in lysis buffer and re-centrifuged at 16000 ***g***. The pellet after the second 16000 ***g*** spin was resuspended in lysis buffer and saved as the low-speed fraction containing plasma membranes. The two supernatants were pooled and centrifuged at 100000 ***g*** in a Ti45 rotor for 90 min at 4 °C. The pellet was resuspended in lysis buffer and saved as the high-speed fraction containing microsomes. Membrane fractions were analysed by SDS/PAGE and immunoblotting as previously described (Goldsmith et al., 1987) using affinity-purified antibodies (1 μg/ml) and horseradish peroxidase-labelled goat anti-rabbit IgG.

RESULTS

Under our experimental conditions, forskolin-stimulated adenylate cyclase activity in RINm5F cell membranes was 520±151 pmol/min per mg (mean±S.E.M.) and was inhibited by galanin with a maximal effect of 25±8% at 100 nM (mean±S.E.M.; $n = 5$) and an IC_{50} of approx. 1 nM (Figure 1). This is consistent with previously reported results (Amiranoff et al., 1988). Preincubation of membranes with control IgG at 4 °C for periods of up to 2 h did not significantly modify forskolin-stimulated adenylate cyclase activity or the ability of 100 nM galanin to inhibit this activity (26±7% and 21±4% inhibition at 0 h and 2 h respectively; not significant by Student's t test; $n = 3$). Preincubation with affinity-purified antibodies also did not significantly modify stimulated adenylate cyclase activity. Preincubation with control IgG for 3 h resulted in a 26% decrease in forskolin-stimulated adenylate cyclase activity and a loss of inhibition by galanin, probably because of spontaneous degradation of adenylate cyclase and/or galanin receptors. We therefore chose a 2 h preincubation to study the effects of antibodies directed against the C-terminus of specific G-protein α-subunits on the coupling of galanin receptors to adenylate cyclase.

RINm5F membranes were preincubated for 2 h at 4 °C with EC, AS7, GO or control IgG, and adenylate cyclase activity was measured in the presence of forskolin and either 0 or 100 nM galanin. Preincubation with EC antibodies (anti-α_{i3}) decreased the inhibitory effect of 100 nM galanin on stimulated adenylate cyclase activity by 45±14% (mean±S.E.M.; $n = 10$) when compared with control IgG (Table 2). Preincubation with either AS7 (anti-$\alpha_{i1,i2}$) or GO (anti-α_o) resulted in no significant decrease in the galanin effect.

Antisense S-ODNs (22-mers) complementary to a region spanning the translational-start sites of α_{i1}, α_{i2}, α_{i3} or α_o were used to specifically lower the expression of each α-subunit in cultured RINm5F cells. S-ODNs containing the sequence of the sense strand for each α-subunit over the same region were used

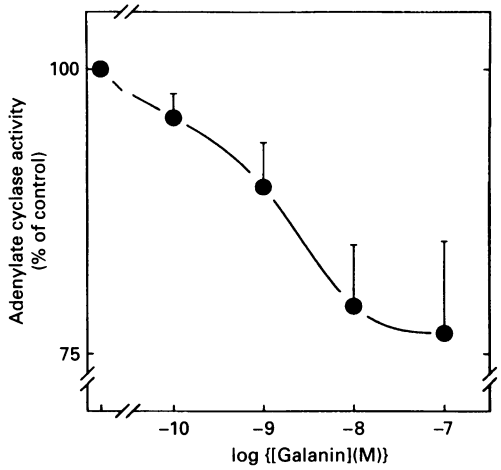

Figure 1 Dose-dependent inhibition of adenylate cyclase activity in RINm5F membranes by galanin

Galanin decreased forskolin-stimulated adenylate cyclase activity (expressed as % of control without galanin) with an IC_{50} of 1 nM. Maximal inhibition was 25±8%. Results are means±S.E.M. of five experiments. Control adenylate cyclase activities for indivdiual experiments (in pmol/min per mg) were: 648, 1651, 371, 733 and 487. The respective maximally inhibited activities were: 496, 1090, 297, 722 and 261.

Table 2 Effect of antibody preincubation of RINm5F membranes on galanin inhibition of adenylate cyclase activity

Inhibition of forskolin-stimulated adenylate cyclase activity by 100 nM galanin was measured in RINm5F membranes preincubated with antibodies for 2 h at 4 °C. Results are means±S.E.M. of ten experiments. $^*P < 0.05$ (Student–Newman–Keuls test).

		Adenylate cyclase activity from one representative experiment (pmol/min per mg)	
Antibody	Inhibition (%)	No galanin	100 nM galanin
IgG	16±2	280±7	225±13
EC	9±2*	256±3	226±17
AS7	17±3	267±7	183±5
GO	21±4	287±11	153±10

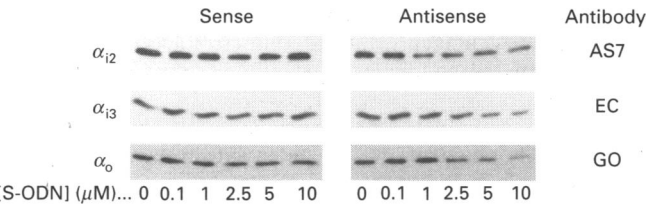

Figure 2 Effect of S-ODN pretreatment on expression of specific G-protein α-subunits in RINm5F membranes

RINm5F membranes from cells exposed on 4 consecutive days to various concentrations (shown below) of S-ODN either sense or antisense to α_{i2}, α_{i3} or α_o were immunoblotted with AS7, EC or GO antibody respectively as described in the Experimental section. One representative experiment is shown. S-ODNs sense to α_{i2}, α_{i3} or α_o had no effect on the levels of the respective membrane protein, whereas each antisense S-ODN lowered the level of its respective targeted α-subunit in a dose-dependent manner. α_{i1} is not detectable in RINm5F membranes (see Figure 4). Therefore the single band detected with AS7 is α_{i2}.

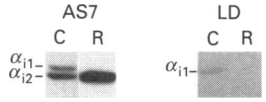

Figure 3 α_{i1} is undetectable in RINm5F membranes

Bovine brain cholate extracts (C) and RINm5F membranes (R) were immunoblotted as described in the Experimental section with AS7 and LD. AS7 (anti-α_{i1}, α_{i2}) detected two bands in the brain cholate extract, a 41 kDa band (α_{i1}) and a 40 kDa band (α_{i2}). In RINm5F membranes, AS7 detected only one strong 40 kDa band (α_{i2}). LD (anti-α_{i1}) detected a 41 kDa band in brain cholate extract but no comparable band in RINm5F membranes.

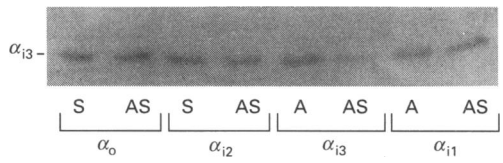

Figure 4 Effect of S-ODN pretreatment on expression of G-protein α-subunits in RINm5F membranes is specific

RINm5F membranes from cells pretreated with S-ODN either sense (S) or antisense (AS) to α_o, α_{i2}, α_{i3} or α_{i1} were immunoblotted with EC (anti-α_{i3}) as described in the Experimental section. Levels of α_{i3} membrane protein were similar in all cells except those pretreated with S-ODN antisense to α_{i3}, in which it was markedly lower.

as controls. As the S-ODNs are directed against a sequence in the 5′ end, treatment with the α_o antisense S-ODN should lower the expression of both alternatively spliced forms of α_o (Tsukamoto et al., 1991). The effect of S-ODN treatment on protein expression was monitored by performing immunoblots of crude membrane fractions using the specific C-terminal antibodies.

In view of previous data from other laboratories suggesting that α-subunits may turn over slowly (Silbert et al., 1990; Levis and Bourne, 1992), we decided to treat the cells with S-ODN for 4 days. On each day, cells were exposed to S-ODN for 12 h in the absence of serum, after which serum was added to the medium to a final concentration of 10% for the second 12 h. We chose to expose the cells to S-ODN in the absence of serum because of the

Table 3 Effect of S-ODN pretreatment of RINm5F cells on galanin inhibition of adenylate cyclase activity

Inhibition of forskolin-stimulated adenylate cyclase activity by 100 nM galanin was measured in RINm5F membranes prepared from cells exposed to S-ODN (5 μM) for 4 days. Results are means ± S.E.M. of four experiments. $^*P < 0.05$ (Student–Newman–Keuls test).

		Adenylate cyclase activity from one representative experiment (pmol/min per mg)	
S-ODN	Inhibition (%)	No galanin	100 nM galanin
G_o sense	16 ± 3	679 ± 14	608 ± 19
G_o antisense	16 ± 1	921 ± 11	769 ± 21
G_{i2} sense	15 ± 3	979 ± 25	875 ± 20
G_{i2} antisense	15 ± 3	1115 ± 55	909 ± 20
G_{i3} sense	15 ± 4	689 ± 20	539 ± 9
G_{i3} antisense	1 ± 2*	987 ± 50	984 ± 47
G_{i1} sense	17 ± 3	788 ± 10	630 ± 11
G_{i1} antisense	20 ± 5	839 ± 27	702 ± 8

short (< 1 h) half-life of S-ODNs in the presence of heat-inactivated fetal calf serum (results not shown). In our initial experiments, cells exposed to S-ODN in the presence of heat-inactivated serum showed a more variable decrease in α-subunit expression (results not shown), perhaps as a result of variable nuclease activity within different lots of serum.

With this protocol, S-ODNs antisense to α_{i2}, α_{i3} and α_o lowered the expression of the targeted α-subunit in a dose-dependent manner (0.1–10 μM) (Figure 2). Treatment with sense S-ODN at a concentration of 10 μM sometimes resulted in decreased α-subunit expression, perhaps as a result of non-specific or toxic effects. Therefore the concentration of S-ODN chosen in experiments to study their effect on the coupling of galanin to adenylate cyclase was 5 μM. At this concentration, the antisense S-ODNs to α_{i2}, α_{i3} and α_o lowered the level of the respective α-subunit protein by 47 ± 7, 45 ± 7 and 41 ± 5% ($n = 2$ for α_{i2} and α_o; $n = 4$ for α_{i3}) when compared with cells treated with the appropriate sense S-ODN. In contrast with a previous report (Cormont et al., 1991), we could not detect α_{i1} in membranes from the clone of RINm5F cells obtained using either AS antibody or LD antibody, which is directed against an internal region of α_{i1} (Figure 3).

The effect of the antisense S-ODNs in suppressing levels of α-subunit was specific. In the experiment shown in Figure 4, α_{i3} expression in RINm5F membranes was decreased by S-ODN antisense to α_{i3} but not by any other sense or antisense S-ODN. After 4 days of treatment with 5 μM S-ODN, membranes were prepared from RINm5F cells and the forskolin-stimulated adenylate cyclase activity was determined in the presence of either 0 or 100 nM galanin. Forskolin-stimulated adenylate cyclase activity in the absence of galanin was not statistically different amongst the membranes derived from cells treated with the different S-ODNs. Pretreatment with the S-ODN antisense to α_{i3} almost totally abolished the inhibitory effect of galanin on adenylate cyclase activity (Table 3). In contrast, membranes treated with either sense S-ODNs or those antisense to α_{i1}, α_{i2} or α_o demonstrated an approximate 20% inhibition of adenylate cyclase activity by galanin ($n = 4$), a result similar to the inhibition seen in membranes from untreated cells.

An incomplete loss of α_{i3} protein after S-ODN treatment was associated with an almost total loss of the inhibitory effect of galanin on adenylate cyclase activity. It seemed likely that this finding may indicate that in RINm5F cells α_{i3} is localized to both

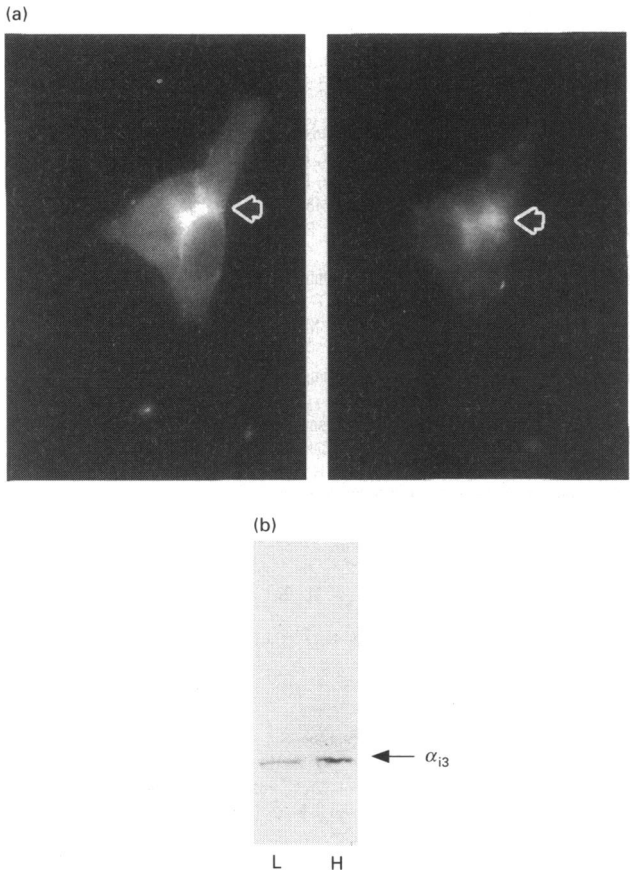

Figure 5 (a) Indirect immunofluorescence of RINm5F cells with affinity-purified EC antibody and (b) immunoblot of RINm5F membrane fractions with EC antibody

(**a**) Labelling of RINm5F cells with affinity-purified EC antibody (5 μg/ml) followed by FITC-labelled goat anti-rabbit IgG is shown on the left. For comparison, labelling of intracellular membranes with WGA–TRITC is shown on the right. Notice the co-localization of intracellular labelling with both reagents (arrows). (**b**) RINm5F membranes were fractionated into a 16000 ***g*** pellet (low-speed fraction) and 100000 ***g*** pellet (high-speed fraction). The fractions were separated by SDS/PAGE (12.5 μg/lane) and immunoblotted with affinity-purified EC antibody (1 μg/ml). Bands were visualized using horseradish peroxidase-labelled goat anti-rabbit IgG. The amount of α_{i3} per mg of protein is much greater in the high-speed fraction (H) than the low-speed fraction (L). The same experiment using AS7 antibody showed a slightly greater amount of α_{i2} in the low-speed fraction than in the high-speed fraction (results not shown).

the plasma membrane and intracellular membranes such as the Golgi. This has already been shown to be true in several other cell types (Stow et al., 1991; Leyte et al., 1992; Hermouet et al., 1992; Aridor et al., 1993; Wilson et al., 1994). We examined the membrane localization of α_{i3} in RINm5F cells using both indirect immunofluorescence and membrane fractionation. Indirect immunofluorescence of RINm5F cells with affinity-purified EC antibody (5 μg/ml) and WGA–TRITC showed significant co-localization of the label in intracellular membranes (Figure 5a). For cell-fractionation experiments, membrane fractions were prepared by differential centrifugation, resulting in a 16000 ***g*** pellet enriched in plasma membranes (low-speed fraction) and a 100000 ***g*** pellet enriched in microsomes (high-speed fraction). Immunoblotting of these membrane fractions (12.5 μg of protein/lane) with affinity-purified EC antibody (1 μg/ml) showed significantly more α_{i3} protein (per mg of membrane protein) in the high-speed fraction than in the low-speed fraction (Figure 5b). Immunoblotting with AS7 antibody showed a slightly higher concentration of α_{i2} in the low-speed fraction (results not shown). The relative proportion of α_{i3} is greater in the high-speed fraction, consistent with localization of a significant amount of α_{i3} to microsomal membranes.

DISCUSSION

Galanin has been shown previously to inhibit adenylate cyclase activity in the rat insulin-secreting cell line RINm5F through a PTX-sensitive mechanism (Amiranoff et al., 1988). Preincubation of RINm5F membranes with specific C-terminal antibodies directed against α_{i3} (EC) decreased the inhibitory effect of galanin on adenylate cyclase activity by 45% whereas preincubation with antibodies directed against α_{i1}, α_{i2} (AS7) or α_o (GO) had no effect. Previous studies demonstrated that C-terminal antibodies are capable of blocking the receptor-mediated activation of G_{i2} and G_o (Simonds et al., 1989a; McFadzean et al., 1989). The lack of effect of AS and GO antibodies, as well as the results after pretreatment with specific S-ODNs, strongly suggests that the other PTX-sensitive G-proteins present in these cells have little or no role in the coupling of galanin receptors to adenylate cyclase.

This study confirms that EC antibody is useful as a functional probe to identify specific receptors and effectors that couple to G_{i3} (Fargin et al., 1991). The inability of EC to abolish completely the galanin-induced effect in our experiments is consistent with previous studies in which the effect of other C-terminal antibodies on receptor–G-protein coupling was incomplete, even at maximal concentrations (McFadzean et al., 1989; Simonds et al., 1989a,b; Shenker et al., 1991). It has been suggested that not all α-subunits within isolated membranes are accessible to C-terminal antibodies (Shenker et al., 1991).

Exposure of RINm5F cells to antisense S-ODNs directed against each PTX-sensitive G-protein α-subunit resulted in a specific and dose-dependent decrease in α-subunit expression. The 40–50% decrease in levels of α-subunit membrane protein observed after 4 days of exposure to antisense S-ODN is consistent with the slow turnover of α-subunit proteins demonstrated in membranes of other cell types (Silbert et al., 1990; Levis et al., 1992). Consistent with the data obtained in experiments using affinity-purified antibodies, only pretreatment of RINm5F cells with S-ODN antisense to α_{i3} disrupted the coupling of galanin receptors to adenylate cyclase. The almost complete loss of galanin inhibition of adenylate cyclase activity after exposure to S-ODN antisense to α_{i3} suggests that this is the only G-protein that mediates this signal-transduction pathway in these cells.

Pretreatment of RINm5F cells with S-ODN antisense to α_{i3} produced an incomplete loss of α_{i3} membrane protein but a virtually complete loss of galanin inhibition of adenylate cyclase activity. It has been shown in several cell lines and tissues that the vast majority of α_{i3} protein is localized to intracellular Golgi membranes, not the plasma membrane (Stow et al., 1991; Leyte et al., 1992; Hermouet et al., 1992; Aridor et al., 1993; Wilson et al., 1994). We have shown in this study that a significant proportion of α_{i3} protein is localized to intracellular membrane compartments in RINm5F cells as well. The C-terminus of α_{i3} has been shown to be important for Golgi targeting (Bruno de Almeida et al., 1994) and it appears that this protein may be localized to the Golgi in all or most cell types. It has been suggested that more than one turnover pool may exist for another G-protein α-subunit, α_s (Levis et al., 1992). Our results suggest that more than one pool of α_{i3} may exist in these cells,

only one of which (presumably located in the plasma membrane) couples to adenylate cyclase.

In contrast with previously published results (Cormont et al., 1991), we could not detect the presence of α_{i1} in our subclone of RINm5F cells. Variable expression of α-subunits has been observed in different RINm5F subclones. In another study in which RINm5F membranes were examined, α_{i1} was present but α_{i3} was absent (Schmidt et al., 1991). Although it is possible that G_{i1} may also couple galanin receptors to adenylate cyclase, the galanin inhibition of this enzyme activity observed here (in membranes lacking α_{i1}) was similar to that observed in previous studies of RINm5F membranes (Amiranoff et al., 1988).

G_{i1} and G_{i2} have been shown previously to be involved in coupling specific receptors to adenylate cyclase (Simonds et al., 1989a; Moxham et al., 1993; Tallent and Reisine, 1992; Bell and Reisine, 1993). Reconstitution and transfection experiments have demonstrated that G_{i3} is also capable of inhibiting adenylate cyclase (Pobiner et al., 1991; Hermouet et al., 1992; Wong et al., 1992; Taussig et al., 1993). Using C-terminal antibodies, 5-hydroxytryptamine 5-HT_{1A} receptors stably transfected into HeLa cells were shown to inhibit adenylate cyclase primarily through G_{i3} (Fargin et al., 1991). This is the first *in situ* demonstration that G_{i3} is involved in receptor-mediated inhibition of adenylate cyclase. In other contexts, activation of G_{i3} by receptors is not necessarily associated with adenylate cyclase inhibition (Law et al., 1993; McClue et al., 1992).

Our study does not determine which PTX-sensitive G-proteins couple galanin receptors to other effectors in RINm5F cells. Cormont et al. (1991) showed that AS7 at a low concentration (10 μg/ml) disrupted high-affinity galanin binding to RINm5F membranes, but that AS7, EC and GO produced a similar effect at a higher concentration (50 μg/ml). These findings suggest that galanin receptors may interact with G_{i1}, G_{i2} or both, as well as G_{i3} and G_o. Galanin also increased photoaffinity labelling of α_{i1}, α_{i2} and α_{o1} in RINm5F membranes (Schmidt et al., 1991). It is therefore likely that in RINm5F cells the other PTX-sensitive G-proteins are involved in the coupling of these receptors to other effectors, such as ATP-sensitive K^+ channels, Ca^{2+} channels and exocytosis. Conversely, our study does not address the question of whether G_{i3} mediates adenylate cyclase inhibition by other agents in RINm5F cells. The somatostatin receptor which is coupled to inhibition of adenylate cyclase (SSTR3) has been shown in other cell types to require G_{i1} to mediate this effect (Tallent and Reisine, 1992; Bell and Reisine, 1993). SSTR3 is the most highly abundant somatostatin receptor subtype in pancreatic β-cells and therefore it is likely that G_{i1} mediates the inhibition of adenylate cyclase by somatostatin in these cells. This evidence and the results of our study suggest that probably more than one G-protein inhibits adenylate cyclase in pancreatic β-cells. Whether G_{i1} and G_{i3} inhibit the same or different adenylate cyclase subtypes in these cells and whether these effects are mediated through α- or $\beta\gamma$-subunits is unknown (Tang and Gilman, 1991, 1992; Taussig *et al.*, 1993). Further studies are necessary to characterize further the coupling specificities in signal-transduction pathways that suppress insulin release in pancreatic β-cells.

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