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The Gonadotrophin-Releasing Hormone Receptor of αT3–1 Pituitary Cells Regulates Cellular Levels of Both of the Phosphoinositidase C-Linked G Proteins, $G_{q\alpha}$ and $G_{11\alpha}$, Equally

BUKHTIAR H. SHAH and GRAEME MILLIGAN

Molecular Pharmacology Group, Departments of Biochemistry and Pharmacology, University of Glasgow, Glasgow, G12 8QQ Scotland, UK

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SUMMARY

The αT3–1 cell line, an immortalized gonadotroph cell line, expresses high levels of the gonadotrophin-releasing hormone (GnRH) receptor. Sustained exposure of these cells to the GnRH receptor agonist des-Gly^{10}-[D-Ala^6]luteinizing hormone-releasing hormone ethylamide resulted in a substantial down-regulation of cellular levels of a combination of the α subunits of the phospholipase C-β_1-linked G proteins G_q and G_{11}, as assessed by immunoblotting with an antiserum able to identify these two proteins equally. This effect was dependent upon the concentration of agonist used (EC_{50} = 4 nM) and on the time of the treatment ($t_{½}$ = 6 hr) when a maximally effective concentration of agonist (1 μM) was used. Comparison of agonist regulation of inositol phosphate generation and $G_{q\alpha}/G_{11\alpha}$ down-regulation demonstrated that effects on inositol phosphate production were approximately 3-fold more potent. In contrast to $G_{q\alpha}/G_{11\alpha}$, membrane-associated levels of $G_{s\alpha}$ and $G_{i2\alpha}$, the G proteins that transduce stimulatory and inhibitory regulation, respectively, of adenylyl cyclase, were not altered by agonist treatment. Analysis of mRNA by reverse transcriptase/polymerase chain reaction indicated the coexpression by αT3–1 cells of mRNA corresponding to both $G_{q\alpha}$ and $G_{11\alpha}$. Immunoblotting with antisera selective for either $G_{q\alpha}$ or $G_{11\alpha}$ confirmed their coexpression. Resolution of membranes from untreated and agonist-treated αT3–1 cells under sodium dodecyl sulfate-polyacrylamide gel electrophoresis conditions able to separate $G_{q\alpha}$ from $G_{11\alpha}$ indicated that $G_{11\alpha}$ was more prevalent than $G_{q\alpha}$ at steady state but that agonist treatment regulated cellular levels of both of these G proteins in a nonselective manner. Sustained activation of protein kinase C with phorbol myristate acetate was unable to mimic agonist regulation of cellular $G_{q\alpha}/G_{11\alpha}$ levels, as was treatment of αT3–1 cells with the selective protein kinase C inhibitor chelerythrine. These data suggest that the GnRH receptor is able to interact functionally with both $G_{q\alpha}$ and $G_{11\alpha}$ in αT3–1 cells and that sustained exposure to a GnRH receptor agonist selectively regulates the cellular levels of the G proteins that interact with the receptor.

The maintained presence of an agonist at a G protein-linked receptor frequently results in a reduction in cellular levels of the receptor. This process is known as down-regulation and contributes to the battery of adaptive changes, collectively called desensitization, that cells and tissues can utilize to prevent chronic full-scale response to a stimulus (1, 2). Although the focus of studies of down-regulation has been at the level of the receptor, due partially to the availability of radiolabeled ligands able to identify and quantitate these polypeptides, it has recently become clear that cellular levels of the G proteins that transduce information from receptors to effector systems can also be regulated in response to the sustained presence of an agonist (3). These effects are noted primarily as a selective down-regulation of the G protein or G proteins with which the receptor for the agonist interacts (3) but in certain cases may also be manifested by an up-regulation of a G protein that opposes the function of the receptor (4).

Receptor regulation of pertussis toxin-insensitive phosphoinositidase C activity is transduced by members of the G_q family of G proteins (5, 6). This family consists of four members, designated G_q, G_{11}, G_{14}, and G_{16} (7, 8). Whereas evidence suggests that the α subunits of all of these G proteins have the potential to activate phosphoinositidase C activity (9, 10), the relative ability of each to activate individual members of the β subfamily of phospholipase C is distinct (11, 12). Current evidence indicates that the profile of expression of G_{14} and G_{16} is relatively restricted (8, 13), and hence these may play relatively specific roles in cellular signaling processes. In contrast, G_q and G_{11} appear to be widely expressed (14–16). Such widespread expression and indeed coexpression of these two G proteins raises questions regarding their individual functions. One possibility is that different receptors can functionally

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ABBREVIATIONS: GnRH, gonadotrophin-releasing hormone; LHRHE, des-Gly^{10}-[D-Ala^6]luteinizing hormone-releasing hormone ethylamide; PMA, phorbol myristate acetate; HBG, Hanks' buffered saline, pH 7.5, containing 1% (w/v) bovine serum albumin and 10 mM glucose; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PCR, polymerase chain reaction.

select between them. Such a possibility is raised by the ability of somatostatin and muscarinic acetylcholine receptors expressed in pituitary GH3 cells to differentially interact with the $G_{o\alpha}$ splice variants G_{o1} and G_{o2} (17), even though agonist stimulation of both receptors results in inhibition of voltage-dependent calcium channels (17). In the only system examined to date, the human muscarinic M1 acetylcholine receptor expressed in Chinese hamster ovary cells was shown to cause down-regulation of both G_q and G_{11} equally and nonselectively (18), and this has been interpreted as evidence that the receptor interacts equally and nonselectively with these two G proteins. To address whether this previous observation might be a reflection of a specific characteristic of the human muscarinic M1 acetylcholine receptor or might be representative of a range of phosphoinositidase C-linked receptors, in this study we have examined the agonist regulation of $G_{q\alpha}$ and $G_{11\alpha}$ in the murine gonadotroph cell line αT3–1. This cell line was selected for study because it has previously been demonstrated that agonists at the GnRH receptor stimulate phosphoinositidase C activity via a member or members of the G_q family of G proteins (19).

Experimental Procedures

Materials. The GnRH analogue LHRHE was from Sigma. Chelerythrine was from Calbiochem and PMA was from Sigma. All materials for tissue culture were from GIBCO/BRL.

Cell culture. αT3–1 cells (20) were grown in Dulbecco's modified Eagle's medium containing pyruvate, supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin, in 5% CO_2 at 37°. Cells were grown in 75-cm^2 tissue culture flasks and were harvested just before confluency. The treatment of cells for different times or with different concentrations of the GnRH analogue was carried out as described in the figure legends.

Preparation of membranes. Membranes were prepared from the cells by homogenization with a Teflon/glass homogenizer and differential centrifugation as described previously (21). Frozen cell pellets were suspended in 5 ml of 10 mM Tris·HCl, 0.1 mM EDTA, pH 7.5 (buffer A), and the cells were ruptured with 25 strokes of the homogenizer. The resulting homogenate was centrifuged at 500 × *g* for 10 min in a Beckman L5–50B centrifuge with a Ti 50 rotor, to remove unbroken cells and nuclei. The supernatant was further centrifuged at 48,000 × *g* for 10 min. The pellet from the second centrifugation was resuspended in buffer A and recentrifuged at 48,000 × *g* for 10 min. Finally the pellet was resuspended in buffer A at a protein concentration of 1–3 mg/ml and was stored at −80° until required. Protein concentration was measured according to the method of Lowry *et al.* (22).

Inositol phosphate assays. Cells were seeded in 24-well plates and labeled to isotopic equilibrium with [^{3}H]inositol (1 μCi/ml), in inositol-free Dulbecco's medium containing 1% dialyzed fetal calf serum, for 48 hr. For measurement of total inositol phosphates, labeling medium was removed and cells were washed twice with 0.5 ml of HBG. The cells were incubated at 37° for 10 min with HBG containing 10 mM LiCl, followed by a 5-min stimulation with agonist in HBG/LiCl. Reactions were terminated by addition of 0.5 ml of ice-cold methanol. The cells in each well were then scraped and transferred to vials. Chloroform was added to a ratio of 1:2 (chloroform/methanol) and the samples were extracted overnight. The phases were split by addition of chloroform and water to final proportions of 10:10:9 (chloroform/methanol/water), and the upper phase was taken for analysis of total inositol phosphates by batch chromatography on Dowex-1 formate, as described previously (23).

Immunological studies. The generation and specifities of the various antisera used in this study are shown in Table 1, except for the $G_{11\alpha}$-specific antiserum E976 (24), which was a kind gift from Dr. John Exton, Vanderbilt University (Nashville, TN). Each antiserum was produced in a New Zealand White rabbit, using a conjugate of a synthetic peptide with keyhole limpet hemocyanin (Calbiochem) as antigen. Details of this process have been described previously (25). Membrane samples were resolved by SDS-PAGE (in 10%, w/v, acrylamide gels overnight at 60 V when resolution of $G_{q\alpha}$ and $G_{11\alpha}$ was not required and in 12.5%, w/v, acrylamide gels, containing a linear gradient of 4–8 M urea, overnight at 155 V to resolve these two polypeptides). Proteins were subsequently transferred to nitrocellulose (Schleicher and Schuell) and blocked for 3 hr with 5% (w/v) gelatin in PBS, pH 7.5. Primary antisera were added in 1% gelatin in PBS containing 0.2% (v/v) Nonidet P-40 and were incubated overnight. The primary antiserum was removed and the blot was washed extensively with PBS containing 0.2% Nonidet P-40. Secondary antiserum (donkey anti-rabbit IgG coupled to horseradish peroxidase) in 1% gelatin/PBS/0.2% Nonidet P-40 was added and left for 3 hr. After removal of the secondary antiserum the blot was washed extensively, as described above, and developed with *o*-dianisidine hydrochloride (Sigma) as substrate for horseradish peroxidase, as described (26). Quantification of such blots was performed by scanning with a Shimadzu CS-9000 dual-wavelength flying-spot laser densitometer at 450 nm. Preliminary experiments demonstrated a linear immunological signal for each primary antiserum with protein amount over the range of membrane protein amounts used for immunoblotting. Apparent molecular mass markers are not recorded for figures in which resolution of membranes were performed in urea-containing SDS-PAGE, because this condition significantly altered the position of migration of the protein standards used.

Reverse transcriptase/PCR. The overall details of the reverse transcriptase/PCR procedure has been described previously (27).

RNA extractions. Total RNA was extracted according to the acid phenol/guanidinium thiocyanate method of Chomcynski and Sacchi (28). The purity and quantity of RNA were assessed by A_{260}/A_{280} ratios.

Reverse transcription. Samples of 1–10 μg of RNA (20 μl) were denatured by incubation at 65° for 10 min, followed by chilling on ice, and were reverse transcribed in 33 μl of reaction mixture using a first-strand cDNA synthesis kit (Pharmacia LKB Biotechnology), as detailed by the manufacturer. Incubation was carried out at 37° for 1 hr. The reactions were terminated by heating of samples at 95° for 5 min, followed by transfer to ice.

PCR. PCR was carried out using the following 24-mer primers: $G_{q\alpha}$-485-sense, 5′-ATGACTTGGACCGTGTAGCCGACC-3′; $G_{11\alpha}$-488-sense, 5′-ACGTGGACCGCATCGCCACAGTAG-3′; $G_{q/11\alpha}$-721-antisense, 5′ CCATGCGGTTCTCATTGTCTGACT 3′. Amplifications were performed in 100 μl of buffer containing 20–40 pmol of primers and 2.5 units of Taq polymerase (Promega). Amplifications were carried out in a HYBAID Omnigene temperature cycler. Cycles were as follows: 95° for 5 min, 60° for 30 sec, and 72° for 1 min (one cycle); 95° for 30 sec, 60° for 30 sec, and 72° for 1 min (25–30 cycles); and 95° for 30 sec, 60° for 30 sec, and 72° for 5 min (one cycle). These conditions ensured a linear relationship between the amount of template mRNA and the amount of amplified product (29). Reaction products were separated by 1.25% agarose gel electrophoresis.

Data analysis. Analysis was performed using the Kaleidagraph (version 2.1) curve-fitting package, with an Apple Macintosh computer.

Results

αT3–1 cells express high levels of a GnRH receptor that exhibits binding characteristics similar to those found in normal mouse and rat pituitary (20, 30). Immunoblotting of αT3–1 cell membranes resolved by 10% (w/v) acrylamide SDS-PAGE, using either the $G_{q\alpha}/G_{11\alpha}$-nonselective antiserum CQ2 (Fig. 1, *left*) or the $G_{q\alpha}$-specific antiserum IQB (Fig. 1, *right*), identified an apparently single 42-kDa polypeptide.

Treatment of αT3–1 cells with the GnRH receptor agonist LHRHE (1 μM) for 16 hr decreased immunodetectable levels of this 42-kDa polypeptide, whether detection was performed with antiserum CQ2 or IQB (Fig. 1; Table 2). In contrast to

TABLE 1

Specificities and generation of anti-G protein antisera

Each antiserum was generated in a New Zealand White rabbit using a conjugate of the synthetic peptide and keyhole limpet hemocyanin, as described in Experimental Procedures. The specificity of these antisera has been established previously (26, 29).

Antiserum	Peptide used	G protein sequence	Antiserum identifies
CQ2	QLNLKEYNLV	$G_{q\alpha}$ 351–360 $G_{11\alpha}$ 350–359	$G_{q\alpha}$, $G_{11\alpha}$
SG1	KENLKDCGLF	Transducin α 341–350	Transducin, $G_{i1\alpha}$, $G_{i2\alpha}$
CS1	RMHLRQYELL	$G_{s\alpha}$ 385–394	$G_{s\alpha}$
BN3	MSELDQLRQE	β_1 1–10	β_1, β_2
IQB	EKVSAFENPYVDAIKS	$G_{q\alpha}$ 119–134	$G_{q\alpha}$

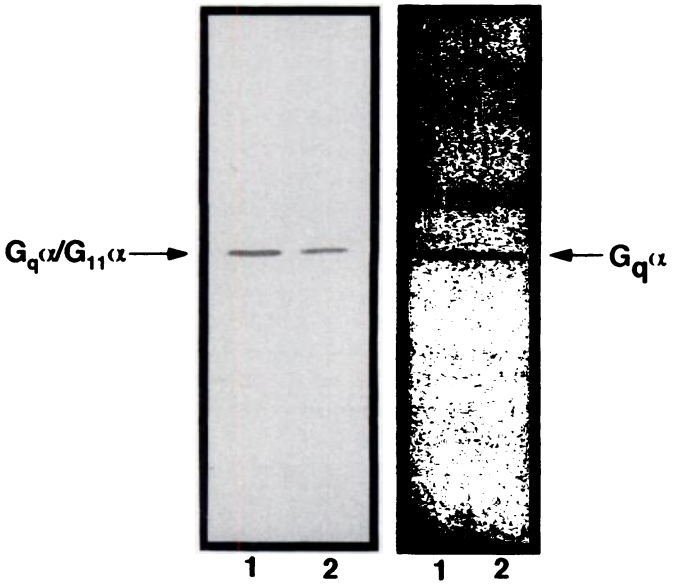

Fig. 1. Immunological detection of $G_{q\alpha}/G_{11\alpha}$ in αT3–1 cells and down-regulation of this immunoreactivity by treatment with LHRHE. Membranes from untreated (*lanes 1*) and LHRHE (1 μM, 16 hr)-treated (*lanes 2*) αT3–1 cells were resolved by SDS-PAGE (10%, w/v, acrylamide) and then immunoblotted with the $G_{q\alpha}/G_{11\alpha}$-common antiserum CQ2 (*left*) or the $G_{q\alpha}$-specific antiserum IQB (*right*). Both antisera identified an apparently single, 42-kDa, polypeptide. In both cases a substantial reduction in immunoreactivity was observed in membranes from agonist-treated cells (see Table 2 for quantitation).

TABLE 2

Evidence that agonist regulation of G proteins in αT3-1 cells is specific for $G_{q\alpha}/G_{11\alpha}$

Membranes from untreated and LHRHE (1 μM, 16 hr)-treated αT3-1 cells were resolved by SDS-PAGE (10%, w/v, acrylamide) and then immunoblotted with the antisera noted. Immunological signals in membranes from untreated cells were set to 100%, and the effect of agonist treatment was then assessed. Data are presented as mean ± standard error from *n* independent experiments.

G protein	Immunologically detected G protein
	% of control
$G_{q\alpha}$ (antiserum IQB)	43 ± 9 (n = 3)
$G_{q\alpha}$ + $G_{11\alpha}$ (antiserum CQ2)	46 ± 8 (n = 4)
$G_{s\alpha}$ (antiserum CS1)	99 ± 6 (n = 5)
$G_{i2\alpha}$ (antiserum SG1)	95 ± 8 (n = 4)
β subunit (antiserum BN3)	97 ± 15 (n = 3)

levels of $G_{q\alpha}/G_{11\alpha}$, immunologically detected levels of the α subunits of both the adenylyl cyclase-stimulatory G protein $G_{s\alpha}$, which in these cells is expressed predominantly as a 45-kDa form of the polypeptide, and the pertussis toxin-sensitive G protein $G_{i2\alpha}$ were not modulated by equivalent agonist treatment when equivalent immunoblots were performed in parallel (Table 2). Similarly, such agonist treatment had no effect on levels of the G protein β subunit (Table 2).

Reverse transcriptase/PCR analysis was used to demonstrate that αT3–1 cells express mRNAs encoding $G_{q\alpha}$ and $G_{11\alpha}$ (Fig. 2A). In each case positive controls for the generation of a fragment of the appropriate size were provided by inclusion of cDNAs encoding these G proteins. To confirm immunologically the expression of both of these polypeptides in αT3–1 cells, we used an SDS-PAGE system that is able to resolve these two polypeptides. In 12.5% (w/v) acrylamide/0.0625% (w/v) bisacrylamide SDS-PAGE with a linear gradient of 4–8 M urea, two

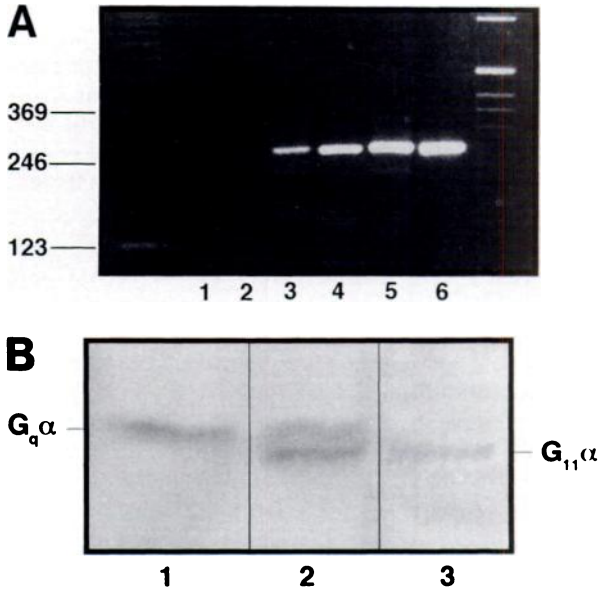

Fig. 2. Expression of both $G_{q\alpha}$ and $G_{11\alpha}$ by αT3–1 cells. A, Reverse transcriptase/PCR demonstration of the presence of mRNAs encoding both $G_{q\alpha}$ and $G_{11\alpha}$ in αT3–1 cells. Reverse transcriptase/PCR was performed, as described in Experimental Procedures, with total RNA isolated from αT3–1 cells (*lanes 3* and *4*) to amplify a fragment of 260 base pairs derived from $G_{q\alpha}$ mRNA (*lane 3*) or a fragment of 257 base pairs derived from $G_{11\alpha}$ mRNA (*lane 4*). Positive controls for these amplifications were provided by concurrent amplification using 35 ng of either a full length murine $G_{q\alpha}$ cDNA in pBluescript II (*lane 5*) or $G_{11\alpha}$ cDNA in pcMV2 (*lane 6*). *Lanes 1* and 2, negative controls for $G_{q\alpha}$ (*lane 1*) and $G_{11\alpha}$ (*lane 2*), in which no template was added. Standards were provided by a 123-base pair ladder (GIBCO/BRL) (*left*) or a 1-kilobase ladder (GIBCO/BRL) (*right*). B, Immunological demonstration of the coexpression of $G_{q\alpha}$ and $G_{11\alpha}$ by αT3–1 cells. Membranes from αT3–1 cells were resolved in the urea gradient SDS-PAGE system described in Experimental Procedures. Segments from this gel were immunoblotted, after transfer to nitrocellulose, with antisera IQB ($G_{q\alpha}$) (*lane 1*), CQ2 ($G_{q\alpha}$ plus $G_{11\alpha}$) (*lane 2*), or E976 ($G_{11\alpha}$) (*lane 3*). Of the two polypeptides identified by antiserum CQ2, the more rapidly migrating was demonstrated to be $G_{11\alpha}$ and the more slowly migrating to be $G_{q\alpha}$.

CQ2-immunoreactive polypeptides were resolved in membranes from αT3–1 cells (Fig. 2B, *lane 2*). The more slowly migrating of these two polypeptides was identified specifically by the $G_{q\alpha}$-specific antiserum IQB, and the more rapidly migrating polypeptide was specifically identified by the $G_{11\alpha}$-specific antiserum E976 (Fig. 2B). These results demonstrate that under these conditions $G_{11\alpha}$ migrates more rapidly through the gel than does $G_{q\alpha}$, and they provide unequivocal identification of the molecular identity of these two CQ2-immunoreactive polypeptides. Because antiserum CQ2 was generated against a region that is completely conserved between these two proteins (Table 1), concurrent detection of the two polypeptides in such immunoblots provided an estimate of the relative steady state levels of the two (see Fig. 2B and below). Membranes from untreated αT3–1 cells possessed somewhat higher steady state levels levels of $G_{11\alpha}$ than of $G_{q\alpha}$ ($G_{11\alpha}$:$G_{q\alpha}$ = 1.3:1.0).

Treatment of αT3–1 cells with different concentrations of LHRHE for 16 hr demonstrated that half-maximal reductions in levels of each of the combination of $G_{q\alpha}$ and $G_{11\alpha}$ measured by immunoblotting with antiserum CQ2 under conditions not able to resolve these polypeptides (Fig. 3A), of $G_{q\alpha}$ measured with the $G_{q\alpha}$-specific antiserum IQB (data not shown), or of both $G_{q\alpha}$ and $G_{11\alpha}$ when the two proteins were detected individually but concurrently (Fig. 3B) were produced with approximately 5 nM ligand. In a number of experiments that used urea-containing SDS-PAGE to resolve $G_{q\alpha}$ from $G_{11\alpha}$, a third CQ2-immunoreactive polypeptide, which migrated in a position very close to that of $G_{q\alpha}$, could be noted. The molecular nature of this polypeptide is unknown, but it was not removed by preparation of cellular membranes in the presence of a cocktail of protease inhibitors and was also identified by immunoblotting with the $G_{q\alpha}$-specific antiserum IQB (data not shown). Quantitation of the agonist-induced down-regulation of $G_{q\alpha}$ and $G_{11\alpha}$ demonstrated that a similar percentage of each G protein polypeptide was lost at each agonist concentration (Fig. 3C). The binding of GnRH and analogues to its receptor in αT3–1 cells leads to activation of phospholipase C, resulting in elevated generation of inositol phosphates (19, 30, 31). LHRHE markedly stimulated inositol phosphate generation in LiCl-treated αT3–1 cells that had been labeled for 48 hr with *myo*-[^{3}H]inositol. Incubation with a maximally effective concentration of the agonist (1 μM) for 5 min resulted in the generation of approximately 7150 dpm above basal levels of inositol phosphates/100,000 dpm of inositol-containing phospholipids. This occurred in a dose-dependent manner, with half-maximal stimulation being produced by approximately 1 nM agonist. This was approximately 3-fold more potent than for agonist-induced down-regulation of $G_{q\alpha}/G_{11\alpha}$ (Fig. 3D).

Time courses for a maximally effective concentration of agonist (1 μM) demonstrated that reduction of levels of the α subunits of both G_q and G_{11} was produced by 6.2 ± 0.7 hr (mean ± standard error, three experiments) and that maximal reduction was achieved by 16 hr (Fig. 4, A and B). Incubation with the ligand for longer time periods did not cause further loss of $G_{q\alpha}/G_{11\alpha}$. At each time point the proportions of steady state $G_{q\alpha}$ and $G_{11\alpha}$ down-regulated were similar (Fig. 4C).

Sustained treatment of αT3–1 cells with the protein kinase C activator PMA (100 nM) (16 hr) was unable to modulate cellular levels of $G_{q\alpha}/G_{11\alpha}$ (Table 3), indicating that agonist-induced down-regulation of $G_{q\alpha}/G_{11\alpha}$ did not occur subsequent to activation of a member or members of this kinase family that are sensitive to phorbl esters. Treatment with the selective protein kinase C inhibitor chelerythrine (10 μM) for 16 hr was also unable to regulate cellular $G_{q\alpha}/G_{11\alpha}$ levels (Table 3), and coincubation of αT3–1 cells with both LHRHE (1 μM) and chelerythrine (10 μM) produced the same levels of $G_{q\alpha}/G_{11\alpha}$ as did LHRHE alone (Table 3).

Discussion

The immortalized, GnRH-responsive, murine gonadotroph pituitary cell line αT3–1, produced by targeted oncogenesis in transgenic mice (20), has been widely used to study cell signaling from the GnRH receptor (18, 20, 30, 31). Even though cDNA species corresponding to the GnRH receptor from a range of species have recently been isolated and demonstrate that the receptor is a serpentine, G protein-linked receptor (32–33), the αT3–1 cell line is at least as useful as stable cell lines that might be generated to express the GnRH receptor after transfection of cells with such cDNAs, because it expresses high levels of the receptor (approximately 1.5 pmol/mg of membrane protein) and would be anticipated to also express the other polypeptides required to generate a cellular response to the presence of an agonist at the GnRH receptor. Previous studies demonstrated a large accumulation of inositol phosphates in LiCl-treated αT3–1 cells and elevations of intracellular calcium levels in response to GnRH receptor agonists (19, 31). These effects are resistant to pretreatment of the cells with pertussis toxin, implying that the G proteins that couple to the GnRH receptor in these cells are not of the G_i family. Pertussis toxin-insensitive activation of phosphoinositidase C activity by G protein-coupled receptors is produced by members of the G_q family of G proteins (5), and GnRH receptor activation of this enzyme in αT3–1 cells has been shown by Hsieh and Martin (19) to be produced by G_q and/or G_{11}. No data have previously been obtained to determine whether the GnRH receptor activates both of these closely related proteins or whether there is selectivity of interaction of the GnRH receptor with one or the other of these G proteins.

In many circumstances, the maintained exposure of cells to an agonist at a G protein-linked receptor results in a reduction in cellular levels of the receptor (1). This process is called down-regulation and contributes to the desensitization mechanisms that cells can utilize to prevent continual response to a stimulus (2). Although not widely examined for a number of years, due largely to the lack of suitably sensitive and discriminatory probes, it has now been firmly established that chronic exposure to a G protein-linked receptor agonist can also frequently result in down-regulation of the G protein or G proteins that interact with the receptor (3). Such effects have been observed for members of each of the G_s, G_i, and G_q families of G proteins and, in the cases in which the mechanisms have been explored, seem to result from enhanced turnover of the G protein (29).

We have recently developed SDS-PAGE conditions that are able to resolve $G_{q\alpha}$ and $G_{11\alpha}$ clearly and unambiguously (34). Thus, by using an antiserum that is targeted to a region that is completely conserved between these two polypeptides, and that hence identifies them equally, relative levels of the two G proteins in a cell can be assessed concurrently (34). Furthermore, by examining possible cellular regulation of levels of these two G proteins by activation of the GnRH receptor, immunoblotting assays can be used to assess the relative degree

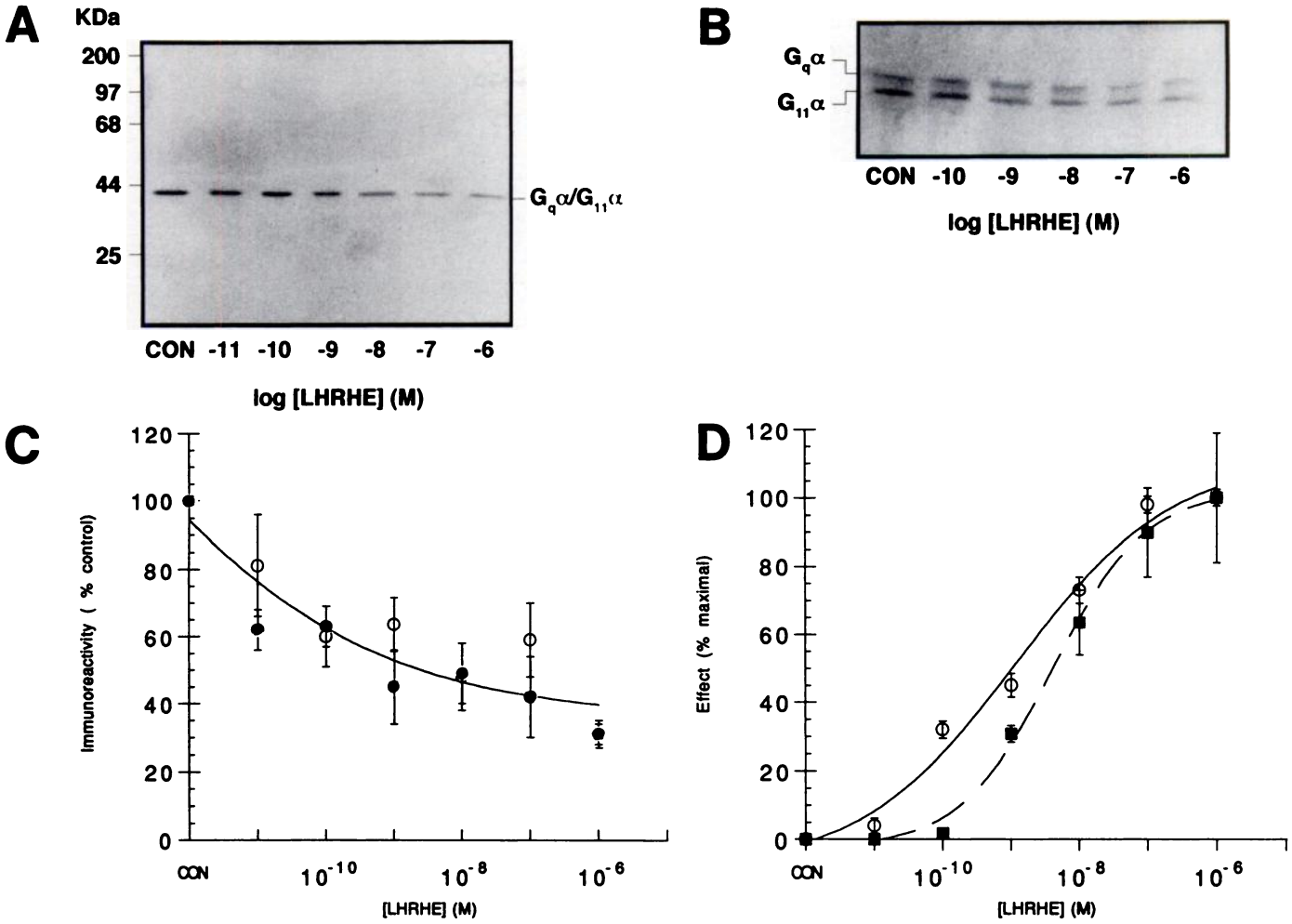

Fig. 3. Down-regulation of both $G_{q\alpha}$ and $G_{11\alpha}$ in αT3–1 cells by treatment with varying concentrations of LHRHE. A, Immunological detection of a combination of $G_{q\alpha}$ and $G_{11\alpha}$. αT3–1 cells were treated with varying concentrations of LHRHE (0–10^{-6} M). Membranes prepared from these cells were then resolved by SDS-PAGE (10%, w/v, acrylamide) and immunoblotted using antiserum CQ2 as primary reagent. Under these conditions $G_{q\alpha}$ and $G_{11\alpha}$ comigrate and thus the immunological signal represents a composite of these two polypeptides. B, Concurrent individual immunological detection of $G_{q\alpha}$ and $G_{11\alpha}$. Membranes (as in A) were resolved by SDS-PAGE (12.5% acrylamide/0.0625% bisacrylamide) with a linear gradient of 4–8 M urea and were immunoblotted with antiserum CQ2. Under these conditions $G_{11\alpha}$ migrates more rapidly than $G_{q\alpha}$ (see Fig. 2B). C, Quantitative analysis of LHRHE-mediated down-regulation of $G_{q\alpha}$ and $G_{11\alpha}$. Relative levels (compared with membranes from untreated cells) of $G_{q\alpha}$ (○) and $G_{11\alpha}$ (●) were measured from the data of B. Results are presented as mean ± standard error of three separate experiments derived from membrane preparations from different cells. D, Comparison of the dose-effect curves for LHRHE-mediated down-regulation of $G_{q\alpha}/G_{11\alpha}$ and stimulation of inositol phosphate generation. LHRHE-mediated down-regulation of $G_{q\alpha}/G_{11\alpha}$ (■) and stimulation of inositol phosphate generation (○) were measured as described in Experimental Procedures. The effect produced by 1 μM agonist was taken to represent the maximal effect. The data were analyzed as described in Experimental Procedures. Estimated EC_{50} values were 1.2 ± 0.9 nM for LHRHE stimulation of inositol phosphate generation and 4.1 ± 1.0 nM for LHRHE-mediated down-regulation of G_q/G_{11}. Data for both curves were derived from four independent experiments. *CON*, control.

of down-regulation of each of these G proteins produced by receptor activation (18). We have argued that such down-regulation reflects activation of the G protein by the receptor (3, 18). This argument is based upon the selectivity of G protein down-regulation, which is restricted to G proteins that can be shown by other approches to be those activated by the receptor (3), and on the observation that receptor stimulation results in an enhanced rate of degradation only of the G proteins that are down-regulated (3, 29). It has further been observed that persistently activated forms of the G protein $G_{s\alpha}$, produced by either mutation or cholera toxin-catalyzed ADP-ribosylation, have a substantially shorter half-life than does the unmodified wild-type protein (35) but that a mutant of G_s that can interact with the receptor but fails to subsequently undergo dissociation of α and βγ subunits does not (35). These observations further support the concept that activation of the G protein is required for down-regulation. It is clearly possible in such a scenario that the real requirement is for activation of a downstream second messenger-activated protein kinase, the activity of which consequently leads to G protein down-regulation. This seems unlikely, however, because analogues of cAMP or treatment with forskolin is unable to mimic receptor-mediated down-regulation of $G_{s\alpha}$ (36) and because we demonstrate herein both that sustained activation of protein kinase C with the phorbol ester PMA is unable to mimic the LHRHE-mediated down-regulation of $G_{q\alpha}$ and $G_{11\alpha}$ and that inhibition of protein kinase C with the selective inhibitor chelerythrine is unable to prevent LHRHE-mediated down-regulation (Table 3). A potential role for other kinases that demonstrate their activity after receptor activation, including the G protein-linked receptor

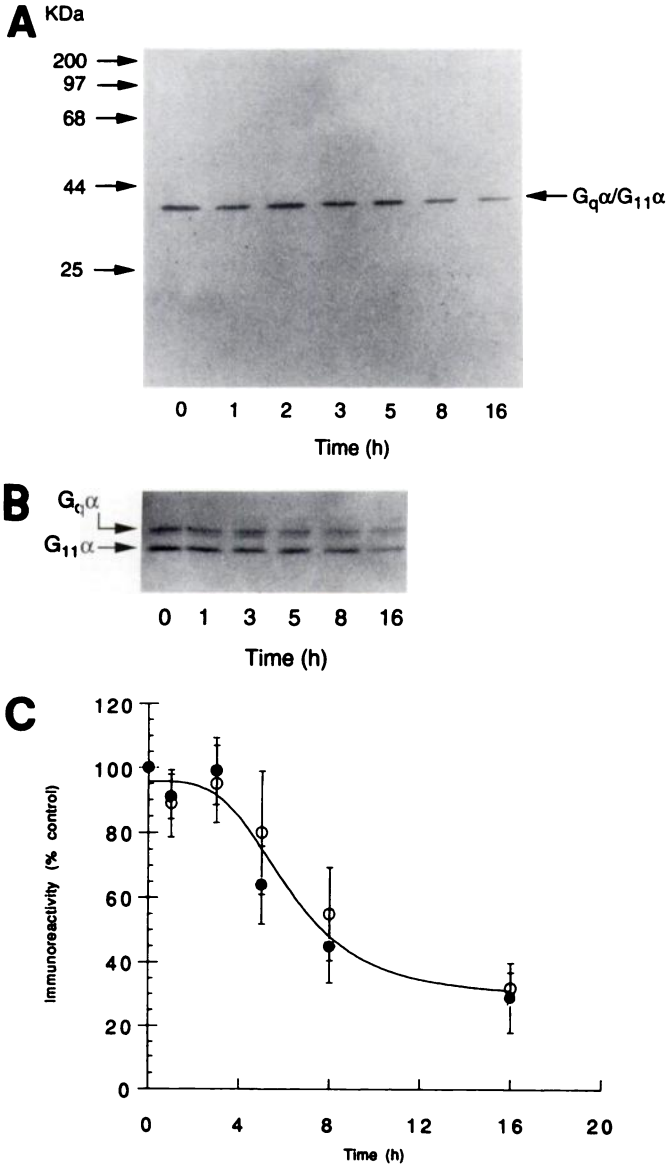

Fig. 4. Down-regulation of both $G_{q\alpha}$ and $G_{11\alpha}$ in αT3–1 cells by treatment with LHRHE for varying times. A, Immunological detection of a combination of $G_{q\alpha}$ and $G_{11\alpha}$. αT3–1 cells were treated for varying times with LHRHE (10^{-6} M). Membranes prepared from these cells were then resolved by SDS-PAGE (10%, w/v, acrylamide) and immunoblotted using antiserum CQ2 as primary reagent. B, Concurrent individual immunological detection of $G_{q\alpha}$ and $G_{11\alpha}$. Membranes (as in A) were resolved by SDS-PAGE (12.5% acrylamide/0.0625% bisacrylamide) with a linear gradient of 4–8 M urea and were immunoblotted with antiserum CQ2. C, Quantitative analysis of LHRHE-mediated down-regulation of $G_{q\alpha}$ and $G_{11\alpha}$. Relative levels (compared with membranes from untreated cells) of $G_{q\alpha}$ (○) and $G_{11\alpha}$ (●) were measured from the data of B. Results are presented as mean ± standard error from three independent experiments.

kinases (37) such as the β-adrenoceptor kinase, has not been investigated in these studies, but because the role of at least the G protein-linked receptor kinase proteins is to produce uncoupling of receptor from G protein (37) these do not appear attractive targets to regulate receptor-mediated G protein down-regulation.

We demonstrated the coexpression of $G_{q\alpha}$ and $G_{11\alpha}$ in αT3–1 cells both by concurrent immunoblotting with antiserum CQ2 under the SDS-PAGE conditions able to resolve the two polypeptides (Fig. 2B) and by detection of mRNA encoding each protein by reverse transcriptase/PCR analysis (Fig. 2A). In these studies the generation of a PCR fragment of the anticipated size must result from the amplification of expressed mRNA rather than contaminating genomic DNA because the primers selected are, based on the known genomic structure of other G proteins, unlikely to lie on the same exon, and thus amplification of genomic DNA would result in the production of a substantially larger fragment. Furthermore, as a contol for these experiments cDNAs corresponding to both murine $G_{q\alpha}$ and $G_{11\alpha}$ were used to provide a positive control (Fig. 2A).

TABLE 3

Lack of a role for protein kinase C in GnRH receptor-mediated down-regulation of $G_{q\alpha}/G_{11\alpha}$

αT3-1 cells were either not treated or treated for 16 hr with LHRHE (1 μM), PMA (100 nM), chelerythrine (10 μM), or combinations of these agents. Membranes derived from these cells were resolved by SDS-PAGE (10%, w/v, acrylamide), transferred to nitrocellulose, and immunoblotted with antiserum CQ2. The signal corresponding to a combination of $G_{q\alpha}/G_{11\alpha}$ was quantitated and compared with the signal from membranes from untreated cells, which was set as 100%. Data are presented as mean ± standard error (five experiments).

Treatment	Immunologically detected G protein
	% of control
LHRHE (1 μM)	58 ± 8
PMA (100 nM)	98 ± 12
Chelerythrine (10 μM)	112 ± 18
LHRHE (1 μM) + chelerythrine (10 μM)	61 ± 12

When αT3–1 cells were maintained in the presence of the GnRH analogue LHRHE (1 μM) for 16 hr, membrane levels of a combination of G_q and G_{11} were decreased markedly; resolution of membranes from untreated and treated cells to detect $G_{q\alpha}$ and $G_{11\alpha}$ individually demonstrated that both polypeptides were down-regulated by the treatment. In contrast, treatment of αT3–1 cells with the GnRH receptor agonist had no effect on membrane levels of either stimulatory ($G_{s\alpha}$) or inhibitory ($G_{i2\alpha}$) (Table 2) G proteins associated with the adenylyl cyclase cascade, demonstrating that this effect is specific for the G proteins that would be anticipated to be activated by a receptor that causes a pertussis toxin-insensitive stimulation of phosphoinositidase C.

When levels of $G_{q\alpha}$ and $G_{11\alpha}$ each were calculated as percentages of levels in membranes from untreated cells, for both dose-response curves for LHRHE (Fig. 3) and time courses (Fig. 4) it was apparent that the degrees of down-regulation of the two individual G proteins were similar in each case. Such results argue not only that the GnRH receptor has the ability to down-regulate both $G_{q\alpha}$ and $G_{11\alpha}$ but also that it shows no inherent selectivity between these two G proteins, interacting with and activating them based solely on their relative levels of expression within the cell. Thus, because the relative steady state levels of G_{11}:G_q in the membranes from these cells were demonstrated to be 1.3:1.0, this also is the ratio for their interaction with the agonist-occupied GnRH receptor. There is little evidence to date to indicate that G_q and G_{11} have distinct profiles for activation of individual forms of the phospholipase C-β family, although this is the case for G_{14} and G_{16} (11–12), the other members of the G_q family. Because we previously demonstrated that, at least when expressed in Chinese hamster ovary cells, the human muscarinic M1 acetylcholine receptor also down-regulates $G_{q\alpha}$ and $G_{11\alpha}$ nonselectively (18), these data pose an unresolved question regarding why these two individual

but very similar proteins are widely coexpressed (34). The data presented herein demonstrate that the GnRH receptor in αT3–1 cells down-regulates $G_{q\alpha}$ and $G_{11\alpha}$ equally and nonselectively, and they extend the concept that agonist control of cellular levels of G proteins that are linked to a receptor is a common mechanistic regulatory phenomenon.

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Send reprint requests to: Graeme Milligan, Department of Biochemistry, University of Glasgow, Glasgow, G12 8QQ Scotland, UK.