Regulation of the GTP-binding protein-based antilipolytic system of sheep adipocytes by growth hormone

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Abstract

Chronic exposure of sheep adipose tissue to growth hormone (GH) *in vitro* decreases the ability of the adenosine analogue, *N*⁶-phenylisopropyladenosine (PIA), to inhibit isoprenaline-stimulated lipolysis by a mechanism which is dependent on both gene transcription and protein serine/threonine phosphorylation. The inhibition is not due to a change in ligand binding to the adenosine receptor, the amounts of the three isoforms of the inhibitory GTP-binding protein, G_i, or the maximum (forskolin-stimulated) adenylate cyclase activity. The ability of GH to modulate the PIA-activated adenosine receptor to stimulate dissociation of heterotrimeric G_i was assessed by measurement of pertussis toxin-catalysed

ADP-ribosylation of G_i; GH does not appear to alter the interaction between the activated receptor and G_i. The ability of GH to alter the ability of activated G_i to inhibit adenylate cyclase activity was assessed by measuring the ability of a GTP analogue, guanosine 5'-[βγ-imido]triphosphate (p[NH]ppG), to inhibit forskolin-stimulated adenylate cyclase activity; chronic exposure to GH prevented this effect of p[NH]ppG. Thus the attenuation of the inhibition of lipolysis by PIA by chronic exposure of adipocytes to GH appears to be due to an impairment in the interaction between adenylate cyclase and the α subunit of one or more isoforms of G_i.

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Introduction

Growth hormone (GH) acts chronically to increase lipolysis in adipocytes by several mechanisms including both an increased response and sensitivity to β-adrenergic agonists and a decreased response and sensitivity to antilipolytic factors such as adenosine and prostaglandin E, the relative importance of these mechanisms depending on the physiological state of the animal (Vernon *et al.* 1987, 1993, Goodman *et al.* 1988, Watt *et al.* 1991, Beauville *et al.* 1992, Yang *et al.* 1995, Doris *et al.* 1994, 1996, Dante *et al.* 1995, Houseknecht *et al.* 1995). The increased response and sensitivity to β-adrenergic agents induced by GH appears to be due to increases in the number of β-adrenergic receptors (Watt *et al.* 1991, Vernon *et al.* 1993, Yang *et al.* 1995, Doris *et al.* 1996) and also a change in the ability of hormone-sensitive lipase to translocate to the fat droplet on activation (Vernon *et al.* 1993).

Locally produced antilipolytic factors such as adenosine and prostaglandin E interact with their own receptors on the cell surface and this results in the dissociation and activation of isoforms of the heterotrimeric inhibitory GTP-binding protein, G_i, into their α and their β plus γ subunits (Birnbaumer *et al.* 1990, Houslay 1994). Three isoforms of G_i have been identified, all having distinct α subunits (G_{i1}α, G_{i2}α and G_{i3}α) and common β and γ subunits (Birnbaumer *et al.* 1990, Houslay 1994). While all

three isoforms are present in adipose tissue (Mitchell *et al.* 1989), G_{i2} appears to be the form primarily involved in transmitting the inhibitory signal from the adenosine (A1) receptor to adenylate cyclase (Houslay 1994, Kalkbrenner *et al.* 1996), although G_{i1} and/or G_{i3} can also transmit signal from the adenosine receptor to some extent, at least in the absence of G_{i2} (Rudolph *et al.* 1996).

Lowering serum GH concentrations in rats with a specific antiserum increased sensitivity (but not maximum response) to the adenosine analogue *N*⁶-phenylisopropyladenosine (PIA), and this was associated with an increase in the amount of the α subunit of G_{i2}; ligand binding to the adenosine receptor and the amounts of the α subunits of G_{i1} and G_{i3} were unchanged (Doris *et al.* 1994). Treatment of such rats with ovine GH reversed the effects of the antiserum on both sensitivity to PIA and amount of G_{i2} (Doris *et al.* 1994).

In contrast to the above, chronic treatment of sheep (Doris *et al.* 1996) or cows (Houseknecht & Bauman 1997) *in vivo* with GH decreased maximum response of adipocytes to PIA but had no effect on either the number of adenosine receptors or the amount of G_{i1} plus G_{i2}. In the present study we show that maintenance of explants of sheep adipose tissue in culture for 24 h with GH mimics the effect of treating sheep *in vivo* with the hormone on the response of lipolysis to PIA, and then we use this culture system to show that GH impairs the ability of one or more

isoforms of G_i to interact with adenylate cyclase by a mechanism that involves both protein serine/threonine phosphorylation and gene transcription.

Materials and Methods

Animals

Finn × Dorset-horn cross-bred, castrated male sheep aged 6–8 months were used and were fed a diet of cereals (300 g/day) plus hay available *ad libitum* for at least 4 weeks before sampling. On the day of killing, sheep were anaesthetised by injection of 20–30 ml Sagatal (Veterinary Drug Co., Falkirk, Scotland, UK) into the jugular vein. They were then exsanguinated and samples of subcutaneous adipose tissue were removed aseptically from the flank fat pads immediately anterior to the hind limbs. The samples were placed immediately in sterile 0.15 M NaCl at 37 °C.

Tissue culture

Explants weighing about 15 mg were cut using scissors and cultured in Medium 199 with Earle's salts, L-glutamine, 25 mM Hepes (pH 7.3) (Gibco Biocult, Paisley, Strathclyde, Scotland, UK), 2.6 mM acetate (final concentration) and antibiotics (Robertson *et al.* 1982). Tissue was preincubated in the culture medium for 22 h after which it was transferred to fresh culture medium plus or minus 4.5 nM GH and other agents as indicated in the text and tables and maintained in culture for a further period of 24 h. Ovine pituitary GH was a gift from NIADDK, Bethesda, MD, USA; H7 was from Scientific Marketing, Barnet, Herts, UK; okadaic acid was from Calbiochem-Novabiochem (UK) Ltd, Nottingham, Notts, UK; phorbol myristyl acetate (PMA) was from Sigma-Aldrich, Poole, Dorset, UK. Okadaic acid and PMA were dissolved in dimethyl sulphoxide (DMSO) and subsequently diluted with Medium 199; maximum DMSO concentration in the culture medium was 1 µl/ml; this concentration had no effect on the variables measured in the study.

Lipolysis

Pieces of adipose tissue before or after maintenance in culture were transferred to flasks containing 2.5 ml Krebs–Ringer bicarbonate buffer (Krebs & Hensleit 1933) (containing 1.22 mM CaCl_2) with 25 mM Hepes buffer, pH 7.4, 5.5 mM glucose, 2 mM acetate and 30 mg/ml BSA (fatty acid-free and dialysed) (Hanson & Ballard 1968). Other agents were added as indicated in the text and tables. Adenosine deaminase, when added, was dialysed overnight before use. Tissue was incubated for 3 h at 37 °C after which samples of medium were removed,

deproteinised and stored at –20 °C until assayed for glycerol (Plested *et al.* 1987). Adipocyte mean cell volume and number/g tissue were determined as described previously (Robertson *et al.* 1982).

Preparation of adipocytes and adipocyte membranes

Adipocytes were prepared by collagenase digestion and washed as described previously (Vernon *et al.* 1995). They were disrupted by suspension in 10–15 ml 10 mM Tris/HCl, pH 7.4, containing 20 mM EDTA, 200 mM sucrose, 17.5 µg/ml benzamidine/HCl and 0.2 mM phenyl-methanesulphonyl fluoride at 40 °C and vortex-mixed for 2 min. This preparation was immediately centrifuged at 2800 g for 5 min at 22 °C; this minimises trapping of membranes within the lipid layer. The infranatant was removed, diluted to 50 ml with ice-cold 10 mM Tris/HCl, pH 7.4, containing 90 mM NaCl, and centrifuged at 42 000 g for 30 min at 4 °C. The membrane pellet was resuspended in 50 mM Tris/HCl, pH 7.4, containing 10 mM MgCl_2 , snap-frozen and stored in liquid N_2 . The protein concentration of the membranes was determined by the Bradford (1976) method using BSA as standard.

Receptor binding and GTP-binding protein concentration of adipocyte membranes

Ligand binding to adenosine receptors was assessed essentially as described previously (Watt *et al.* 1991). Adipocyte membranes (200 µg protein/ml) were pre-incubated in 50 mM Tris/HCl (pH 7.4) plus 10 mM MgCl_2 containing 1 µg/ml adenosine deaminase for 10 min at 37 °C to remove endogenous adenosine, and then incubated at 37 °C for a further 10 min with either 20 nM [^3H]PIA or a range of [^3H]PIA concentrations in a final volume of 0.2 ml. [^3H]PIA, 49 Ci/mmol, was from Amersham International, Amersham, Bucks, UK. Non-specific binding was determined by the addition of 100 µM PIA. Incubations were stopped by the addition of 5 ml ice-cold incubation buffer. The contents of each assay tube were then immediately filtered under vacuum through Whatman GF/C filter discs (2.5 cm diameter). The filters were washed with 2 × 5 ml ice-cold incubation buffer and dried under vacuum. Filters were placed in 10 ml Opti-Fluor scintillant to measure radioactivity.

The amounts of G-protein α subunits were determined by immunoblotting after separation by SDS–PAGE as described previously (Strassheim *et al.* 1990, Vernon *et al.* 1995). Anti-peptide sera (CSI, SGI and 13B) that specifically recognised the two isoforms (42 and 45 kDa) of the stimulatory (with respect to adenylate cyclase) GTP-binding protein, G_s (CSI), G_{i1} plus G_{i2} (SGI) and G_{i3} (13B) were raised in house and characterised as described previously (Mitchell *et al.* 1989, Vernon *et al.* 1995). After autoradiography, the relative amounts of the G-protein α subunits were determined by densitometry.

ADP-ribosylation of membrane proteins

Pertussis toxin-stimulated ADP-ribosylation of G_i was performed as described by McKenzie (1992) with modifications. Pertussis toxin (1 µg/ml) was activated by preincubation with 50 mM dithiothreitol plus 0.08 mM ATP for 60 min at room temperature. ADP-ribosylation was performed using 250 mM sodium phosphate, pH 7.0, 20 mM thymidine, 1 mM ATP, 0.1 mM GTP, 20 mM arginine, 10 µg/ml activated pertussis toxin and 10 µM [32 P]NAD; incubation was for 2 h at 37 °C. The reaction was terminated by the addition of 1 ml ice cold 50 mM Tris/HCl, pH 7.5, containing 100 µM phenylmethylsulphonyl fluoride and 1 mg/ml leupeptin. Labelled membranes were collected by centrifugation (14 000 g, for 10 min at 4 °C); they were separated by SDS-PAGE, detected by autoradiography (McKenzie 1992) and quantified by densitometry.

Assay of adenylate cyclase activity

This was determined essentially as described by Heyworth & Houslay (1983). Briefly, the reaction conditions employed the use of a medium containing 5 mM $MgSO_4$, 10 mM theophylline, 1 mM EDTA, 22 mM disodium phosphocreatine, 1.5 mM ATP, 1 mM dithiothreitol, 1 mg creatine kinase/ml and 25 mM triethanolamine/KOH, pH 7.4. Incubations were carried out over 10 min, using a final volume of 0.1 ml with 3–7 µg protein at a temperature of 30 °C. In experiments where PIA was employed, theophylline was omitted from the assays and the non-methylxanthine cAMP phosphodiesterase inhibitor Ro-07-2956 was added at 0.1 mM, together with 1 unit of adenosine deaminase/ml. Other agents were added as described in the text and tables. The amount of cAMP produced was measured as described by Whetton *et al.* (1983).

Statistical analysis

Each value contributing to a mean was obtained from a different sheep. Results were analysed by ANOVA or Student's *t*-test for paired observations, and are presented as means $\pm$ S.E.M. or with S.E.D. (standard error of difference) as indicated in the text and tables or figure legends.

Results

Effects of GH on response of lipolysis to PIA and isoprenaline

The addition of PIA markedly inhibits isoprenaline-stimulated lipolysis in sheep adipose tissue with an ED_{50} of about 2 nM (Fig. 1). Maintenance of tissue in culture for 48 h in the absence of exogenous hormones resulted in a small decrease ($P < 0.05$; ANOVA) in response to PIA

Figure 1 Effect of chronic exposure to GH on the ability of PIA to inhibit isoprenaline-stimulated lipolysis in sheep adipose tissue. Explants of sheep adipose tissue were maintained in culture for 22 h in the absence of exogenous hormones and then for a further 24 h with or without 4.5 nM GH. Subsequently the ability of various concentrations of PIA to inhibit the rate of lipolysis stimulated by 10^{-5} M isoprenaline plus adenosine deaminase was assessed. ●, Tissue before culture; ■, tissue cultured without GH; ▲, tissue cultured with GH for the last 24 h. Values are means of four observations, each from a different sheep; S.E.D., from ANOVA, 9.8 (values for 100 nM PIA are given as means $\pm$ S.E.D.).

(Fig. 1). The addition of GH (4.5 nM) during the last 24 h of culture markedly decreased the response to PIA ($P < 0.001$; ANOVA) (Fig. 1).

Pooling of results from a number of experiments, including that shown in Fig. 1, showed that tissue culture with GH for 24 h also induced a small but significant increase in the rate of basal (unstimulated) lipolysis ($P < 0.001$) and that stimulated by isoprenaline plus adenosine deaminase ($P < 0.001$); values were 4.51 and 3.86 µmol/3 h per 10^6 cells for stimulated lipolysis after culture with and without GH respectively (S.E.D. 0.16) and 1.61 and 0.86 µmol/3 h 10^6 cells for basal lipolysis after culture with and without GH respectively (S.E.D. 0.15). Results are means of 26 observations.

A time course study showed that effects of GH on the response of lipolysis to PIA developed gradually over a 24 h period (Fig. 2). Basal lipolysis increased in response to GH with a similar time course to the change in response to PIA (results not shown). The effects of GH were lost if actinomycin D, an inhibitor of transcription, was included during culture (Table 1).

Effect of GH on components of the adenosine signal-transduction system

[3 H]PIA binding to the adenosine receptor was measured in adipocyte membrane preparations from tissue cultured with and without GH (Fig. 3); ANOVA showed that culture with GH had no effect on [3 H]PIA binding.

Figure 2 Time course of the development of the effect of GH on the response of lipolysis in sheep adipose tissue to PIA. Explants of sheep adipose tissue were maintained in culture for 22 h in the absence of exogenous hormones and then for periods up to 24 h with (●) or without (○) 4.5 nM GH. Subsequently the ability of 100 nM PIA to inhibit lipolysis stimulated by 10^{-5} M isoprenaline plus adenosine deaminase was assessed as described in the text. Values are means of three observations each from a different sheep; S.E.D. from ANOVA, 5.6 (values for 24 h given as means $\pm$ S.E.D.).

Scatchard plots of the results presented in Fig. 3 were linear (not shown) and gave K_d values of 1.5 ± 0.04 and 2.3 ± 0.09 nM respectively for membranes from control and GH-treated adipocytes; corresponding B_{max} values were 228 ± 12 and 240 ± 37 fmol PIA bound/mg protein for membranes from control and GH-treated adipocytes respectively (means $\pm$ S.E.M. obtained from regression analysis of Scatchard plots). Figure 3 shows that essentially maximum binding was achieved with concentrations of [3 H]PIA of 20 nM, as found previously with sheep adipocyte membranes (Vernon *et al.* 1995). [3 H]PIA

Figure 3 Effect of chronic exposure to GH on binding of [3 H]PIA to adenosine receptors. Explants of sheep adipose tissue were maintained in culture for 46 h in the absence (○) or presence (●) of 4.5 nM GH during the last 24 h. Subsequently adipocyte membranes were prepared and the amount of [3 H]PIA bound determined. Results are means of three observations, each from a different sheep; S.E.D. from ANOVA, 33 (values for 50 nM PIA given as means $\pm$ S.E.D.).

binding was also assessed in three further experiments using a single concentration of 20 nM PIA; pooling these values with corresponding values from Fig. 3 gave values of 211.1 and 215.2 fmol PIA bound/mg protein for membranes from control and GH-treated adipocytes respectively; values are means of six observations, S.E.D. 8.3. Thus GH does not appear to alter maximum ligand binding to the adenosine receptor, suggesting that the number of adenosine receptors does not change in response to chronic exposure to GH. Tissue culture with GH also had no effect on the amount of G_i1+G_i2 or G_i3 (Table 2). These observations suggest that GH may exert its effects on the G_i -based antilipolytic system by altering

Table 1 Effect of actinomycin D on the ability of GH to modulate the lipolytic system of sheep adipose tissue. Explants of adipose tissue were maintained in culture for 22 h without added agents and then for a further 24 h with and without combinations of GH (4.5 nM) and actinomycin D (80 nM). Subsequently, the rates of basal lipolysis and that stimulated by isoprenaline (Isop.) (10^{-5} M) plus adenosine deaminase (0.8 μ g/ml) were measured and the ability of 100 nM PIA to inhibit isoprenaline-plus-adenosine deaminase-stimulated lipolysis determined. Results are means of four observations

Addition to culture	Inhibition of Isop.-stimulated lipolysis by PIA (%)	Rate of lipolysis (μ mol glycerol/3 h per 10^6 cells)	
		Isop.-stimulated	Basal
None	71.2 ^a	2.83 ^a	0.60
GH	42.5 ^b	3.57 ^b	0.99
Actinomycin D	60.6 ^a	2.97 ^a	0.60
GH+actinomycin D	60.6 ^a	2.82 ^a	0.67
S.E.D.	7.3	0.27	0.21

Values in a column without the same suffix (a, b) differ significantly ($P < 0.05$) (ANOVA).

Table 2 Effect of culture with GH hormone on the amounts of G-proteins of sheep adipocyte membranes. Explants of adipose tissue were maintained in culture for 22 h in the absence of exogenous hormones and then for a further 24 h with and without 4.5 nM GH, after which adipocyte membranes were prepared and the amount of G-proteins assessed by immunoblotting and densitometry. Results are means $\pm$ S.E.M. for three observations

Treatment	Amount of G-protein (arbitrary units)			
	G _i 1+2	G _i 3	G _s (45 kDa)	G _s (42 kDa)
Control	259 $\pm$ 66	555 $\pm$ 93	1150 $\pm$ 39	2287 $\pm$ 55
GH	207 $\pm$ 40	496 $\pm$ 128	1271 $\pm$ 103	2539 $\pm$ 66

the ability of G_i to interact with either the adenosine receptor or adenylate cyclase, or both.

To explore the mechanism of the GH effect further, adipocyte membrane preparations were obtained from adipose tissue maintained in culture with and without GH. As shown in Table 3, culture with GH had no effect on basal or forskolin-stimulated (maximum) adenylate cyclase activity, nor was the ability of isoprenaline to activate adenylate cyclase altered. However, the ability of 10^{-7} M PIA to inhibit isoprenaline-plus-GTP-stimulated adenylate cyclase activity was completely lost after culture with GH, whereas with membranes from control tissue, PIA caused a significant ($P<0.05$), $26.2 \pm 4.0\%$, decrease in activity (Table 3). Thus the change elicited by GH in intact adipocytes is retained in membrane preparations.

Culture with GH had no effect on the amount of pertussis toxin-catalysed ADP-ribosylation of G_i (3154 ± 267 and 3472 ± 267 c.p.m./mg protein for membranes from cells after culture with and without GH; results are means $\pm$ S.E.M. for five observations). The addition of $1 \mu\text{M}$ PIA during the ADP-ribosylation reaction decreased ($P<0.01$) the amount of ribosylation of G_i by $36.8 \pm 5.9\%$ and $37.0 \pm 3.9\%$ of these membranes

Table 3 Effect of culture with GH on the adenylate cyclase activity of adipocyte membranes. Explants of adipose tissue were maintained in culture for 22 h and then for a further 24 h in the presence or absence of 4.5 nM GH. Adipocyte membranes were then prepared and the adenylate cyclase activity assessed in the presence or absence of various effectors. Results are means $\pm$ S.E.M. for four observations

Effector	Adenylate cyclase activity (pmol/min per mg protein)	
	Control	GH
Basal (none)	62 $\pm$ 13	72 $\pm$ 10
+ Isoprenaline (10^{-4} M)	216 $\pm$ 46	225 $\pm$ 76
+ Forskolin (10^{-4} M)	805 $\pm$ 49	865 $\pm$ 85
+ GTP (10^{-5} M)	88 $\pm$ 6	76 $\pm$ 3
+ Isoprenaline+GTP	359 $\pm$ 27	401 $\pm$ 101
+ Isoprenaline+GTP+PIA (10^{-7} M)	262 $\pm$ 13	424 $\pm$ 97

from cells after culture with and without GH respectively (means $\pm$ S.E.M. for five observations; no significant difference). The lack of effect of GH on the ability of PIA to decrease ADP-ribosylation was confirmed in a further experiment using a range of PIA concentrations (Fig. 4).

To assess G_i activity independently of receptor activation, we measured the ability of guanosine 5'-[β -imido]triphosphate (p[NH]ppG), a non-hydrolysable analogue of GTP, to inhibit forskolin-stimulated adenylate cyclase activity (Strassheim *et al.* 1990). At low concentrations, p[NH]ppG is thought to activate only G_i whereas at higher concentrations it activates both G_i and G_s (Strassheim *et al.* 1990). As shown in Fig. 5, with membranes from control tissue, low concentrations of p[NH]ppG inhibited forskolin-stimulated adenylate cyclase activity ($P<0.001$; ANOVA). In contrast, with

Figure 4 Effect of chronic exposure to GH on the ability of PIA to decrease pertussis toxin-catalysed ADP-ribosylation of sheep adipocyte membranes. Explants of sheep adipose tissue were maintained in culture for 46 h in the absence (○) or presence (●) of 4.5 nM GH during the last 24 h. Subsequently adipocyte membranes were prepared and the amount of pertussis toxin-catalysed ADP-ribosylation determined in the presence and absence of various concentrations of PIA. Results are means of three observations, each from a different sheep; S.E.D. from ANOVA, 5.7 (values for 10^{-6} M PIA given as means $\pm$ S.E.D.).

Figure 5 Effect of chronic exposure to GH on the ability of the GTP analogue, p[NH]ppG, to inhibit forskolin-stimulated adenylate cyclase activity of sheep adipocyte membranes. Explants of sheep adipose tissue were maintained in culture for 46 h in the absence (○) or presence (●) of 4.5 nM GH during the last 24 h. Subsequently adipocyte membranes were prepared and the ability of various concentrations of p[NH]ppG to inhibit forskolin (10⁻⁴ M)-stimulated adenylate cyclase activity was determined. Values are means of four observations, each obtained from a different sheep; S.E.D. from ANOVA, 5.4 (values for 1 nM p[NH]ppG given as means ± S.E.D.).

membranes from tissue cultured with GH, p[NH]ppG had no inhibitory effect on stimulated adenylate cyclase activity.

Effects of inhibitors of protein serine kinases and phosphatases

Evidence for a role for protein phosphorylation was sought using a protein serine kinase inhibitor, H7, and a protein serine phosphatase inhibitor, okadaic acid; the concentration of inhibitors used was based on findings from an earlier study (Vernon 1996) investigating the mechanism

of the inhibition of lipogenesis by GH in sheep adipose tissue. At a concentration of 100 µM, H7 during culture prevented the effect of GH on PIA inhibition of lipolysis and also GH stimulation of basal lipolysis (Table 4). In the absence of GH, H7 had no effect on the response to PIA, basal lipolysis or isoprenaline-stimulated lipolysis (Table 4). Okadaic acid at 10 nM mimicked the effect of GH on the response to PIA (Table 4); however, the effects of the two agents were additive (Table 4).

Effect of PMA

Acute exposure to PMA results in activation of some isoforms of protein kinase C (PKC), while chronic exposure to PMA leads to their downregulation and hence loss of activity (Dekker & Parker 1994). Culture of explants of sheep adipose tissue with 10 µg/ml PMA, in the presence and absence of GH, for 5 h after the usual 22 h pre-incubation, had no effect on either basal or isoprenaline-stimulated lipolysis or the response to PIA (n=3, results not shown). Addition of 10 µg/ml PMA during either the second 24 h period of culture or during the whole 46 h period (i.e. added during the 22 h preincubation period as well as the 24 h culture period) had no effect on the changes in basal or isoprenaline-stimulated lipolysis or response to PIA induced by GH (Table 5); indeed, PMA partly mimicked the effects of GH on basal lipolysis and response to PIA (Table 5).

Discussion

Chronic treatment with GH *in vitro* decreases the antilipolytic effect of PIA on sheep adipose tissue, and thus mimics the effect of chronic treatment with GH *in vivo* of sheep (Doris *et al.* 1996) and cows (Dante *et al.* 1995,

Table 4 Effects of H7 and okadaic acid on the ability of GH to modulate the lipolytic system of sheep adipose tissue. Explants of adipose tissue were maintained in culture for 22 h without added agents and then for a further 24 h with and without combinations of GH (4.5 nM), H7 (100 µM) and okadaic acid (10 nM). Subsequently, the rates of basal lipolysis and that stimulated by isoprenaline (Isop.) (10⁻⁵ M) plus adenosine deaminase (0.8 µg/ml) were measured and the ability of 100 nM PIA to inhibit Isop.-plus-adenosine deaminase-stimulated lipolysis determined. Results are means of four observations

	H7			Okadaic acid		
	Inhibition of Isop.-stimulated lipolysis by PIA (%)	Rate of lipolysis (µmol glycerol/3 h per 10 ⁶ cells)		Inhibition of Isop.-stimulated lipolysis by PIA (%)	Rate of lipolysis (µmol glycerol/3 h per 10 ⁶ cells)	
		Isop.-stimulated	Basal		Isop.-stimulated	Basal
Addition to culture						
None	26.1 ^a	4.23 ^a	0.90 ^a	44.0 ^a	6.24	1.80 ^a
GH	10.8 ^b	4.87 ^{ab}	1.89 ^b	22.8 ^b	6.70	3.75 ^b
Inhibitor	36.0 ^a	4.44 ^a	0.87 ^a	18.8 ^b	6.62	2.57 ^a
GH+inhibitor	34.2 ^a	5.83 ^b	1.20 ^a	4.3 ^c	6.30	4.17 ^b
S.E.D.	7.2	0.34	0.25	7.3	0.70	0.54

Values in a column without the same suffix (a, b, c) differ significantly (P<0.05) (ANOVA).

Table 5 Effect of phorbol ester (PMA) on the ability of GH to modulate the lipolytic system of sheep adipose tissue. Explants of adipose tissue were maintained in culture either without exogenous agents or with PMA (10 µg/ml) for 22 h and then for a further 24 h with combinations of GH (4.5 nM) and PMA (10 µg/ml). Subsequently, the rates of basal lipolysis and that stimulated by isoprenaline (Isop.) (10^{-5} M) plus adenosine deaminase (0.8 µg/ml) were measured and the ability of 100 nM PIA to inhibit Isop.-plus-adenosine deaminase-stimulated lipolysis determined. Results are means of four observations

Additions to culture		Inhibition of Isop.-stimulated lipolysis by PIA (%)	Rate of lipolysis (µmol glycerol/3 h per 10^6 cells)	
0–22 h	22–46 h		Isop.	Basal
None	None	60.3 ^a	3.78	0.47 ^a
None	GH	38.5 ^b	4.67	0.95 ^b
None	PMA	44.5 ^{ba}	3.67	0.56 ^{ac}
None	PMA+GH	36.0 ^b	4.54	0.94 ^b
PMA	PMA	50.8 ^{ab}	3.60	0.86 ^{bc}
PMA	PMA+GH	40.5 ^b	4.52	1.12 ^b
S.E.D.		7.6	0.58	0.16

Values in a column without the same suffix (a, b, c) differ significantly ($P < 0.05$) (ANOVA).

Houseknecht *et al.* 1995, Houseknecht & Bauman 1997) on this system. Furthermore the effects of GH *in vitro* on the antilipolytic effects of PIA do not appear to involve a change in ligand binding to adenosine receptors or the amount of G_i1+G_i2 or G_i3 , again in agreement with our previous studies with sheep (Doris *et al.* 1996) and cows (Houseknecht & Bauman 1997) in which animals were chronically treated with GH *in vivo*. As forskolin-stimulated adenylate cyclase activity did not change on GH treatment, it would appear that the amount of adenylate cyclase also is unchanged by GH. These observations suggested that GH may exert its effects on the G_i -based antilipolytic system by altering the ability of G_i to interact with either the adenosine receptor or adenylate cyclase, or both. As the effect of GH on the ability of PIA to inhibit the lipolytic system was retained in isolated membranes, it allowed further dissection of the basis of the GH effect.

Pertussis toxin elicits the ADP-ribosylation of the undissociated G_i heterotrimer (Tsai *et al.* 1984, Wong *et al.* 1985); interaction of G_i with the activated receptor causes dissociation of G_i into the α subunit and the $\beta\gamma$ subunit with a concomitant decrease in pertussis toxin-catalysed ADP-ribosylation. Thus pertussis toxin-catalysed ADP-ribosylation along with determination of the total amount of G_i by immunoblotting can provide an indication of the proportion of the heterotrimer that has dissociated into α and $\beta\gamma$ subunits. The decrease in pertussis toxin-catalysed ADP-ribosylation induced by PIA (Fig. 4) is consistent with PIA activation of the adenosine receptor, resulting in dissociation of heterotrimeric G_i . As GH did not alter the ability of PIA to decrease pertussis toxin-catalysed ADP-ribosylation, it would appear that the interaction of G_i with the agonist-activated receptor is not altered by culture with GH.

In contrast with the above, a recent study in which lactating cows were treated chronically with GH *in vivo* reported a diminished pertussis toxin-stimulated ADP-ribosylation of one or more G_i isoforms of adipose tissue (Houseknecht & Bauman 1997); these authors did not, however, examine the effects of GH on the ability of PIA to decrease pertussis toxin-stimulated ADP-ribosylation of G_i . This finding of Houseknecht & Bauman (1997) is surprising as it suggests an enhanced dissociation of G_i into its subunits in the GH-treated animals. The reason for the difference between their study and the data presented here is not clear; however, their membranes were prepared from whole adipose tissue taken by biopsy so it is possible that the enhanced dissociation of G_i in the GH-treated cattle may reflect altered concentrations of agonists acting via G_i in the tissue at the time of sampling. Whatever, if there was more undissociated G_i in the GH-treated cattle, it must presumably be less active to account for the decreased antilipolytic effect of PIA that they observed.

Assessment of G_i activity independently of receptor activation using p[NH]ppG showed that, with membranes from control tissue, low concentrations of p[NH]ppG inhibited forskolin-stimulated adenylate cyclase activity to a similar extent to that found previously with rat adipocyte membranes (Strassheim *et al.* 1990, 1991). In addition to the present study, a diminished ability of G_i to inhibit adenylate cyclase activity of adipocyte membranes has been found previously with preparations from streptozotocin-diabetic rats (Strassheim *et al.* 1990) and from obese (*fa/fa*) Zucker rats (Strassheim *et al.* 1991). As p[NH]ppG had no effect on forskolin-stimulated adenylate cyclase activity in membranes from tissue cultured with GH, it appears that the diminished ability of PIA, and probably other antilipolytic agents such as prostaglandin E, to inhibit lipolysis after chronic exposure

of adipose tissue to GH is due to an impaired ability of G_i to interact with adenylate cyclase. This suggests a covalent change, such as phosphorylation, to either isoform of G_i (Houslay 1991, 1994, Morris *et al.* 1996) which couple to adenylate cyclase or the catalytic unit of adenylate cyclase itself (Taussig & Gilman 1995).

The present study indicates involvement of at least one protein serine/threonine phosphorylation in the mechanism by which GH attenuates response to PIA. The identity of the protein serine/threonine kinases involved, however, is not clear. H7 can inhibit a number of protein kinases including protein kinase A (PKA) and PKC isoforms. PKA mediates the stimulation of lipolysis by isoprenaline. As H7 had no effect on either basal or isoprenaline-stimulated lipolysis, it appears that PKA was not inhibited by the concentration of H7 used. A concentration of 1 mM H7 was found to be required to inhibit basal lipolysis in sheep adipose tissue (Vernon 1996). Gorin *et al.* (1990) have suggested that the effects of GH on lipolysis in rat adipose tissue involve activation of PKC. This would not appear to be the case in sheep adipose tissue, as a 5 h exposure to PMA, which enhances the rate of lipogenesis in sheep adipose tissue (Vernon 1996), had no effect on the lipolytic system, while chronic exposure to PMA, which should lead to a loss of most, but not all, PKC isoforms, did not prevent the effects of GH.

While a protein serine/threonine phosphorylation is supported by the studies with H7 and okadaic acid, the mechanism of GH action on the system is clearly complex, developing slowly over a 24 h period and involving an actinomycin D-sensitive step, presumably gene transcription. Earlier studies, carried out before the discovery of the adenosine-antilipolytic system, also showed that lipolytic effects of GH developed slowly and were prevented by inhibitors of gene transcription (Fain *et al.* 1965).

The effects of GH on the response to PIA resemble its chronic inhibitory effects on lipogenesis, which also develop slowly, and are prevented by actinomycin D and H7, again implicating gene transcription and protein serine/threonine phosphorylation (Vernon 1996). However, mechanisms involved in the chronic modulation of lipogenesis and lipolysis by GH in sheep adipose tissue are not identical, as okadaic acid mimicked the effect of GH on lipolysis but prevented the effect of GH on lipogenesis (Vernon 1996). In addition, chronic exposure to PMA partly prevented the effects of GH on lipogenesis (Vernon 1996) but tended to mimic the effects of GH on lipolysis.

Thus this and our previous study (Doris *et al.* 1994) show that GH can alter the ability of antilipolytic agents, which act via G_i , to inhibit lipolysis by at least two mechanisms: through a change in the concentration of G_{i2} and through a change in the ability of one or more isoforms of G_i to interact with adenylate cyclase. The latter involves a mechanism that appears to involve both gene transcription and protein serine/threonine phosphorylation.

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