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Insulin and insulin-like growth factor-I responsiveness and signalling mechanisms in C_2C_{12} satellite cells: effect of differentiation and fusion

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Abstract

In proliferating C_2C_{12} myoblasts, serum and physiological concentrations of insulin and IGF-I stimulated protein synthesis and RNA accretion. After fusion, the multinucleated myotubes remained responsive to serum but not to insulin or IGF-I, even though both insulin and type-1 IGF receptor mRNAs increased in abundance. Protein synthetic responses to insulin and IGF-I in myoblasts were not inhibited by dexamethasone, ibuprofen or Ro-31-8220, thus phospholipase A_2, cyclo-oxygenase and protein kinase C did not appear to be involved in the signalling mechanisms. Neither apparently were polyphosphoinositide-specific phospholipase C or phospholipase D since neither hormone increased inositol phosphate, phosphatidic acid, choline or phosphatidylbutanol production. Only the phosphatidylinositol-3-kinase inhibitor, wortmannin, and the 70 kDa S6-kinase inhibitor, rapamycin, wholly or partially blocked the effects of insulin and IGF-I on protein synthesis. 2-deoxyglucose uptake remained responsive to insulin and IGF-I after fusion and was also inhibited by wortmannin. The results suggest that the loss of responsiveness after fusion is not due to loss of receptors, but to the uncoupling of a post-receptor pathway, occurring after the divergence of the glucose transport and protein synthesis signalling systems, and that, if wortmannin acts at a single site, this is prior to that point of divergence.

Keywords: Insulin; Insulin-like growth factor I; Myoblast; Differentiation; Intracellular signalling; mRNA

1. Introduction

Following the loss of skeletal muscle through injury or disease, new fibres can be formed by proliferation, fusion and maturation of satellite cells [1,2]. An understanding of the mechanisms regulating these processes may be of great benefit in combatting the effects of sepsis and trauma and in states such as cachexia. To investigate these events we have used the pure, immortalised mouse satellite cell line, C_2C_{12} [3], which is morphologically similar to primary satellite cell cultures [4]. Using these cells in the unfused, myoblast state, we have shown that protein and DNA synthesis are stimulated by Epidermal growth factor (EGF), basic Fibroblast growth factor (bFGF), 12-*O*-tetradecanoylphorbol 13-acetate (TPA) and exogenous phospholipase D (PLD) and that PLD-dependent and independent pathways are involved in these events [5].

Knowledge of the pathways which control protein synthesis and hypertrophy following fusion of my-

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oblasts into multinucleated myofibres is equally important in assessing a cell line as a model for myogenesis. The C_2C_{12} cell line forms myotubes in culture and can participate in muscle regeneration in vivo [6]. However, we have shown that, upon differentiation, the protein synthetic response to EGF, bFGF, TPA and PLD is lost [5]. This is not surprising with regard to EGF and bFGF as receptors for both are permanently down-regulated during terminal differentiation of several skeletal muscle cell lines, including C_2C_{12} cells [7]. In contrast, both TPA and PLD elicit second messenger production in C_2C_{12} myotubes suggesting that uncoupling occurs downstream of the receptor [5].

Mature skeletal muscle is one of the major targets for action of anabolic hormones such as insulin and insulin-like growth factor-I (IGF-I). For example, in vivo, muscle protein synthesis is highly sensitive to physiological concentrations of insulin in overnight-fasted rats [8], whilst studies in vitro have demonstrated that insulin is mitogenic at high concentrations [9], and affects cell metabolism and stimulates protein synthesis at a concentration closer to the physiological range in isolated muscle [10] and in L6 myoblasts [11]. Furthermore, unlike the receptors for EGF and bFGF, those for insulin and IGF-I increase upon fusion [12–14], suggesting an important role for these two agonists, in keeping with their function in vivo. Studies both in vivo and in vitro have implicated phospholipase A_2 (PLA_2) and the release of arachidonic acid in the stimulation of muscle protein synthesis by insulin. Conversion of arachidonate to cyclo-oxygenase metabolites appears to play an important role in the control of translation in isolated muscle [10] and in L6 myoblasts [15], whereas arachidonate activation of protein kinase C (PKC) may be the mechanism by which insulin stimulates transcription in L6 cells [16]. Thus skeletal muscle and the L6 cell line have similarities in their insulin signal transduction pathways. In other respects, such as the extent to which fusion occurs, the C_2C_{12} line may be a preferable model.

The aims of this study were to investigate the ability of insulin and IGF-I to stimulate protein synthesis in C_2C_{12} cells before and after fusion and to evaluate the suitability of the C_2C_{12} cell line as a model for the action of anabolic hormones in skeletal muscle by determining if the signal transduction mechanisms involved in the action of insulin and IGF-I are the same as those employed in L6 myoblasts and intact muscle.

2. Materials and methods

2.1. Materials

[9,10(*n*)-^{3}H]oleic acid, [*methyl*-^{3}H]choline chloride, [^{3}H]inositol, L-[2,6^{3}H]phenylalanine, [^{14}C]2-deoxy-D-glucose, gamma-CT^{32}P, Hyperfilm MP and Megaprime random priming kit were from Amersham International (Amersham, Bucks., UK). Other suppliers were: EN^{3}HANCE spray, N.E. Technologies (Stevenage, Herts., UK); IGF-I, Bachem (Saffron Walden, Essex, UK); insulin, human actrapid, Novo Nordisk A/S (Bagsvaerd, Denmark); Silica gel 60 A K6 plates, Whatman (Maidstone, Kent, UK); Ultima Gold Scintillation fluid, Packard (Pangbourne, Berks., UK); tissue culture plasticware, Greiner (Dursley, Glos., UK); Superscript reverse transcriptase and all media and sera, Gibco/BRL Life Technologies (Paisley, Scotland); Ro-31-8220 and rapamycin Calbiochem (Beeston, Notts., UK). All other chemicals were from Sigma (Poole, Dorset, UK).

2.2. Protein synthesis, RNA accretion, and second messenger production

C_2C_{12} cells (American Type Culture Collection) were grown on 60 mm Petri dishes in 2.5 ml of Dulbecco's modified Eagle's medium (DMEM) +12% foetal calf serum [17]. They were used either before the onset of fusion, when approx. 75% confluent, or 4–5 days after the onset of fusion, induced by incubation with DMEM +10% horse serum. Before each experiment, subconfluent or fused cells were stepped down to 2 ml of serum-free DMEM. Then, 18 h later, insulin, IGF-I or TPA were added for up to 6 h and at concentrations described in the text. Dexamethasone was dissolved in ethanol and added to give a final concn. in the medium of 100 nM, it was added 18 h before insulin, i.e. when the medium was changed, because its inhibitory effects are not apparent in the short term [18]. All other inhibitors, ibuprofen (in ethanol, final concn. 1 μM); Ro-31-8220 (in DMSO, final concn. 1 μM); wortmannin

and rapamycin (both in DMSO, final concn. 100 nM) were added 10 min before insulin or IGF-I. Equal volumes (maximally 4 μl) of carrier were added to controls. Protein synthesis was measured by adding L-[2,6^{3}H]phenylalanine for the final 60 min of incubations [19]. This method, a modification of a technique widely used in vivo [20] involves elevating the phenylalanine concentration of the medium by adding, per ml of medium, 750 nmol L-phenylalanine specific radioactivity 3,000 dpm/nmol. 60 min later cells were rapidly washed 3 times in ice-cold PBS, pH 7.4. Protein bound and free intracellular phenylalanine were separated by incubating the cells in each dish in 1 ml of 0.3 M-NaOH at 37°C for 30 min. Protein was then precipitated with 0.5 ml of 2 M perchloric acid. Following centrifugation, the supernatant was used to determine total RNA [21] and the pellet was washed twice in 0.2 M-PCA, redissolved in 1 ml of 0.3 M-NaOH and aliquots were analysed for total protein content [22] and by scintillation counting to determine the specific radioactivity (d.p.m./μg protein).

Measurements of total inositol phosphates (IP), phosphatidic acid (PA), phosphatidylbutanol (PtdBuOH) and choline release were performed as described previously [5,19], on cells prepared as above and labelled for 18 h in serum-free medium with [^{3}H]inositol, [^{3}H]oleic acid or [^{3}H]choline chloride, respectively.

2.3. 2-Deoxyglucose uptake

For glucose uptake experiments, cells were grown under the same conditions but medium was replaced with 2 mls Kreb's-Ringer phosphate [23]. Insulin (0.5 nM–1 μM) or IGF-I (1–100 nM), 20 μl of a solution containing 2-deoxyglucose (10 μCi per ml) and glucose (final concn. 5 μM) were added. Uptake of glucose was measured over periods of 15 min-1 h, after which the cells were washed 3 times in ice-cold PBS, solubilised in 0.3 M-NaOH and uptake was determined by scintillation counting.

2.4. Extraction and resolution of mRNAs and Northern blotting

Cells were grown in 90 mm dishes containing 8 ml medium. Either before or after fusion, induced as described above, total RNA was extracted [24,25] and RNA (20 μg) was resolved in a 1.2% agarose gel containing formaldehyde and transferred to a nylon membrane (Genescreen, Dupont) by overnight capillary blotting. Two cDNAs; a 1.5 kb *Eco*RI/*Pst*I fragment of the human insulin receptor gene corresponding to 1,101–2,602 which can be found in the extracellular domain [26] and a 2 kb *Bgl*II fragment of the human GLUT-1 gene [27] were then labelled with gamma-CT^{32}P using the Megaprime random priming kit. After Northern hybridisation [24], the membranes were exposed to pre-flashed autoradiography film (Hyperfilm MP; 10 days at −70°C).

2.5. Reverse transcriptase polymerase chain reaction (RT-PCR)

cDNAs were prepared from 5 μg of total RNA using an oligo-dT-primer and Superscript reverse transcriptase. The respective cDNAs were then amplified using specific primers (IGF-1 forward primer CTCGGATCCTCCTCGCATCTCTTCTACC; reverse primer CTCAAGCTTCTTGTTTCCTG-CACTCCCTC: IGF-1 receptor forward primer GC-CTCTCCGGGTTTGAAAATG; reverse primer CCGGAGCCAGACTTCATTCCT) and the resulting PCR products were resolved in a 1.8% agarose gel, stained with ethidium bromide and visualised under ultraviolet light. PCR products of 381 bp and 333 bp were obtained using the IGF-1 and IGF-1 receptor specific primers, respectively.

2.6. Statistics

Each experiment was performed 2 or 3 times, with 4 or 5 replicates per treatment. Data were analysed by two way analysis of variance (using set as a blocking factor) with the Genstat 5 version 3.1 statistics package. Results of experiments which were not significantly different were combined and a Student's *t*-test was used to assess the level of significance of effects of the various agonists.

3. Results

3.1. Protein synthesis

When protein synthesis was measured in subconfluent myoblasts during the final hour of a 6 h

Table 1
Effect of 6 h exposure to insulin and IGF-I on protein synthesis and RNA accretion in fused and unfused C_2C_{12} cells

	Protein-bound phenylalanine — specific radioactivity			
	Unfused (dpm/μg protein)	Fused (dpm/μg protein)	Unfused (RNA; μg/dish)	Fused (RNA; μg/dish)
Control	9.2 ± 0.2	10.2 ± 0.3	25.8 ± 1.7	114.6 ± 2.4
Insulin				
0.5 nM	11.1 ± 0.2 ***	10.5 ± 0.3	28.2 ± 2.0	113.4 ± 2.5
5 nM	12.4 ± 0.1 ***	10.7 ± 0.4	36.8 ± 1.9 **	114.6 ± 1.9
50 nM	13.4 ± 0.2 ***	10.8 ± 0.3	37.5 ± 0.7 ***	115.2 ± 1.7
500 nM	14.6 ± 0.2 ***	10.6 ± 0.2	44.1 ± 0.7 ***	113.8 ± 0.8
1 μM	14.6 ± 0.4 ***	10.1 ± 0.4	44.0 ± 1.0 ***	117.4 ± 2.2
IGF-I				
1 nM	12.9 ± 0.3 ***	10.6 ± 0.2	29.5 ± 0.5 *	113.4 ± 0.6
5 nM	13.8 ± 0.1 ***	10.1 ± 0.2	34.8 ± 0.6 ***	116.0 ± 2.1
100 nM	14.1 ± 0.2 ***	10.4 ± 0.3	37.9 ± 0.6 ***	117.0 ± 2.0
12% FCS	14.8 ± 0.1 ***	15.3 ± 0.3 ***	29.5 ± 0.3 ***	116.5 ± 1.1

Cells were used either before reaching confluence or 4–5 days after the onset of fusion. Insulin, IGF-I or foetal calf serum (FCS) were added for 6 h, incorporation of [^{3}H]phenylalanine into protein was measured over a 1 h period commencing 5 h after adding the agonist. Values are means of 10 replicates ± S.E.M. (2 identical experiments each with $n = 5$).
Significance of differences (by Student's *t*-test); *, **, ***; $P < 0.05$, 0.01, 0.001 vs. control, respectively.

incubation, insulin at a concentration of 0.5 nM (a value close to the physiological range; ca 70 μU/ml) increased [^{3}H]phenylalanine incorporation by 21%. Higher concentrations of insulin induced greater stimulation of both protein synthesis and RNA accretion (46% and 45%, respectively, at 50 nM; 59% and 71%, respectively, at 1 μM; Table 1). IGF-I gave similar results, increasing protein synthesis by 53% and RNA accretion by 47% at the highest concentration (100 nM).

In differentiated cells, used 4–5 days after the onset of fusion, all these effects were lost, neither insulin nor IGF-I stimulated protein synthesis or RNA accretion (Table 1). However the ability to respond to repletion with foetal calf serum was not lost, protein synthesis was increased by 61% and 50% respectively in the subconfluent and the fused cells (Table 1).

Briefer (90 min) exposure to 0.5 nM insulin stimulated protein synthesis in the unfused myoblasts by 19% and 1 nM IGF-I gave an 18% increase (Table 2). Higher concentrations of insulin (5 and 50 nM) and 5 nM IGF-I enhanced protein synthesis further, from 9.0 ± 0.2 to 11.8 ± 0.1, 12.0 ± 0.3 and 12.2 ± 0.1 respectively (all n = 10; P < 0.001). Thus the maximal increase in protein synthesis induced by insulin was 32% and by IGF-I was 34%.

Again, after fusion to myotubes the ability to respond to 0.5 nM insulin and 1 nM IGF-I was lost (Table 2). Higher concentrations were equally ineffective; 5 and 50 nM insulin and 5 nM IGF-I gave specific radioactivities of 9.6 ± 0.2, 9.9 ± 0.2 and 10.0 ± 0.4 respectively (all $n = 10$) and not significantly different from the control (10.0 ± 0.3).

3.2. Insulin and type-I IGF receptor and IGF-I mRNAs

The second series of experiments was designed to investigate the possibility that the loss of response

Table 2
Effect of 90-min exposure to insulin and IGF-I on protein synthesis in fused and unfused C_2C_{12} cells

	Specific radioactivity (dpm/μg) protein	
	Unfused	Fused
Control	9.1 ± 0.2	10.0 ± 0.3
Insulin (0.5 nM)	10.8 ± 0.3 *	9.6 ± 0.3
IGF-I (1 nM)	10.7 ± 0.3 *	10.1 ± 0.3

Details as in Table 1 but insulin or IGF-I were added for 90 min, phenylalanine incorporation was measured over a 1-h period commencing 30 min after adding insulin. Values are means of 10 replicates ± S.E.M. (2 identical experiments each with $n = 5$); *, $P < 0.001$ vs. control.

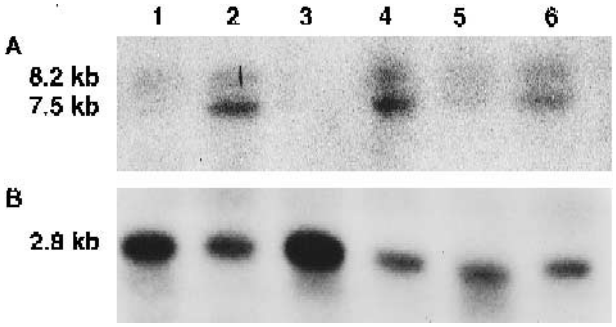

Fig. 1. Northern hybridisation of total RNA with (a) insulin receptor and (b) GLUT-1 specific cDNAs. Results are shown for total RNA (20 μg) extracted from triplicate cultures of subconfluent myoblasts (lanes 1,3,5) or myotubes, 5 days after the onset of fusion (lanes 2,4,6).

related to a loss of insulin and IGF-I receptors. However, a Northern blot of the insulin receptor mRNA (Fig. 1A) revealed that it had increased markedly on fusion. Furthermore, mRNAs for the IGF-I receptor and IGF-I itself were both detected by RT-PCR in the cells before and after fusion and whilst the IGF-I mRNA changed little in abundance on fusion (Fig. 2A), the type 1-IGF receptor mRNA, like that for the insulin receptor, increased in the differentiated cells (Fig. 2B).

3.3. Glucose transport

Additional evidence for the presence of receptors was provided by the observation that not all of the metabolic responses to insulin and IGF-I were lost on

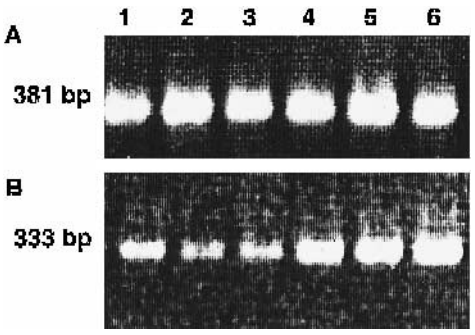

Fig. 2. RT-PCR of the IGF-I and type 1 IGF receptor specific genes. Messenger RNAs for IGF-I (A) and the type 1 IGF-I receptor (B) were detected by visualisation of a specific PCR product in triplicate cultures of subconfluent myoblasts (lanes 1,2,3) or myotubes, 5 days after the onset of fusion (lanes 4,5,6).

Table 3
Effect of insulin and IGF-I on 2-deoxyglucose uptake in fused and unfused C_2C_{12} cells

	Glucose uptake, cpm/μg protein	
	Unfused	Fused
Control	78 ± 4 (12)	9.2 ± 0.2 (12)
Insulin		
0.5 nM	98 ± 5 ** (12)	10.3 ± 0.5 * (12)
5 nM	110 ± 4 *** (8)	12.2 ± 0.6 *** (12)
1 μM	122 ± 4 *** (8)	13.9 ± 0.2 *** (8)
IGF-I		
1 nM	120 ± 5 *** (8)	11.9 ± 0.9 ** (12)
5 nM	119 ± 5 *** (12)	12.4 ± 0.5 *** (8)
100 nM	124 ± 3 *** (8)	13.9 ± 0.5 *** (8)
100 nM Wortmannin	47 ± 6 *** (8)	4.7 ± 0.3 *** (8)
Insulin 5 nM + 100 nM Wortmannin	53 ± 2 (8)	5.1 ± 0.2 (8)

Medium was replaced with 2 ml Kreb's-Ringer phosphate and experiments were performed 1 h later by adding insulin (0.5 or 5 nM) or IGF-I (1 or 5 nM) and 20 μl of a solution containing 2-deoxyglucose (10 μCi/ml) and glucose to obtain a concentration in the medium of 5 μM. Values are means ± S.E.M. for the no. of replicates shown in parentheses (two or three identical experiments each with 4 dishes of cells).
Significance of differences (by Student;s *t*-test); *, **, ***; $P < 0.05$, 0.01, 0.001 vs. control, respectively.

fusion. In myotubes, in sharp contrast to the response of protein synthesis, insulin and IGF-I both significantly increased glucose transport (both by 51% at the maximum concentration in a 1 h incubation; Table 3). The linearity of the response was examined by measuring glucose transport after 15 and 30 min. Increases of 37 ± 1% and 57 ± 3% were obtained at 15 and 30 min respectively (both $n = 8$, $P < 0.01$).

To compare the time-course of effects on protein synthesis under similar conditions, protein synthesis was measured 15 and 30 min after adding insulin (1 μM) and [^{3}H]phenylalanine simultaneously. Insulin increased phenylalanine incorporation from 2.6 ± 0.1 to 3.9 ± 0.1 d.p.m. per μg of protein at the end of a 15-min incubation; corresponding values at 30 min were 4.9 ± 0.1 and 7.1 ± 0.2 d.p.m. per μg (all $P < 0.001$; $n = 8$).

3.4. PLA_2, cyclo-oxygenase and PKC inhibition

In the third series of experiments, the potential role of a number of signal transduction pathways was

Table 4
Effect of dexamethasone, ibuprofen, Ro 31-8220, wortmannin and rapamycin on the stimulation of protein synthesis in C_2C_{12} myoblasts by insulin and IGF-I

Addition	Specific radioactivity (dpm/μg protein)		
	control	0.5 nM insulin	1 nM IGF-I
None	16.3 ± 0.2	18.9 ± 0.2 **	n.d.
Dexamethasone 100 nM	14.9 ± 0.1 ††	17.3 ± 0.3 **	n.d.
None	16.5 ± 0.4	19.5 ± 0.2 **	19.6 ± 0.2 **
Ibuprofen 1 μM	16.8 ± 0.3	18.9 ± 0.3 **	19.3 ± 0.3 **
None	14.9 ± 0.3	17.4 ± 0.2 **	17.3 ± 0.2 **
Ro 31-8220 1 μM	12.6 ± 0.2 ††	14.8 ± 0.3 **	14.7 ± 0.5 **
None	15.8 ± 0.5	19.7 ± 0.1 **	n.d.
Wortmannin 100 nM	13.6 ± 0.5 †	14.9 ± 0.3	n.d.
None	17.0 ± 0.4	21.7 ± 0.6 **	21.2 ± 0.3 **
Rapamycin 100 nM	15.3 ± 0.8 ††	18.1 ± 0.2 *	17.9 ± 0.4

Dexamethasone was added to sub-confluent cultures in serum free DMEM 18 h before insulin. Ibuprofen, Ro-31-8220, wortmannin or rapamycin were added 10 min before insulin or IGF-I which were added for 6 h, incorporation of [^{3}H]phenylalanine into protein was measured over the final 1 h
Values are means of 10 replicates ± S.E.M. (2 identical experiments each with $n = 5$). Significance of effect of insulin or IGF-I vs. corresponding control ± inhibitor *, ** $P < 0.01$, $P < 0.001$. Significant inhibition of basal rate of protein synthesis †, ††; $P < 0.05$, $P < 0.001$.

investigated, firstly, by examining effects of specific inhibitors on the stimulation of protein synthesis.

Dexamethasone, an inhibitor of PLA_2, did not inhibit the ability of insulin to stimulate protein synthesis in the unfused cells, whilst ibuprofen, a cyclooxygenase inhibitor, and the PKC inhibitor, Ro-31-8220, also failed to block the effects of either agonist (Table 4).

Lack of involvement of PLA_2 was also indicated by the failure of insulin or IGF-I to increase glycerophosphocholine release during a 30-min incubation (542 ± 60 and 518 ± 28, respectively; control = 536 ± 24; $n = 4$).

3.5. Metabolites of phospholipases C and D

The involvement of polyphosphoinositide-specific phospholipase C (PI-PLC) and PLD in the response of myoblasts to insulin and IGF-I was also investigated by measuring metabolites of PI-PLC (i.e., IP) and of PLD (PA and choline). Production of the stable transphosphatidylation product, PtdBuOH, in the presence of 0.5% butanol was also measured. Neither insulin nor IGF-I increased IP production, whereas AlF_4^-, an activator of G-proteins coupled to PI-PLC, gave a 177% increase (Table 5). Similarly, insulin and IGF-I both failed to increase the production of PtdBuOH or PA or the release of choline, whilst TPA, used as a positive control to stimulate PLD, elicited a more than 20-fold increase in PtdBuOH and increases of 40% and 80%, respectively, in PA and choline release (Table 5).

3.6. Involvement of 70 kDa S6-kinase and phosphatidylinositol-3-kinase

Two inhibitors did affect the ability of insulin to stimulate protein synthesis. A partial inhibition of the response was obtained with rapamycin, an inhibitor of the 70 kDa S6-kinase ($p70^{S6K}$; Table 4). Insulin stimulation was decreased from 28% in the absence of rapamycin to 18% in its presence. IGF-I-induced stimulation was also reduced, from 25 to 17% (Table 4). Complete inhibition was obtained with wortmannin, an inhibitor of phosphatidylinositol-3-kinase (PI-3 kinase). This was apparently not due to a general toxic effect of wortmannin as the basal rate of protein synthesis was reduced by only 12–17%. As further evidence for a specific effect of wortmannin, the ability of 1 nM TPA to stimulate protein synthesis during a 90-min incubation was undiminished by this inhibitor (TPA increased the specific radioactivity of protein-bound phenylalanine from 19.9 ± 0.4 to 22.8 ± 0.4 d.p.m./μg in the absence of wortmannin and

Table 5
Release of metabolites of PI-PLC and PLD in response to insulin and IGF-I

	Total dpm released			
	Inositol phosphates	Choline	PA	PtdBuOH
Control	1364 ± 110	1 298 ± 45	10 993 ± 463	1 942 ± 456
Insulin	1536 ± 84	1 157 ± 94	11 985 ± 464	1 954 ± 296
IGF-I	1486 ± 88	1 457 ± 150	11 267 ± 376	1 704 ± 272
AlF_4^-	3784 ± 183 *	1 508 ± 84	12 274 ± 376	1 897 ± 84
TPA	n.d.	2 371 ± 158 *	15 336 ± 655 *	42 807 ± 4 310 *

Cells were labelled for 18 h in serum free medium with [^{3}H]inositol, [^{3}H]choline chloride or [^{3}H]oleic acid. Release of metabolites was measured over a 30-min period following addition of 0.5 nM insulin or 1 nM IGF-I. There were no significant effects of either agonist. 1 nM TPA or AlF_4^- (aqueous solutions of $AlCl_3$ and NaF to give final concentrations of 100 μM and 10 mM respectively) were used as positive controls. Values are means of 10 replicates ± S.E.M. (2 identical experiments each with $n = 5$) except for choline release data where data are from a single experiment, $n = 5$; by Student's t-test *, $P < 0.001$.

from 16.7 ± 0.7 to 19.4 ± 0.3 in its presence). Wortmannin also inhibited glucose uptake, reducing the basal rate by 60% and 50%, respectively, in the unfused and fused cells, and completely blocking the ability of insulin to stimulate glucose uptake in both myoblasts and myotubes (Table 3).

4. Discussion

Insulin and IGF-I both induced a significant increase in protein synthesis in myoblasts within 90 min, an effect which was probably due to an increase in the rate of translation, since it was not blocked by actinomycin D [5]. Similar effects were observed at the end of a 6 h incubation, by which time the enhanced phenylalanine incorporation was accompanied by an increase in RNA content of the cells suggesting that the effects were due, at least in part, to an increase in transcription. The complete inability of insulin and IGF-I to increase protein synthesis after fusion, suggests that both agonists lose their ability to stimulate transcription and translation following differentiation.

It has previously been shown that fusion of C_2 cells results in a 5- to 10-fold increase in insulin receptor number and ^{125}I-insulin binding [12], whilst terminal differentiation of BC3 H-1 myocytes results in similar changes in insulin receptor number and binding. Other studies in C_2 cells have also shown increases in IGF-I mRNA, IGF-I itself and the number of both types of IGF receptor after differentiation [13,14]. Another widely used muscle cell line, L6, also exhibited a two-fold increase in receptor number and ^{125}I-insulin binding as an early differentiation event [28]. In this latter case, the increase in insulin receptor number correlated well with increased ability to stimulate glucose and amino acid uptake. Therefore, although we have not demonstrated that the mRNAs for the two receptors are being translated, published evidence, and that presented here, suggests that the failure of insulin and IGF-I to stimulate processes after fusion which they were capable of increasing in the myoblast is not likely to be due to a loss of the insulin or type 1 IGF receptors. Nor is there any evidence to suggest autocrine or paracrine effects of IGF-I after fusion, since the IGF-I mRNA changed little, if at all, on fusion, and glucose uptake remained sensitive to exogenous IGF-I in the fused cells. This latter observation also supports the conclusion that functional receptors are not lost following differentiation. Glucose transport is probably mediated by translocation of GLUT 1 in C_2/C_{12} cells, as it is in BC3H-1 myocytes, since both these cell lines lack GLUT 4 [23]. Stimulation of glucose transport of similar magnitude to that found in the present study has been reported previously in differentiated C_2C_{12} cells [29]. Although there was little effect of fusion on the ability of insulin or IGF-I to stimulate glucose uptake, it was noticeable that, in sharp contrast to the essentially similar rates of protein synthesis before and after fusion, the unfused cells had a basal rate of 2-deoxy glucose uptake per unit of protein 9 times that in the myotubes. This would appear to be consistent with the apparent

reduction in the mRNA for GLUT 1 upon fusion (Fig. 1B).

To examine the possibility that, following fusion, the persistence of the stimulation of glucose uptake measured after 1 h, and the loss of the protein synthetic effect, measured over a longer time period, was due to degradation of the hormone, both measurements were made 15 and 30 min after the addition of insulin. Using these same time periods, the fact that the protein synthetic response was still absent, whilst the glucose transport effect appeared to be approximately linear, suggests that insulin degradation is not the reason for the different responses in myotubes.

The fact that the rate of incorporation of phenylalanine into protein was similar before fusion and 4–5 days after the onset of fusion, and that cells responded to repletion with foetal calf serum at both these times, makes the complete loss of response to insulin and IGF-I surprising. Our data are in agreement with the hypothesis that the insulin-insensitivity of protein synthesis is probably due to events that occur after receptor binding. In this respect, they agree with the observation that glucocorticoid-induced insulin resistance is due to a post-receptor defect [30], possibly involving PKC [31]. The mitogenic effects of insulin e.g. effects on creatine kinase and DNA labelling [9], like the effect on glucose uptake reported here, either persist or increase on fusion, again suggesting that the hormones still bind to their receptors and therefore the loss of effects in the present study is due to the uncoupling or loss of part of the signal transduction mechanism. Several possible such pathways have been investigated. Dexamethasone, an inhibitor of PLA_2, has been shown to inhibit the ability of insulin to stimulate protein synthesis in L6 myoblasts [11] and in rat muscle in vivo [32]. Similarly, ibuprofen, a cyclo-oxygenase inhibitor, blocked the effect of insulin on translation in L6 myoblasts [15] and other non-steroidal anti-inflammatory drugs such as indomethacin and meclofenamate have been shown to block insulin-stimulated protein synthesis in isolated muscle [10] and in vivo [33]. It was therefore surprising that, in the present experiments, inhibition of PLA_2 and cyclo-oxygenase, with dexamethasone or ibuprofen respectively, was ineffective in blocking the effects of insulin in C_2C_{12} myoblasts. Insulin also failed to activate PI-PLC or PLD although it has been shown to stimulate PI-PLC in rat epididymal fat pads [34] and PLD in rat hepatocytes [35]. PI-PLC and PLD are clearly present in C_2C_{12} cells as in both the present study and in previous work [5], AlF_4^- and TPA produced large increases in IP and PtdBuOH, respectively.

We have previously shown that protein synthesis in C_2C_{12} cells is stimulated by both PLD-dependent and -independent routes and demonstrated that EGF and bFGF stimulation of protein synthesis is PLD-independent [5]. It is now apparent that the stimulation of protein synthesis and RNA accretion by insulin in this cell line is independent not only of PLD but of PLA_2 and PI-PLC as well. This prompted the series of experiments in which the involvement of PI-3 kinase was investigated. PI-3 kinase is considered to play a pivotal role in insulin intracellular signalling [36] and wortmannin inhibits PI 3-kinase by association with the 110 kDa catalytic subunit [37]. The ability of wortmannin to block insulin stimulation of both glucose uptake and protein synthesis in unfused myoblasts could suggest that it is a non-specific inhibitor, or that it acts on two PI-3 kinase isoforms involved in these two signal transduction pathways. With regard to protein synthesis, non-specific inhibition is unlikely as wortmannin did not block the action of TPA. Furthermore, at the concentration used here, its action seems to be specific as it does not inhibit phosphatidylinositol 4-kinase [38], cyclic nucleotide dependent kinases or PKC [39]. An alternative explanation is that a single PI-3 kinase is a very early participant in the insulin signalling system, acting before the glucose transport and protein synthetic signals diverge. Previous published data suggest that this is the case; PI3-kinase couples the insulin receptor to downstream signalling components thought to be involved in regulating protein synthesis such as $p70^{S6k}$ and is also involved in the translocation of glucose transporters [40,41]. In this case, the ability of insulin to continue to stimulate glucose uptake, and of wortmannin to block this in the terminally differentiated myotube suggests that the uncoupling of protein synthesis occurs after divergence of the two pathways. The ability of rapamycin, an inhibitor of the $p70^{S6k}$ [42], to block the effect of insulin by about 50%, suggests that this enzyme and PI-3 kinase may be involved in a com-

mon pathway by which insulin stimulates protein synthesis in the C_2C_{12} myoblast.

The C_2C_{12} cell line does not appear to be a good model for studying metabolic effects of anabolic hormones such as insulin and the IGFs on skeletal muscle, because insulin and IGF-I cease to stimulate protein synthesis in fused myotubes and signalling pathways involving PLA_2 and cyclo-oxygenase which mediate insulin action in muscle do not appear to do so in the C_2C_{12} cell.

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