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Acute and long-term effects of insulin-like growth factor I on glucose transporters in muscle cells

Translocation and biosynthesis

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Insulin-like growth factor I (IGF-I) rapidly (<10 min) stimulated glucose uptake into myotubes of the L6 muscle cell line, at concentrations that act specifically on IGF-I receptors. Uptake remained stimulated at a steady level for 1–2 h, after which a second stimulation occurred. The first phase was insensitive to inhibition of protein synthesis. Subcellular fractionation demonstrated that it was accompanied by translocation of glucose transporters (both GLUT1 and GLUT4) to the plasma membrane from intracellular membranes. Translocation sufficed to explain the first phase increase in glucose transport, and there was no change in the total cellular content of GLUT1 or GLUT4 glucose transporters. The second phase of stimulation was inhibitable by cycloheximide, and involved a net increase in either GLUT1 or GLUT4 transporter content, which was reflected in an increase in transporter number in plasma membranes. These results define a cellular mechanism of metabolic action of IGF-I in muscle cells; furthermore, they suggest that IGF-I has acute metabolic effects that mimic those of insulin, bypassing action on the insulin receptor.

Sugar transport; Glucose transporter; Insulin-like growth factor; L6 muscle cell

1. INTRODUCTION

Insulin-like growth factor I (IGF-I) is a potent growth factor [1] inducing both muscle growth and differentiation [2]. These effects are extremely important during fetal maturation, but also during post-natal growth and for muscle regeneration. Recently, effects of IGF-I other than its growth and differentiation promoting actions were recognized, specifically the ability to lower glycemia when perfused in rats [3] or dogs [4]. This effect was associated with enhanced glucose utilization by skeletal muscle [4] and indeed was reflected in increased hexose uptake by muscles in vivo [5]. This metabolic effect of IGF-I is specific and independent of actions on the insulin receptor, and it has been proposed that IGF-I stimulation of glucose uptake can bypass insulin resistance in certain animal models of diabetes [3,6]. The molecular mechanism underlying such stimulation of glucose influx into muscle is unknown. In the present study we examined the effect of acute (5–45 min) and prolonged (2–8 h) effects of IGF-I on glucose transport and glucose transporters (subcellular distribution and biosynthesis) in differentiated L6 muscle cells in culture.

Abbreviations: αMEM, α Minimal Essential Medium; TM, total membranes; IM, intracellular light microsomes; PM, isolated plasma membranes; WGA, wheat germ agglutinin; GLUT1, brain/HEP-G2 glucose transporter; GLUT4, muscle/fat glucose transporter.

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2. EXPERIMENTAL

2.1. *Cell cultures, incubations and hexose uptake assays*

A clonally selected line of L6 muscle cells (selected for high fusion potential into myotubes) was grown in culture and allowed to fuse and differentiate, essentially as reported earlier [7]. Cells were studied at the myotube stage, when >90% fusion was attained. Monolayer cultures of myotubes in 3.5 cm diameter wells were incubated for 5 h in αMEM containing 25 mM D-glucose in the absence of serum. This results in low and steady basal glucose uptake rates [8]. Human recombinant IGF-I was then added at the indicated concentrations and for the indicated times, at 37°C, followed by removal of the medium and assay of hexose (2-deoxy-D-glucose) transport as described earlier [9]. Under the conditions of the assay (5 min, 10 μM hexose) transport is rate limiting over phosphorylation [9]. Non-carrier-mediated hexose uptake was measured in parallel as the cytochalasin B-insensitive component and was subtracted from all measurements.

2.2. *Membrane isolation*

Total membranes from L6 myotubes were prepared as described earlier [7]. Plasma membranes (PM) and intracellular light microsomes (IM) were isolated by a modification of a previously published procedure [10] as follows: cells of twelve 10 cm dishes of L6 myotubes in incubation medium were gently scraped with a rubber policeman, centrifuged (700 × *g* for 10 min), the cell pellet was resuspended in 16 ml of ice-cold buffer I (250 mM sucrose, 20 mM HEPES, pH 7.4, 2 mM EGTA, 3 mM NaN_3 containing freshly added protease inhibitors: 200 μM PMSF, 1 μM leupeptin, 1 μM pepstatin A, 10 μM E-64) and homogenized in a 40 ml glass Dounce type A homogenizer (20 strokes). All subsequent steps were conducted at 4°C. The homo-

genate was centrifuged at 700 × g for 5 min and the resultant supernatant (SN1) was collected and set on ice. The pellet (P1) was resuspended in 8 ml of buffer I and re-homogenized followed by 700 × g 5 min centrifugation. Pellet P2 was discarded, and supernatants SN2 and SN1 were pooled and centrifuged at 31,000 × g for 60 min. Supernatant SN3 was used to collect IM by centrifugation at 177,000 × g for 60 min and resuspension in 0.5 ml buffer I. The 31,000 × g pellet (P3) was resuspended in buffer I to 3 ml using a Wheaton 5 ml teflon on glass homogenizer, and layered on a discontinuous sucrose gradient (32% w/w, 40% w/w and 50% w/w, in 20 mM HEPES, pH 7.4, 3 ml each). After centrifugation at 210,000 × g for 2.5 h, membranes were collected through the side of the gradient tube by puncture with a syringe from the 32% layer and 32%/40% and 40%/50% interfaces. Each fraction was diluted 20-fold with 5 mM HEPES, pH 7.4, centrifuged at 31,000 × g for 60 min and resuspended in 0.5 ml buffer I. Membrane protein was de termined by the bicinchoninic acid method (Pierce). To prepare total membranes (TM), the mixture SN1+SN2 was centrifuged at 177,000 × g for 60 min.

The PM, identified by their enrichment in α1 Na/K ATPase subunit and retention of label after biotinylation of intact cells, were found only on the 32% sucrose layer (see Results). IM were largely depleted of α1 Na/K ATPase and did not retain biotin label. Biotinylation was carried out by labelling whole cells with NHS-LC-biotin (Pierce), and detection on Western blots using avidin-coupled alkaline phosphatase.

2.3. *Western blotting*

GLUT1 and GLUT4 glucose transporter isoforms were detected by Western blot analysis essentially as described earlier [7] using C-terminus specific antibodies to each isoform (RaGLUTRANS and RaIRGT, respectively, from East Acres Biologicals). α1 Na/K ATPase was detected with hybridoma culture supernatant (1:100) containing the isozyme-specific monoclonal antibody McK1 and ^{125}I- labelled sheep anti-mouse IgG (0.1 μCi/ml). Statistic analysis was by Student's *t*-test.

3. RESULTS AND DISCUSSION

3.1. *Dose-dependence and time-course of IGF-I stimulation of hexose transport*

Fig. 1A shows a representative IGF-I dose–response curve of the stimulation of 2-deoxyglucose uptake in L6 myotubes exposed to the growth factor for 45 min. Maximal stimulation was ~300% above basal transport, and it was attained with 1–3 nM IGF-I. Half-maximal stimulation was obtained with 0.1 nM IGF-I* These concentrations are far lower than those at which IGF-I interacts with the insulin receptor in L6 muscle cells [11,12]. L6 muscle cells have been shown to express IGF-I receptors [11,13] and insulin receptors ([12] and R.J. Smith, personal communication). Based on binding and immunoprecipitation studies in WGA-purified membrane proteins, an insulin receptor with high affinity for IGF-I [13] was also proposed to exist in these cells. However, since 10 nM IGF-I did not displace bound ^{125}I-insulin from intact L6 cells at all, and 100 nM IGF-I displaced only 10% of the bound insulin [11],

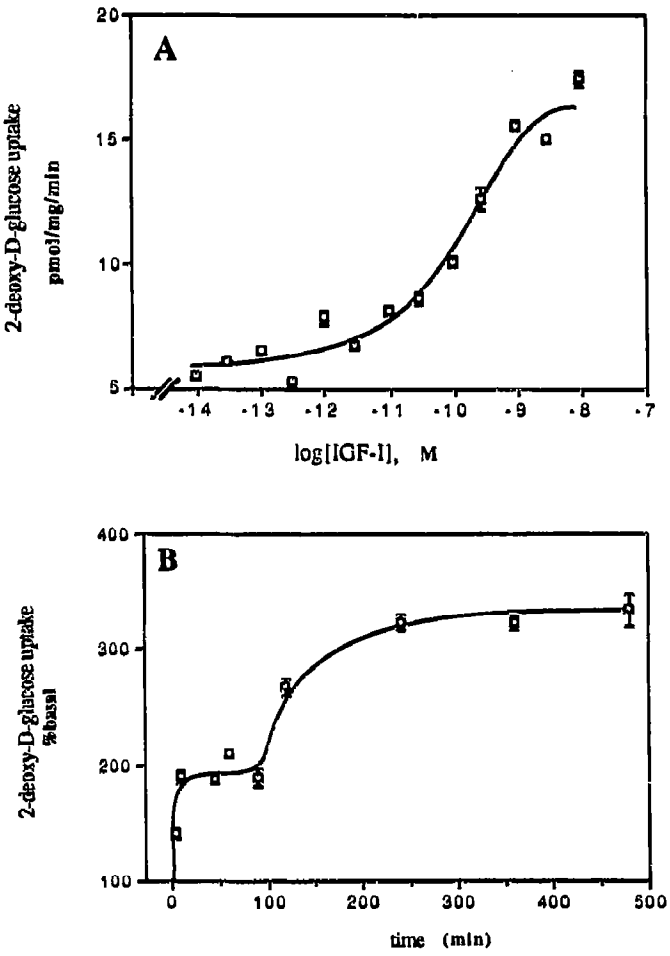

Fig. 1. (A) Dose–response curve of the stimulation of 2-deoxy-D-glucose uptake by IGF-I. L6 myotubes were pre-incubated for 5 h in αMEM containing 25 mM glucose in absence of serum, and then exposed to the indicated concentrations of IGF-I and 5 mg/ml BSA in the above medium for 45 min. This was followed by rapid rinsing and determination of initial rates of carrier-mediated 2-deoxy-D-glucose uptake in HEPES-buffered saline solution (see Experimental). Results are the mean of six replicates in one experiment representative of 4 with average maximal stimulation of 210±30% and average ED_{50} of 0.07±0.03 nM. (B) Time–course of IGF-I action on 2-deoxy-D-glucose uptake. Incubations were as in A except that cells were exposed to 10 nM IGF-I for the indicated time periods. Results are the mean of three replicates in one experiment representative of 3.

it can be considered that, at concentrations at or below 10 nM, IGF-I is bound exclusively to the IGF-I receptor and not to the classical insulin receptor.

Fig. 1B illustrates the time–course of stimulation of hexose uptake by 10 nM IGF-I. Surprisingly, the growth factor had a temporal bimodal effect: a rapid stimulatory phase was completed in 10 min, followed by a plateau period and a secondary stimulatory phase after 1–2 h which lasted for up to 8 h. The second phase was not due to a glucose-deprivation effect caused by glucose exhaustion from the medium, since similar results were observed when the medium was changed

*The dose–response curve does not fit a perfect sigmoidal curve. This could indicate that more than one binding site is responsible for stimulation of glucose transport. Alternatively, the shape of the curve may be governed by the amplification process of the IGF-I signalling pathway.

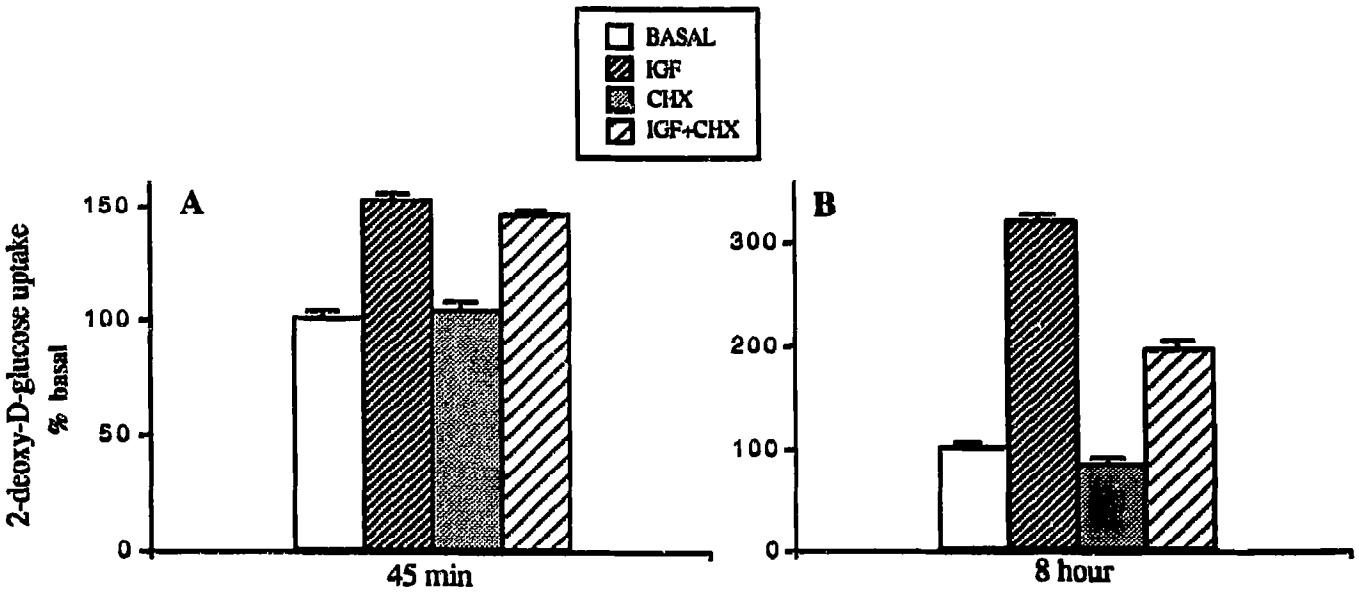

Fig. 2. Effect of cycloheximide on the early and late phases of IGF-I stimulation of hexose transport. L6 myotubes were pre-incubated in serum-free αMEM containing 25 mM glucose for 4.5 h, followed by a 30 min incubation in the absence or presence of 5 μg/ml cycloheximide (CHX). Incubation with 3 nM IGF-I followed, in otherwise the same media, for (A) 45 min or (B) 8 h. Hexose uptake was subsequently determined as in Fig. 1A. Results are the mean of six replicates in one experiment representative of 2, expressed as % of basal uptake. CHX (5 μg/ml) lowered ^{35}[S]methionine incorporation into protein to 6% of its value in control cells.

several times during the 8 h incubation (not shown). The fast stimulatory phase was not inhibited by pre-incubation with the protein synthesis inhibitor cyloheximide (Fig. 2A), whereas the second stimulation was largely prevented by this treatment (Fig. 2B). Thus, the second stimulatory phase depends on protein synthesis.

3.2. *The rapid phase: recruitment of glucose transporters*

The mechanism underlying the fast phase was investigated by assaying the content of glucose transporter isoforms in subcellular membrane fractions (PM and IM). L6 myotubes were previously shown to express both the GLUT1 and GLUT4 isoforms of glucose transporters [7,14]. Subcellular fractions were isolated as described in Experimental. The purity of the membrane fractions was confirmed by an enrichment in α1 Na/K ATPase (a plasma membrane marker) in the PM but not in the IM relative to TM. Western blot analysis of GLUT1 and GLUT4 transporter content are illustrated in Fig. 3A. The GLUT1 isoform was more abundant in the PM than in the IM, whereas the reverse was true for the GLUT4 isoform. IGF-I treatment (10 nM for 45 min) prior to subcellular fractionation resulted in a gain in GLUT1 transporters in the PM fraction and a concomitant loss in the IM fraction. A qualitatively similar observation was made for the GLUT4 isoform. These results suggest that both isoforms translocate from the IM to the PM in response to the growth factor. The specificity of this response was underscored by the lack of change in content of α1 Na/K ATPase subunit in response to cellular treatment with IGF-I (Fig. 3A). The other membrane fractions isolated by the subcellular fractionation procedure are membranes banding atop 40% and 50% sucrose. Glucose transporters were present at a low level in these fractions, but importantly neither fraction showed a change in GLUT1 or GLUT4 content in response to IGF-I (not shown). Fig. 3B presents the results of 7 Western blot studies of GLUT1 analogous to that illustrated in Fig. 3A, quantitated by laser scanning densitometry. Fig. 3C shows the equivalent results of GLUT4 immunoreactivity. The results are in arbitrary densitometric units per mg protein in each fraction. It can be appreciated that IGF-I caused a 1.22±0.09-fold increase in GLUT1 protein ($P<0.005$) and a 1.27±0.10-fold increase in GLUT4 protein ($P<0.001$) in the PM, and both isoforms decreased significantly in the IM fraction ($P<0.025$ for both isoforms), whereas α1 Na/K ATPase in the PM was not significantly affected (1.04±0.2). In contrast, neither GLUT1 nor GLUT4 content changed in the TM of IGF-I treated (45 min) cells.

D-Glucose-protectable binding of cytochalasin B is a recognized quantitative measurement of glucose transporters, and this approach was used to calculate the actual number of pmol of glucose transporters in the membrane fractions, irrespective of their isoform. In experiments not shown, we observed that the% increase in cytochalasin B binding in the PM fraction closely matched the% increase in hexose transport caused by

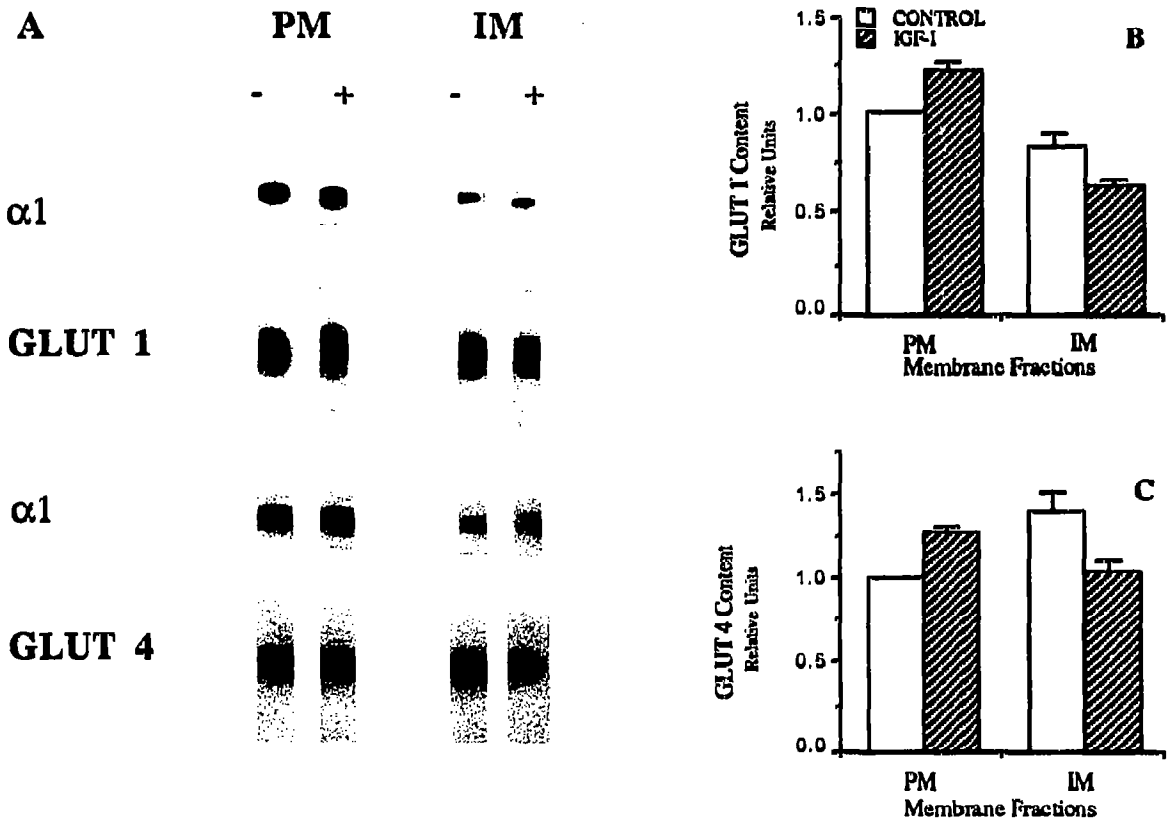

Fig. 3. (A) Rapid effect of IGF-I on GLUT1 and GLUT4 glucose transporters. L6 myotubes were serum-depleted for 16 h in αMEM containing 25 mM glucose, then incubated without (&min;) or with (+) 10 nM IGF-I for 45 min. Purified plasma membranes (PM) and intracellular light microsomes (IM) were prepared and GLUT1 and GLUT4 glucose transporters were detected by Western blot analysis as described in Experimental. The content of immunoreactive α1 Na/K ATPase subunit in each gel is given for assessment of membrane purity and specificity of IGF-I action. (B and C) Densitometric analysis of 7 experiments (mean±S.E.M.) similar to that illustrated in Fig. 3A. The content in control PM was assigned a value of 1.0 and other values are expressed in relative units.

IGF-I. These results indicate that translocation of glucose transporters alone may suffice to account for the rapid stimulation of transport activity caused by the growth factor. Such a comparison cannot be made from Western blot analysis of the individual isoforms, since the actual amount of each transporter isoform cannot be quantitated on a molar basis.

3.3. *The second phase: synthesis of glucose transporters*

The second (protein synthesis-dependent) phase of stimulation of hexose transport by IGF-I was also investigated. In contrast to the observations made of the rapid stimulatory phase, treatment with IGF-I for 8 h resulted in a gain in both GLUT1 and GLUT4 in TM (Fig. 4). Scanning densitometry of the fractions isolated from 5 experiments indicated that the increase in GLUT1 transporters was 1.27±0.11-fold ($P<0.05$) and the increase in GLUT4 transporters was 1.15±0.05-fold ($P<0.05$) in the TM (Fig. 4C). Isolated membrane fractions showed that both GLUT1 and GLUT4 increased in the PM (by 1.92±0.13 and 1.25±0.17, respectively) without significant decrease in the IM (Fig. 4A,B). Since the increase in the PM is higher for GLUT1 than for GLUT4, it is proposed that the stimulation of glucose transport by long-term exposure to IGF-I is largely due to the presence in the PM of newly synthesized GLUT1 transporters, and that the newly synthesized GLUT1 transporters are mostly rou ted to the cell surface.

In conclusion, IGF-I was here demonstrated to have acute and prolonged effects on glucose transport in muscle cells. These responses arose through different mechanisms. The rapid stimulatory phase was independent of ongoing protein synthesis and was due to recruitment of glucose transporters to the cell surface from an intracellular membrane organelle. This is similar to the action of insulin in skeletal muscle in vivo [15,16] and in L6 muscle cells [10]. Recruitment involved both the GLUT1 and GLUT4 transporter isoforms. The slower phase was largely dependent on new protein synthesis, and involved increases in cellular content of GLUT1 and GLUT4 transporters which raised the levels of these transporters in the cell surface. This is similar to the effect of prolonged insulin treatment of L6 cells, which elevated GLUT1 content biosynthetically

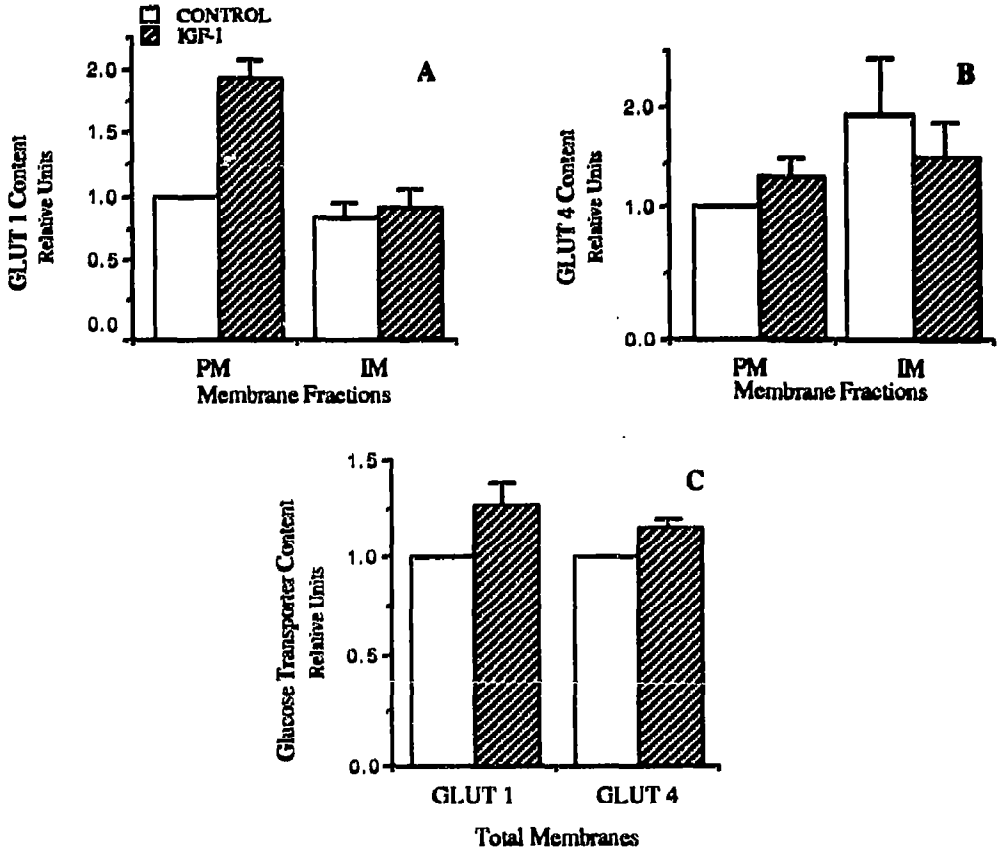

Fig. 4. Late effects of IGF-I on (A) GLUT1 and (B) GLUT4 glucose transporters. Incubations were as in Fig. 3 except that exposure to IGF-I was for 8 h. Results are of 4 experiments, expressed as mean±S.E.M. in units relative to the content in the control PM for each transporter. (C) Content of GLUT1 and GLUT4 proteins in total (unfractionated) membranes of 5 experiments. Control fractions were assigned a value of 1.0 for each transporter.

[9,14] and GLUT4 content through post-translational mechanisms [14,17].

3.4. *Nature of the responding receptor*

The above actions of IGF-I are not likely to be mediated by the classical insulin receptor in L6 cells, since the ED_{50} for stimulation of glucose transport by IGF-I was 0.1 nM, about two orders of magnitude lower than the ED_{50} for insulin (28 nM). However, one cannot distinguish operationally whether IGF-I responses occur (i) through the atypical insulin receptor with very high affinity for IGF-I proposed by Burant et al. [13], or (ii) through a bona fide IGF-I receptor**. Regardless of this uncertainty, it remains clear that IGF-I can elicit responses through receptors that bind IGF-I other than the typical insulin receptor. It is speculated that defects in insulin action that involve binding to and signalling from the insulin receptor could potentially be bypassed by judicious administration of IGF-I.

**There is presently no *functional* evidence for the existence of an abnormal insulin receptor binding IGF-I with high affinity in intact L6 cells. Hybrid receptors remain a viable explanation for the results observed in immunoprecipitated receptor studies. Resolution of the two alternatives stated would require selective inhibition of the classical IGF-I receptor with a non-penetrating, specific probe such as an anti-IGF-I receptor antibody that could block IGF-I binding to intact rat cells. Such antibodies are currently unavailable.

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