IGF-I and insulin induce different intracellular calcium signals in skeletal muscle cells

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Abstract

We studied the effect of IGF-I and insulin on intracellular Ca^{2+} in primary cultured myotubes. IGF-I induced a fast and transient Ca^{2+} increase, measured as fluo-3 fluorescence. This response was blocked by both genistein and AG538. IGF-I induced a fast inositol-1,4,5-trisphosphate (IP_3) increase, kinetically similar to the Ca^{2+} rise. The Ca^{2+} signal was blocked by inhibitors of the IP_3 pathway. On the other hand, insulin produced a fast (<1 s) and transient Ca^{2+} increase. Insulin-induced Ca^{2+} increase was blocked in Ca^{2+} -free medium and by either nifedipine or ryanodine. In the normal muscle NLT cell line, the Ca^{2+} signals induced by both hormones resemble those of primary myotubes. GLT cells, lacking the $\alpha 1$ -subunit of

dihydropyridine receptor (DHPR), responded to IGF-I but not to insulin, while GLT cells transfected with the $\alpha 1$ -subunit of DHPR reacted to both hormones. Moreover, dyspedic muscle cells, lacking ryanodine receptors, responded to IGF-I as NLT cells, however they show no insulin-induced calcium increase. Moreover, G-protein inhibitors, pertussis toxin (PTX) and $\text{GDP}\beta\text{S}$, blocked the insulin-induced Ca^{2+} increase without major modification of the response to IGF-I. The different intracellular Ca^{2+} patterns produced by IGF-I and insulin may improve our understanding of the early action mechanisms for these hormones in skeletal muscle cells.

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Introduction

Skeletal muscle is a target tissue for the action of both insulin-like growth factor I (IGF-I) and insulin (Adams 2002, Kimball *et al.* 2002). Some of the intracellular signaling pathways activated by these hormones are similar; however, the global effects in muscle cells are different (Crown *et al.* 2000). IGF-I regulates several adaptive processes in skeletal muscle such as growth (Coolican *et al.* 1997), differentiation (Tureckova *et al.* 2001) and hypertrophy (Semsarian *et al.* 1999) while insulin, whose receptors have a similar heterotetrameric structure to those of the IGF-I receptor, is involved in metabolic processes including glucose transporter (GLUT)-4 translocation (Ryder *et al.* 2001) and protein synthesis (Kimball *et al.* 2002). Moreover, IGF-I and insulin receptors are composed of two α - and two β -subunits and share 80% structural homology in their β -subunits. The binding of IGF-I or insulin to its receptors promotes dimerization of receptors and tyrosine autophosphorylation. This event produces the subsequent phosphorylation of proteins such as insulin receptor substrate 1 (IRS-1) and docking of proteins that contain Src homology (Shc) (Blakesley *et al.* 1996, Kaburagi *et al.* 1999). On the other hand, recent evidence indicates that IGF-I and insulin receptors require a heterotrimeric G-protein for some of their biological effects (Kanouh *et al.*

2002). Imamura *et al.* (1999) have described in adipocytes that G-protein $\text{G}\alpha\text{q}/11$ associates with the insulin receptor to cause phosphatidylinositol 3-kinase (PI3K) signals, producing GLUT-4 translocation (Imamura *et al.* 1999). Moreover in intestinal smooth muscle, IGF-I mediates mitogen-activated protein kinase (MAPK) activation through a pathway that is, at least in part, $\text{G}\alpha\text{i}$ dependent and promotes cell growth (Kuemmerle & Murthy 2001). The signal transduction mechanisms responsible for the differential effects of these two hormones and their receptor-mediated pathways are poorly understood. A pathway of divergence for the effects of these hormones could be the generation of different patterns of calcium signals and, consequently, differential activation of Ca^{2+} -dependent pathways by either IGF-I or insulin. In skeletal muscle cells Ca^{2+} regulates contractile activity as well as different energetic pathways (Dulhunty *et al.* 2002). In several cell models, IGF-I binds to a receptor (IGF-IR) with intrinsic tyrosine kinase activity and activates several substrates; an example is phospholipase C- γ (PLC- γ), regulating cardiac muscle differentiation (Hong *et al.* 2001). Activation of PLC through IGF-I generates inositol-1,4,5-trisphosphate (IP_3) and produces Ca^{2+} release from endoplasmic reticulum (Takasu *et al.* 1989, Guse *et al.* 1992). Furthermore, IGF-I may elicit Ca^{2+} influx from the extracellular medium by a variety of mechanisms, which seem to depend on the cell

type (Delbono *et al.* 1997, Solem & Thomas 1998). In fresh mouse muscle fibers, insulin increases calcium concentration in the cytoplasm located near the plasma membrane through the entry of calcium from outside the cell rather than release from the sarcoplasmic reticulum (Bruton *et al.* 2001). The interplay between Ca^{2+} release from IP_3 -sensitive stores and Ca^{2+} influx through the plasma membrane may result in a variety of Ca^{2+} signals, which can be important for differential generation of patterns of calcium transients and also in the interaction between different Ca^{2+} -dependent pathways (Foskett *et al.* 1991).

In skeletal muscle cells, calcium has been mainly associated with excitation–contraction coupling (Dulhunty *et al.* 2002); nevertheless in our laboratory we have described a role for Ca^{2+} as second messenger in the activation of MAPK, transcription factors and induction of early genes (Carrasco *et al.* 2003). Some hormones induce intracellular Ca^{2+} increases in muscle cells independently of contraction, by a mechanism mediated by IP_3 -dependent Ca^{2+} release from intracellular stores (Estrada *et al.* 2000, 2003). The slow Ca^{2+} signal related to regulation of gene expression can be activated by membrane depolarization and has been shown to involve the voltage sensor, dihydropyridine receptor (DHPR) (Araya *et al.* 2003). IGF-I has been shown to produce activation of voltage-gated Ca^{2+} channels and to produce Ca^{2+} entry in chondrocytes (Poiraudou *et al.* 1997) and cardiomyocytes (Solem & Thomas 1998); IP_3 -dependent Ca^{2+} release has recently been described in cardiomyocytes in response to IGF-I (Ibarra *et al.* 2003). Moreover Delbono *et al.* (1997) have shown a modulation by IGF-I of both activity of DHPR and an increase in the number of DHPRs expressed in transgenic mice that overexpress human IGF-I (Renganathan *et al.* 1997). A detailed study of Ca^{2+} signals induced by IGF-I and insulin in skeletal muscle cells has not been reported. In the present study, we investigated the effects produced by both IGF-I and insulin on intracellular Ca^{2+} changes in skeletal muscle cells and we explored the mechanisms involved in these responses. We focused our study on the kinetics of the Ca^{2+} signals and on the Ca^{2+} release mechanisms involved. Moreover, we evaluated the Ca^{2+} signals induced by both hormones in different cell lines: NLT, from normal skeletal muscle cells; GLT, dysgenic cell line lacking the $\alpha 1$ -subunit of the DHPR (Powell *et al.* 1996); GLT cells transfected with the $\alpha 1$ -subunit of DHPR; and 1B5, dyspedic cells lacking all three subtypes of ryanodine receptor (RyR). Our results show that IGF-I and insulin use different signaling pathways to elicit Ca^{2+} signals in skeletal muscle cells.

Material and Methods

Chemical reagents

IGF-I and insulin (recombinant human) were purchased from Invitrogen. U73122, ryanodine, nifedipine and

verapamil were purchased from Sigma. Fluo-3 acetoxymethyl ester (fluo-3 AM) was purchased from Molecular Probes (Eugene, OR, USA). *Bordetella pertussis* toxin (PTX), GDP β S, AG538, genistein and xestospongine C were from Calbiochem (La Jolla, CA, USA). Anti-IGF-I receptor against β -subunit and anti-insulin receptor antibodies were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Enhanced chemiluminescence reagents and horseradish peroxidase-linked antirabbit immunoglobulin G (IgG) were obtained from Pierce Chemical Co. (Rockford, IL, USA). Other reagents were of analytical grade.

Primary cell cultures

Rat myotubes kept in primary culture were used for measurement of intracellular calcium levels as described previously (Jaimovich *et al.* 2000, Estrada *et al.* 2003). Briefly, skeletal muscle tissue from hindlimbs was isolated from rats aged 12–24 h. After dissection, the tissue was mechanically dispersed and then treated with 0.2% (w/v) collagenase for 30 min, at 37 °C, under mild agitation. The dissociated tissue was filtered through lens tissue paper and spun down at low speed. Cells were plated onto either gelatin-coated glass coverslips at a density of 350×10^3 cells per dish (35 mm) for cytosolic calcium fluorescence measurements or at a density of 9.5×10^5 cells onto gelatin-coated plates (60 mm) for IP_3 radioreceptor assay determination. Culture medium was Dulbecco's modified Eagle's medium (DMEM)/F12, 10% bovine serum, 2.5% fetal calf serum, 100 mg/l penicillin, 50 mg/l streptomycin and 2.5 mg/l amphotericin B. The medium was then replaced by a medium without serum. Myotubes with an estimated purity of more than 90% were visible after the fifth day of culture. Unless indicated, we used 6- to 8-day-old cultures exhibiting a fairly homogeneous population of myotubes with large central nuclei and measuring 200–300 μm long and 20–40 μm wide, corresponding to young, not fully differentiated cells. Such cells allowed us to obtain proportionally large signals from the nuclei. Myotubes used for calcium signal measurements did not spontaneously contract or exhibit spontaneous calcium transients.

Cell lines

We used the mutant mouse cell line GLT (muscular dysgenic, *mdg/mdg*, line transfected with the Large T antigen), and the wild-type cell line NLT, prepared from dysgenic and normal cultures respectively. GLT $\alpha 1$ corresponds to GLT cells transfected with the $\alpha 1$ -subunit DNA of skeletal DHPR as described by Powell *et al.* (2001). Myoblasts of the immortalized dyspedic cell line 1B5 (Moore *et al.* 1998), kindly provided by Dr Paul Allen (Women and Children Hospital, Boston, MA, USA), were used to determine the role of RyRs in the calcium

responses to hormones. All muscle cell lines were cultivated in DMEM (1 g of glucose/l), 2.5% heat-inactivated fetal calf serum and 10% bovine serum (all Life Technologies) in gelatin-covered dishes at 37 °C in 5% CO_2 . The serum was reduced to 2% horse serum after 2 days to induce cell maturation and fusion, and cells were studied 5–7 days after differentiation was initiated.

Intracellular calcium signals at single-cell level

For intracellular calcium measurements, myotubes were cultured on glass coverslips until they reached 80% confluence. Calcium images were obtained from non-contracting myotubes pre-loaded with the fluorescence Ca^{2+} dye fluo-3 AM (Molecular Probes), using either an inverted confocal microscope (Carl Zeiss Axiovert 135 M–LSM Microsystems), or a fluorescence microscope (Olympus Diaphot-TMD, Nikon Corporation), equipped with a cooled charge-coupled device camera and image acquisition system (Spectra Source MCD 600). Myotubes were washed three times with Krebs buffer (145 mM NaCl, 5 mM KCl, 2.6 mM CaCl_2 , 1 mM MgCl_2 , 10 mM Hepes–Na, 5.6 mM glucose, pH 7.4), to remove serum, and loaded with 5.4 μM fluo-3 AM (coming from a stock in 20% pluronic acid–dimethyl sulfoxide), for 30 min at room temperature. After loading, myotubes were washed for 10 min to allow de-esterification of the dye, and were used within 2 h. The cell-containing coverslips were mounted in a 1 ml capacity plastic chamber and placed in the microscope for fluorescence measurements after excitation with a 488 nm wavelength argon laser beam or filter system. Time series of images were recorded before and after cell stimulation; 80–100 images were recorded for each experiment, each series containing images recorded at time intervals of 1–2 s and analyzed frame by frame with the image data acquisition program (Spectra-Source) of the equipment. A PlanApo $\times 60$ (numerical aperture 1.4) objective lens was generally used. In most of the acquisitions, the image dimension was 512×120 pixels. The inhibitors were added during the dye incubation, times and concentrations are indicated in the Results. To assess the role of G-proteins, myotubes were incubated with either 1 $\mu\text{g}/\text{mL}$ PTX for 5 h, or in a permeabilization solution (100 mM KCl, 20 mM NaCl, 5 mM MgSO_4 , 1 mM NaH_2PO_4 , 25 mM NaHCO_3 , 3 mM EGTA, 1 mM CaCl_2 , 20 mM Tris–HCl (pH 7.4), 0.1% BSA, 1 mM ATP, 0.1% glucose and 40 mg/ml saponin), for 5 min in the presence or absence of 100 nM $\text{GDP}\beta\text{S}$, a non-hydrolysable analog of GDP, before hormone stimulation. Intracellular Ca^{2+} was expressed as a percentage of fluorescence intensity relative to basal fluorescence (a value stable for at least 5 min in resting conditions). The fluorescence intensity increase is proportional to the rise in intracellular Ca^{2+} level (Minta *et al.* 1989).

Measurements of IP_3 mass

Myotubes were rinsed and pre-incubated at room temperature for 20 min with a saline solution of the following composition: 145 mM NaCl, 5 mM KCl, 2.6 mM CaCl_2 , 1 mM MgCl_2 , 10 mM Hepes–Na, 5.6 mM glucose, pH 7.4. Cells were stimulated by replacing this solution with IGF-I (1–100 nM)–containing solution in the presence or absence of inhibitors. At the times indicated the reaction was stopped by rapid aspiration of the stimulating solution, addition of 0.8 M ice-cold perchloric acid and freezing with liquid nitrogen. Samples were allowed to thaw and cell debris was spun down for protein determination. The supernatant was neutralized with a solution of 2 M KOH, 0.1 M 2-*N*-morpholinoethanesulfonic acid (MES), and 15 mM EDTA. The neutralized extracts were frozen until required for IP_3 determination. IP_3 mass measurements were carried out by radioreceptor assay (Bredt *et al.* 1989).

IGF-I and insulin receptor immunodetection

Homogenate proteins were resolved in 7% SDS–polyacrylamide electrophoresis gels and transferred to nitrocellulose membranes for 2 h at 0.4 A. Primary antibody incubations using dilutions of 1:1000 of antibodies against subunit β , either of IGF-I receptor or insulin receptor (IR), were carried out at 4 °C overnight. After incubation with horseradish peroxidase-conjugated secondary antibodies for 2 h at room temperature, the membranes were developed by enhanced chemiluminescence according to the manufacturer's instructions. Membranes were stripped and re-probed with an antibody which detects β -actin in order to control for protein loading.

Statistics

All data are expressed as means $\pm$ S.D. Differences between basal and post-stimulated points were determined using a paired Student's *t*-test. $P < 0.05$ was considered statistically significant.

Results

Effects of IGF-I on intracellular calcium in myotubes

Using the fluorescence dye fluo-3 AM, intracellular Ca^{2+} measurements were performed at the single-cell level in myotubes. In Fig. 1A a representative gallery of fluorescence images acquired at indicated times is shown. IGF-I at concentrations (10 nM) in the physiological range induced a fast and transient Ca^{2+} increase in myotubes. The fluorescence changes began during the first second after hormone addition with a maximal fluorescence peak at 6.3 ± 1.6 s (range 4–11 s, $n = 39$ cells, from 23 different cultures; Fig. 1A and B). The response was transient and fluorescence returned towards basal values

Figure 1 IGF-I induces intracellular calcium increase in myotubes. (A) Series of fluorescence images acquired every 5 s. Cells were incubated for 30 min with fluo-3 AM. T, transmitted image (bright field); B, basal fluorescence at time zero; fluorescence levels are shown at the times indicated after addition of 10 nM IGF-I. Note that during the peak, fluorescence in the nuclei is higher than that of the cytoplasm. (B) Fluorescence time course for a representative experiment ($n=39$) of myotubes stimulated with either IGF-I (hormone was added at the arrow) or vehicle ($<0.01\%$ acetic acid, $n=5$) as indicated. (C) Kinetics for Ca^{2+} increase in regions of interest (ROI) located in either nuclei or cytoplasm as indicated. Inset, transmitted image indicating the position of chosen ROIs in the myotube. (D) IGF-I induces a concentration-dependent increase in Ca^{2+} . The graph represents the peak value obtained with concentrations of 1, 10, 50 and 100 nM IGF-I ($n=3$ for each point, $P<0.05$).

after 60 s. Calcium increased in the whole myotube, but fluorescence was more intense in the nuclei; we analyzed whether there was any difference in the kinetics of Ca^{2+} signals between nucleus and cytoplasm. Regions of interest (ROI) were delimited in the cytoplasm and nucleus of the myotube (Fig. 1C, inset) and a sum of all pixels of these areas was performed. ROI analyses show that fluorescence

changes elicited by IGF-I began at the same time and reached maximal values simultaneously in both cell compartments (Fig. 1C). A maximal fluorescence increase in myotubes induced by IGF-I was concentration dependent. Increasing concentrations of IGF-I were applied to primary myotubes and the percentage of fluorescence increase referred to basal values was plotted (Fig. 1D).

Figure 2 Effect of two inhibitors of tyrosine kinase receptors on calcium increase induced by IGF-I. Myotubes were incubated for 15 min with 100 μM genistein (A) or 400 nM AG538 (B), a competitive inhibitor of IGF-I receptors. Both genistein and AG538 blocked the Ca^{2+} increase induced by IGF-I ($n=9$ and 6 respectively). Control curves represent the effect of 10 nM IGF-I without inhibitors.

In the concentration range studied (1–100 nM) an apparent K_d of 4.4 nM was estimated; 10 nM IGF-I was routinely used in all subsequent experiments.

To determine whether IGF-I receptor activation is involved in the Ca^{2+} signals induced by IGF-I, we used 100 μM genistein, a tyrosine kinase inhibitor (Fig. 2A). Addition of genistein completely blocked IGF-I-induced

Ca^{2+} increase ($n=9$). We further tested the effects of AG538, a competitive inhibitor of IGF-I R kinase auto-phosphorylation (Blum *et al.* 2000); 400 nM AG538 produced almost complete inhibition of IGF-I-induced Ca^{2+} increase in the myotubes (Fig. 2B; $n=6$). These results indicate that the tyrosine kinase domain of the IGF-I receptor is required to induce IGF-I-stimulated intracellular Ca^{2+} increases in myotubes.

In order to investigate whether the IGF-I-induced Ca^{2+} increase was due to Ca^{2+} influx, two types of experiments were performed. First, myotubes were incubated and stimulated in a nominally Ca^{2+} -free medium (1 mM EGTA added) (Fig. 3A). In these conditions, IGF-I produced an intracellular Ca^{2+} increase in 78.9% of myotubes (45 of 57); the fluorescence peak was slightly reduced (8.1% as a mean) and the time to peak was longer (10 ± 0.6 s; $n=42$, $P<0.05$ vs 6.3 ± 1.6 s) in the Ca^{2+} condition. These results suggest that extracellular Ca^{2+} has some role in the early part of the Ca^{2+} signal induced by IGF-I. The second type of experiment was designed to identify whether IGF-I-induced Ca^{2+} influx was mediated by voltage-gated Ca^{2+} channels (VGCC). Myotubes were pretreated with nifedipine (Fig. 3B) or verapamil (Fig. 3C), two specific L-type VGCC antagonists. With both antagonists the IGF-I-induced calcium response was both smaller and slower than control. The fluorescence peak value was reached at 16 ± 3.4 ($n=22$) and 13 ± 7.5 s ($n=9$) respectively.

In order to determine whether Ca^{2+} is being released from RyRs, we used 20 μM ryanodine (Fig. 3D). Treatment of myotubes with ryanodine reduced the IGF-I-induced Ca^{2+} spike and produced a delay in time to fluorescence peak, which was similar to that seen with L-type VGCC antagonists (Fig. 3B and C). Nevertheless, the main part of the Ca^{2+} signal evoked by IGF-I in myotubes was still seen in the presence of the inhibitors. These data together suggest that IGF-I-induced intracellular Ca^{2+} increase is complex and could be understood as a two-phase response: an early component appears to be blocked by either L-type VGCC antagonists or ryanodine, and a late component appears to be insensitive to these inhibitors.

In many cell types the rapid increase in intracellular Ca^{2+} levels is associated with IP_3 receptors, present in the intracellular Ca^{2+} stores (Berridge 1995). In myotubes, hormone- and membrane potential-stimulated Ca^{2+} increases are mediated by activation of IP_3 receptors (Jaimovich & Rojas 1994, Jaimovich *et al.* 2000, Powell *et al.* 2001). To test the possible involvement of this pathway in the calcium responses to IGF-I in myotubes, inhibitors of the IP_3 pathway were used: 10 μM U73122, a specific inhibitor of PLC, and 5 μM xestospongine C, a blocker of IP_3 receptors, were tested. Myotubes were pre-incubated with either inhibitor, in medium both with and without Ca^{2+} . With both inhibitors the response was almost completely abolished in Ca^{2+} -free medium

Figure 3 Role of external Ca^{2+} , DHPR and RyR in IGF-I-induced calcium increase. (A) Fluorescence changes after addition (arrow) of 10 nM IGF-I, with either 2.6 mM Ca^{2+} or Ca^{2+} -free (1 mM EGTA) medium as indicated ($n=29$ and 35 respectively). Effect of two inhibitors of DHPR. In cells previously incubated with 20 μM nifedipine (B) or 20 μM verapamil (C), a fast component of the signal induced by IGF-I was inhibited ($n=22$ and 9 respectively). (D) Ryanodine (20 μM) produced both slowing and partial reduction of the fluorescence increase ($n=8$). Each graph illustrates representative kinetics of experiments performed with each inhibitor. Control curves represent the effect of 10 nM IGF-I without inhibitors.

(Fig. 4A and B). Nevertheless in normal Ca^{2+} IGF-I produced a fast and transient response lower in magnitude and duration than the control without inhibitors (Fig. 4A and B). These experiments suggest that a major, relatively slow component of the IGF-I-induced Ca^{2+} transient is mediated by IP_3 . In order to confirm a role for IP_3 in this signal, we measured the mass of IP_3 in cells after stimulation. The effect of 10 nM IGF-I on the IP_3 levels in rat myotubes at different times is depicted in Fig. 4C. IGF-I induced a nearly 3-fold transient increase in the IP_3 mass. This increase was initially fast with a peak that reached a maximum 15 s after stimulus. A similar result was obtained when the experiment was performed in the absence of extracellular Ca^{2+} (Fig. 4C, inset). The effect of IGF-I on the IP_3 mass increase was dependent on IGF-I concentration (Fig. 3D).

Effects of insulin on intracellular calcium in myotubes

Insulin effects on intracellular Ca^{2+} signals were tested. Stimulation of myotubes with 50 nM insulin resulted in a mean fluorescence increase in 77.7% of cells tested (28 of 36 from 18 different cultures) (Fig. 5A). Insulin response was somehow variable in intensity, ranging from 50 to 200 times the basal value of fluorescence (Fig. 5A–D). The kinetics of this response was different to that of IGF-I; insulin-induced Ca^{2+} increase was faster, reaching a peak at 2 s and returning to basal value 10 s after insulin addition. In 43.6% of myotubes, two to four oscillations were seen around 40 s after stimulus (Figs 5C and 7B). The involvement of genistein-sensitive tyrosine kinase, related to insulin receptor, was evaluated. Myotubes were stimulated with insulin in the presence or absence of

Figure 4 Involvement of IP_3 pathway in the IGF-I-triggered calcium increase. Effect of 10 μM U73122 (A) and 5 μM xestospongine C (B; xpg C) on fluorescence changes elicited by 10 nM IGF-I in myotubes. Inhibitors were tested in both calcium and calcium-free medium ($n=12$ for each condition). (C) IP_3 production in myotubes at different times, after stimulation with IGF-I. Total IP_3 mass was measured as described in Materials and Methods. Inset, measurement of IP_3 production in a calcium-free medium. (D) IP_3 increase depends on IGF-I concentration, the measurement was performed 15 s after hormone addition (the IP_3 mass corresponds to the mean $\pm$ S.D. of three experiments performed in triplicate).

genistein. Genistein blocked the insulin-induced Ca^{2+} increase (Fig. 5B), indicating tyrosine kinase receptor participation ($n=12$). We examined the effects of various inhibitors on the Ca^{2+} signal induced by insulin. Unlike the signal produced by IGF-I, the insulin-induced intracellular Ca^{2+} increase was almost completely blocked in either Ca^{2+} -free medium (Fig. 5C) or in medium containing 20 μM nifedipine (Fig. 5D). The use of 20 μM ryanodine (Fig. 5D) also completely eliminated the fast Ca^{2+} response. On the other hand, inhibitors of the IP_3 pathway, U73122 (Fig. 5E), xestospongine C or 2-aminoethoxydiphenyl borate (2-APB) (data not shown) did not produce any inhibitory effect on the Ca^{2+} increases evoked by insulin. Accordingly, myotubes treated with insulin exhibit no detectable modifications of intracellular IP_3 mass (data not shown).

Effects of IGF-I and insulin in different skeletal muscle cells

To further explore the role of DHPRs and RyRs on the IGF-I- and insulin-mediated Ca^{2+} signals, we worked

with skeletal muscle cell lines. A normal skeletal muscle cell line (NLT) was used as control; a dysgenic skeletal muscle cell line (GLT), which does not express the $\alpha 1$ -subunit from DHPRs, and myotubes from the dyspedic skeletal muscle cell line (1B5), which lacks RyRs, were also used. In all three muscle cell lines, both IGF-I receptor as well as insulin receptor were present (Fig. 6E). Fluorescence increased in NLT cells in response to IGF-I (Fig. 6A). In GLT myotubes, the fluorescence increase was $29 \pm 6\%$ less than in NLT cells and the kinetics of the signal appear to be different; the fluorescence peak value was reached at 15 ± 6 s ($n=9$) in NLT cells and at 20 ± 7.5 s ($n=10$) in GLT cells. In transfected GLT $\alpha 1$ cells, IGF-I-induced Ca^{2+} increases were similar both in kinetics and magnitude to NLT cells (Fig. 6A; peak value was reached at 14 ± 3.6 s; $n=6$). Consistent with the signal dependence on RyRs, in dyspedic myotubes (1B5), the fast component of the calcium signal in response to IGF-I was not present (Fig. 6B; $n=14$). However, a slow increase in fluorescence was evident after hormone addition (Fig. 6B). Together these results suggest the

Figure 5 Insulin produces an intracellular calcium increase. (A) Fluorescence changes elicited by 50 nM insulin; series of fluorescence images acquired every 5 s. Fast and transient (<2 s) calcium increase was produced. T, transmitted image (bright field); B, basal fluorescence. Insulin was added at the time indicated by Ins ($n=28$). (B) Kinetics of the fluorescence increase produced by insulin, the moment when insulin was added is indicated by the arrow, this response was inhibited by pre-incubation with 100 μM genistein as indicated ($n=12$). (C) Insulin failed to increase fluorescence in a Ca^{2+} -free medium. (D) After pre-incubation with either 20 μM nifedipine or 20 μM ryanodine, insulin failed to elicit a response. (E) Effect of insulin with or without 10 μM U73122 ($n=3$). Unless indicated, all experiments were performed in Ca^{2+} medium.

participation of both DHPR and RyR in the fast phase of IGF-I-induced signal; however the main part of this signal is related to IP_3 -mediated Ca^{2+} increase.

When insulin-induced Ca^{2+} increases were evaluated in these muscle cell lines, 72% (18 out of 25 cells, from 9 different cultures) of NLT cells responded. Insulin produced a fast and transient Ca^{2+} increase (Fig. 6C), with up-rise kinetics similar to those of primary myotubes, but generally displaying a slower falling face. In GLT cells the fast part of the Ca^{2+} rise evoked by insulin was either absent (10 out of 14 experiments) or greatly reduced (4 out of 14 experiments) (Fig. 6C). In GLT $\alpha 1$ cells, the insulin response was partly recovered, a fast rise was clearly present (Fig. 6C, $n=7$) but the signal was long lasting. When insulin was added to 1B5 cells (Fig. 6D, $n=12$) the fast component observed in NLT cells and primary myotubes was either absent or very small; normally, a sustained low level of fluorescence was seen.

Effect of PTX on IGF-I-induced calcium release

To determine whether a G-protein was involved in either IGF-I- or insulin-induced Ca^{2+} signals, we used PTX and GDP β S, a non-hydrolysable GDP analog, before

stimulating with hormones in primary myotubes. The Ca^{2+} signal elicited by IGF-I was slowed down in the presence of 1 $\mu\text{g}/\text{ml}$ PTX. The maximum value of fluorescence was reached 16 ± 5 s after stimulus ($n=9$), nevertheless the magnitude of the signal was not significantly altered (Fig. 7A). The same effect was observed with GDP β S in which the peak value was reached after 15 ± 6 s ($n=8$). The kinetics of this response, observed with both inhibitors, was similar to that in the presence of either nifedipine or ryanodine. In all of these cases the peak value of fluorescence was reached around 15 s after stimulus. By contrast, insulin-induced intracellular Ca^{2+} increases were almost completely blocked in the presence of either PTX or GDP β S ($n=8$ and $n=5$ respectively; Fig. 7B). These data suggest that a G-protein is involved in the Ca^{2+} response to insulin in myotubes.

Discussion

In this study we have demonstrated that both IGF-I and insulin produce fast and transient Ca^{2+} increases in myotubes. We routinely used 1–10 nM IGF-I, this concentration corresponding to 7.6–76 ng/ml, a value higher

Figure 6 IGF-I and insulin effects in dysgenic and dyspedic cells. (A) Kinetics of Ca^{2+} changes elicited by 10 nM IGF-I both in a normal myoblast cell line (NLT) and in dysgenic cells (GLT). In GLT cells, the fast component is reduced and was partly recovered in GLT cells transfected with the α 1-subunit (GLT α 1) of DHPR. (B) Effect of IGF-I on dyspedic (1B5) cells. The fluorescence increase was different to that of NLT cells; only a slow Ca^{2+} increase was observed. (C) Calcium increase elicited by insulin in NLT cells, GLT cells and GLT α 1 cells. Note that GLT cells did not show a fast fluorescence increase with insulin, but the transfected cell line presented the fast component of the signal. (D) Effect of 50 nM insulin on calcium increase in both NLT cells and 1B5 cells, the fluorescence increase was minimal in dyspedic cells. (E) Western blot analysis of IGF-IR and IR. Cell lysates from all three cell lines presented both IGF-I and insulin receptors.

than that reported by Le Roith *et al.* (2001) in rodent blood (5.05 ± 1.2 ng/ml). Nevertheless, the local concentration of IGF-I could be higher, since it can emulate the action of mechano growth factor on the myocyte. This is an autocrine/paracrine IGF-I splice variant, detectable upon both mechanical and electrical stimulation (McKoy *et al.* 1999). Normal plasma levels of insulin in rodents are of the order of 0.5–2 ng/ml (Morgan *et al.* 1963). The insulin concentrations we are using are higher than physiological plasma levels and we cannot ascertain whether the final concentrations we used are of physiological significance, although they are in the lower range of

those reported for *in vitro* studies in the literature (Imamura *et al.* 1999, Dalle *et al.* 2001); in any case, it is interesting to study the possible cross-reactivity of IGF-I receptors for insulin at high concentrations.

IGF-I-induced intracellular Ca^{2+} increase is complex, involving at least two processes. The main part of the Ca^{2+} signal appears to be due to Ca^{2+} release from intracellular stores through IP_3 receptors. An early component appears to be sensitive to both dihydropyridines and ryanodine. On the other hand, the insulin response was completely dependent on both extracellular Ca^{2+} and on RyRs, resembling the first phase of the response to IGF-I.

Figure 7 G-protein inhibitors partly inhibit the IGF-I effect and totally block insulin-induced calcium increase. (A) Cells were either pre-incubated for 8 h with 1 $\mu\text{g}/\text{ml}$ PTX or permeabilized and incubated for 5 min with 100 nM GDP β S. Both inhibitors produced a delayed calcium signal when incubated with IGF-I. (B) Similar treatment with 1 $\mu\text{g}/\text{ml}$ PTX and 100 nM GDP β S totally abolished the fast signal induced by insulin in myotubes. Control for permeabilized cells (not shown) was similar to the untreated control cells.

IGF-I is a factor required for myogenic differentiation and subsequent growth and hypertrophy of myofibers (Coleman *et al.* 1995, Musaro *et al.* 1999). These effects are triggered by activation of well-known signal transduction pathways such as MAPK, PI3K and calcineurin (Coolican *et al.* 1997, Semsarian *et al.* 1999). The mechanisms of activation for these pathways are not totally clear. Ca^{2+} increase has been postulated as a possible mediator in these

signals (Poiraudou *et al.* 1997; Semsarian *et al.* 1999). If we analyze the Ca^{2+} response elicited by IGF-I and we separate the signal into two components: an early, fast Ca^{2+} transient remains after we use IP_3 pathway inhibitors (xestospongine C and U73122); on the other hand, a delayed response can be seen in isolation after we blocked the faster component using nifedipine, ryanodine, or G-protein inhibitors. The peak value of fluorescence from the second response was reached at times compatible with the maximum value of IP_3 production elicited by IGF-I. It is possible then to consider that the actual response of IGF-I receptor to its ligand is relatively slow and that it is mediated by IP_3 . IGF-I-induced IP_3 production has been shown in rat cardiac myocytes (Guse *et al.* 1992) and porcine thyroid cells (Takasu *et al.* 1989). In C_2C_{12} myotubes in which IGF-I production was over-expressed, a fast calcium signal has been recently described involving the participation of store-operated Ca^{2+} channels (SOCCs) (Ju *et al.* 2003). In our work, we show that IGF-I produced a Ca^{2+} release from internal stores through IP_3R activation; it is possible that when IP_3 -dependent Ca^{2+} stores are depleted, SOCC activation will occur.

The kinetics of the fluorescence changes produced by insulin were different from those changes produced by IGF-I. Calcium increase was fast and transient, returning to basal values in a few seconds. Moreover, the response was completely dependent on extracellular Ca^{2+} . A recent study on fresh mouse muscle fibers showed that insulin increased the calcium concentration in the cytoplasm located near the plasma membrane (Bruton *et al.* 2001). These results suggest that insulin triggers an entry of calcium from outside the cell rather than a release from the sarcoplasmic reticulum (SR). The signal triggered by insulin in our model is explained by calcium release from SR, through RyRs. The kinetics of this signal is similar to the calcium transient involved in skeletal muscle contraction. In adult muscle, either exercise or insulin induces translocation of GLUT-4 to the membrane (Ryder *et al.* 2001). It is possible that the calcium signal we described could be involved in this type of insulin effect.

Since both IGF-I and insulin receptors share a similar structure, it is plausible that IGF-I could also interact with insulin receptors. Moreover, a hybrid receptor to both IGF-I and insulin has been reported to exist (Pandini *et al.* 2002). The Ca^{2+} increase produced via insulin receptors could represent the first part of the response to IGF-I. Also the signal induced by insulin in NLT cells (Fig. 6C) shows prolonged kinetics that may be attributable to an effect of insulin on either IGF-I receptors or hybrid receptors. When we used either nifedipine or ryanodine, both blockers of the insulin receptor-associated signal, they turned the IGF-I-induced Ca^{2+} signal into a single slow Ca^{2+} rise. The fact that the inhibitory effect of ryanodine was larger than that of nifedipine can be explained by intracellular Ca^{2+} depletion mediated by ryanodine, impairing the IP_3 -induced calcium release.

In order to confirm the role of both DHPRs and RyRs on IGF-I- and insulin-regulated signals, we tested different muscle cell lines that express both IGF-I and insulin receptors. GLT cells do not express the $\alpha 1$ -subunit of the DHPR; insulin elicited no Ca^{2+} signals in these cells, but upon transfection with the $\alpha 1$ -DHPR-subunit, the Ca^{2+} signal was recovered (Fig. 6D). The fact that a dihydropyridine such as nifedipine blocks the insulin-induced calcium signal does not necessarily mean that calcium influx is needed for this signal. The DHPRs have been shown to mediate excitation-contraction coupling in the absence of calcium influx (Dulhunty *et al.* 2002). Moreover, in addition to antagonizing calcium flux, the dihydropyridines can act as an immobilizing part of the charge movement of the $\alpha 1$ -subunit of DHPRs, resulting in a reduction of intracellular calcium (Lamb 1986). When we used a cell line that lacks expression of RyRs (1B5), no significant Ca^{2+} increase upon insulin addition was observed. Both GLT and 1B5 cells do respond to IGF-I stimulation, indicating that the IGF-I receptor acts through a pathway that is essentially independent of both Ry and DHP receptors.

The effect of IGF-I on intracellular Ca^{2+} increase in myotubes is not blocked by PTX, but the kinetics of the response was slower than control, suggesting that a G-protein modulates the first part of the signal. We have demonstrated that the insulin receptor mediates a G-protein-dependent signal, because both PTX and GDP β S completely block insulin-induced calcium release. This evidence again favors the idea that the first part of the signal is dependent on insulin receptors.

In skeletal muscle cells, DHPRs are linked to RyRs, to produce a fast calcium release signal to trigger excitation-contraction coupling. Although no G-protein has been shown to be involved in this mechanism (Lamb & Stephenson 1991), it might well be that a G-protein-component connects insulin receptors with DHPRs. It has been reported in the literature that G $\beta\gamma$ can modulate a DHPR channel in rat vein myocytes (Viard *et al.* 1999), producing calcium entry. It has also been reported that insulin augments the inward calcium current and that this effect can be abolished by PTX and GDP β S (Nie *et al.* 1998).

As mentioned above, it is possible that IGF-I also interacts with insulin receptors; we cannot rule out that IGF-I receptor could also mediate DHPR regulation. Delbono *et al.* (1997) have shown that IGF-I facilitates skeletal muscle DHPR activity, producing opening of this Ca^{2+} channel. It is possible then that the early part of the calcium signal induced by IGF-I could be due to calcium release from RyR after IGF-I activation of DHPR.

We propose an alternative model which explains our results (Fig. 8). IGF-I can interact with both insulin receptor (producing a fast calcium signal component) and its own receptor (producing a slow calcium signal component); likewise, insulin can interact with both receptors

Figure 8 Simplest model proposed to explain calcium increases elicited by both IGF-I and insulin. The scheme shows that IGF-I binds to IGF-I receptors and triggers PLC activation. IP_3 is produced and calcium is released through IP_3 receptors (IP_3Rs). This mechanism is probably responsible for the slow component of the IGF-I calcium response in myotubes. The IGF-I response in the absence of inhibitors probably represents its action on both receptors. The graph (upper-left panel) shows a representative experiment in the presence of an inhibitor of PLC (U73122) (●). This curve probably represents the effect of IGF-I acting on insulin receptors. On the other hand, insulin binds to its receptor and triggers an interaction between DHPRs and RyRs. This interaction produces calcium release through the latter. A G-protein mediates the interaction between insulin receptors and DHPRs. The curve seen in the presence of U73122 was probably produced through this mechanism by IGF-I acting on insulin receptors. The IGF-I-induced signal in the presence of PTX (upper-left panel, ○) probably represents the effect of IGF-I acting on its receptor alone. Addition of the two curves (dashed line), one with an inhibitor of the first part of the signal (PTX) and the other with an inhibitor of the second part of the signal (U73122), gives a curve not unlike those seen after IGF-I addition alone (upper-right panel and Fig. 1B).

(producing both fast and slow signals), both molecules having higher affinity for their own receptor. When activated, the IGF-I receptor will in turn activate PLC to produce IP_3 , which will release calcium from SR. On the other hand, when activated, the insulin receptor will interact with a G-protein and this will result in activation of DHPR, which in turn will activate RyRs, producing Ca^{2+} release. In fact, if we add the fast component of the IGF-I-induced signal obtained in the presence of U73122 to the slow component of this signal, obtained in the presence of PTX, the curve representing the sum of these two components does not differ from the signal evoked by IGF-I alone (Fig. 8). The first part of the curve obtained in

the presence of U73122 is kinetically similar and could be replaced by the signal induced by insulin.

Our simple model (Fig. 8) proposes that each stimulus will activate different signal transduction pathways, both involving Ca^{2+} increase in skeletal muscle cells. Both pathways are dependent on tyrosine kinase activity, but one of them (insulin) depends on a PTX-sensitive G-protein and the other one (IGF-I) is mediated by PLC activation. A more complex explanation would be for IGF-I receptor to trigger responses, i.e. calcium release, by an increase of IP_3 production and calcium release by activation of RyR. The sum of both mechanisms could be the final curve shown for IGF-I. This suggestion is supported by a dual mechanism for calcium increase that has been proposed in cultured articular chondrocytes (Poiraudou et al. 1997).

In previous studies we have demonstrated the presence of different IP_3 receptor isoforms in skeletal muscle cells and we have explained the function of these receptors both in calcium release and regulation of gene expression (Powell et al. 2001, Carrasco et al. 2003). It is likely that calcium signals elicited by both insulin and IGF-I would be involved in regulation of gene expression as well. IGF-I induced calcium signals due to calcium release and related to activation of transcription factors in cardiomyocytes (Ibarra et al. 2004). In a similar cell model, myosin light chain 2 and troponin I expression was up-regulated by IGF-I and this response was calcium dependent (Huang et al. 2002). In skeletal myoblasts, IGF-I activates signaling pathways that increase the expression of muscle-specific genes such as α -actin, in a Ca^{2+} -dependent manner (Spangenburg et al. 2004). Other studies have shown that external calcium is required for markers of skeletal myoblast differentiation expression in C_2C_{12} cells (Porter et al. 2002).

Since we are using partly differentiated myotubes from neonatal rats, we cannot ascertain whether the signal we described participates in the differentiation process or whether it also plays a role in adult muscle tissue. Further studies, using a developmental approach will be needed to address this issue.

The fact that IGF-I receptors and insulin receptors appear to elicit calcium signals that differ both in kinetics and intracellular release mechanisms could provide future clues on how calcium signals can differentially regulate gene expression in response to different hormones.

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