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 α1-subunit of dihydropyridine receptor (DHPR), responded to IGF-I but not to insulin, while GLT cells transfected with the α1-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 GDPβS, 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.


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 2 (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 (Kanoh et al. 2002). Imamura et al. (1999) have described in adipocytes that G-protein G\(_{\alphaq/11}\) 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, G\(_{\alpha}\) 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, Somel & Thomas 1998). In fresh
mouse muscle fibers, insulin increases calcium concentra-
tion in the cytoplasm located near the plasma membrane
through the entry of calcium from the cell rather than release from the sarcoplasmic reticulum (Bruton et al.
2001). The interplay between Ca\textsuperscript{2+} release from IP\textsubscript{3}-
sensitive stores and Ca\textsuperscript{2+} influx through the plasma mem-
brane may result in a variety of Ca\textsuperscript{2+} signals, which can be
important for differential generation of patterns of calcium
transients and also in the interaction between different
Ca\textsuperscript{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\textsuperscript{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\textsuperscript{2+} increases in muscle cells independently
of contraction, by a mechanism mediated by IP\textsubscript{3}–
dependent Ca\textsuperscript{2+} release from intracellular stores (Estrada
et al. 2000, 2003). The slow Ca\textsuperscript{2+} signal related to regula-
tion 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\textsuperscript{2+} channels and to produce Ca\textsuperscript{2+} entry in
chondrocytes (Poiraud et al. 1997) and cardiomyocytes
(Somel & Thomas 1998); IP\textsubscript{3}–dependent Ca\textsuperscript{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\textsuperscript{2+}
signals induced by IGF-I and insulin in skeletal muscle
cells has not been reported. In the present study, we investi-
gated the effects produced by both IGF-I and insulin on
intracellular Ca\textsuperscript{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\textsuperscript{2+} signals and on
the Ca\textsuperscript{2+} release mechanisms involved. Moreover,
we evaluated the Ca\textsuperscript{2+} signals induced by both hormones in
different cell lines: NLT, from normal skeletal muscle cells;
GLT, dysgenic cell line lacking the \textalpha{}1-subunit of the
DHPR (Powell et al. 1996); GLT cells transfected with the
\textalpha{}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\textsuperscript{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 acetoxy-
methyl ester (fluo-3 AM) was purchased from Molecular
Probes (Eugene, OR, USA). Bordetella pertussis toxin
(PTX), GDP\textsubscript{58S}, AG538, genestein and xestospongin C
were from Calbiochem (La Jolla, CA, USA). Anti-IGF-I
receptor against \textbeta{}-subunit and anti-insulin receptor anti-
bodies 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\textsuperscript{3}
cells per dish (35 mm) for cytosolic calcium fluorescence
measurements or at a density of 9.5 × 10\textsuperscript{3} cells onto
gelatin–coated plates (60 mm) for IP\textsubscript{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 strepto-
mycin 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, corre-
sponding to young, not fully differentiated cells. Such cells
allowed us to obtain proportionally large signals from the
tissue. 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, \textmdg/\textmdg, line transfected with the Large T
antigen), and the wild-type cell line NLT, prepared from
dysgenic and normal cultures respectively. GLT/\textmdg corre-
sponds to GLT cells transfected with the \textalpha{}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
(Children and Women Hospital, Boston, MA, USA),
were used to determine the role of RyRs in the calcium

responses to hormones. All muscle cell lines were cultured 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% CO2. 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% confluency. Calcium images were obtained from noncontracting myotubes pre-loaded with the fluorescence probe Ca2+ 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 CaCl2, 1 mM MgCl2, 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 × 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 µg/mL PTX for 5 h, or in a permeabilization solution (100 mM KCl, 20 mM NaCl, 5 mM MgSO4, 1 mM NaH2PO4, 25 mM NaHCO3, 3 mM EGTA, 1 mM CaCl2, 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 GDP β(S), a non-hydrolysable analog of GDP, before hormone stimulation. Intracellular Ca2+ 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 Ca2+ level (Minta et al. 1989).

Measurements of IP3 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 CaCl2, 1 mM MgCl2, 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 IP3 determination. IP3 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 ± 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 Ca2+ 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 Ca2+ 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.
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\textsuperscript{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 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\textsuperscript{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\textsuperscript{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).
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 ($10 \pm 0·6 \mu s$; $n=42$, $P<0·05$ vs $6·3 \pm 1·6 \mu s$) 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 $16 \pm 3·4 \mu s$ ($n=22$) and $13 \pm 7·5 \mu s$ ($n=9$) 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 xestospongin 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.
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\(_3\) mass 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

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**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 \(\mu\)M nifedipine (B) or 20 \(\mu\)M verapamil (C), a fast component of the signal induced by IGF-I was inhibited (n=22 and 9 respectively). (D) Ryanodine (20 \(\mu\)M) 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.
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), xestospongin C or 2-aminoethoxydiphenyl borate (2-APB) (data not shown) did not produce any inhibitory effect on the Ca\(^{2+}\) increase 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 α1-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±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 GLTα1 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

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**Figure 4** Involvement of IP\(_3\) pathway in the IGF-I-triggered calcium increase. Effect of 10 µM U73122 (A) and 5 µM xestospongin 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 ± s.o. of three experiments performed in triplicate).
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 IP3-mediated Ca2+ increase.

When insulin-induced Ca2+ 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 Ca2+ 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 Ca2+ rise evoked by insulin was either absent (10 out of 14 experiments) or greatly reduced (4 out of 14 experiments) (Fig. 6C). In GLT/afii9825 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 Ca2+ signals, we used PTX and GDPβS, a non-hydrolysable GDP analog, before stimulating with hormones in primary myotubes. The Ca2+ signal elicited by IGF-I was slowed down in the presence of 1 µg/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 GDPβS 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 Ca2+ increases were almost completely blocked in the presence of either PTX or GDPβS (n=8 and n=5 respectively; Fig. 7B). These data suggest that a G-protein is involved in the Ca2+ response to insulin in myotubes.

Discussion
In this study we have demonstrated that both IGF-I and insulin produce fast and transient Ca2+ increases in myotubes. We routinely used 1–10 nM IGF-I, this concentration corresponding to 7·6–76 ng/ml, a value higher...
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 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 α1-subunit (GLTα1) of DHPR. (B) Effect of IGF-I on dysgenic (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 GLTα1 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 dysgenic cells. (E) Western blot analysis of IGF-IR and IR. Cell lysates from all three cell lines presented both IGF-I and insulin receptors.
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\textsuperscript{2+} increase has been postulated as a possible mediator in these signals (Poiraudeau et al. 1997; Semsarian et al. 1999). If we analyze the Ca\textsuperscript{2+} response elicited by IGF-I and we separate the signal into two components: an early, fast Ca\textsuperscript{2+} transient remains after we use IP\textsubscript{3} pathway inhibitors (xestospongin 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\textsubscript{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\textsubscript{3}. IGF-I-induced IP\textsubscript{3} production has been shown in rat cardiac myocytes (Guse et al. 1992) and porcine thyroid cells (Takasu et al. 1989). In C\textsubscript{2}C\textsubscript{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\textsuperscript{2+} channels (SOCCs) (Ju et al. 2003). In our work, we show that IGF-I produced a Ca\textsuperscript{2+} release from internal stores through IP\textsubscript{3}R activation; it is possible that when IP\textsubscript{3}-dependent Ca\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{2+} signal into a single slow Ca\textsuperscript{2+} rise. The fact that the inhibitory effect of ryanodine was larger than that of nifedipine can be explained by intracellular Ca\textsuperscript{2+} depletion mediated by ryanodine, impairing the IP\textsubscript{3}-induced calcium release.

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**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 μg/ml PTX or permeabilized and incubated for 5 min with 100 nM GDPβS. Both inhibitors produced a delayed calcium signal when incubated with IGF-I. (B) Similar treatment with 1 μg/ml PTX and 100 nM GDPβS totally abolished the fast signal induced by insulin in myotubes. Control for permeabilized cells (not shown) was similar to the untreated control cells.
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 α1-subunit of the DHPR; insulin elicited no Ca\textsuperscript{2+} signals in these cells, but upon transfection with the α1-DHPR-subunit, the Ca\textsuperscript{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 α1-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\textsuperscript{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\textsuperscript{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\textsubscript{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 Gβγ 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\textsubscript{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\textsuperscript{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 (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\textsubscript{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\textsuperscript{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 (Poirauadeau 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 (Spangenberg et al. 2004). Other studies have shown that external calcium is required for markers of skeletal myoblast differentiation expression in C$_2$C$_{12}$ 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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