Insulin-like Growth Factor-1 Induces an Inositol 1,4,5-Trisphosphate-dependent Increase in Nuclear and Cytosolic Calcium in Cultured Rat Cardiac Myocytes*

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The abbreviations used are: IGF-1, insulin-like growth factor 1; PI3K, phosphatidylinositol 3-kinase; PLC, phospholipase C; IP₃, inositol 1,4,5-trisphosphate; PTX, pertussis toxin; MES, 2-(N-morpholino)ethanesulfonic acid; Adenosinoviridae; [Ca²⁺], intracellular [Ca²⁺]; TBST, Tris-buffered saline with Tween 20; ROI, region of interest; BAPTA, 1,2-bis-(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid.

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In the heart, insulin-like growth factor-1 (IGF-1) is a pro-hypertrophic and anti-apoptotic peptide. In cultured rat cardiomyocytes, IGF-1 induced a fast and transient increase in Ca²⁺/ levels apparent both in the nucleus and cytosol, releasing this ion from intracellular stores through an inositol 1,4,5-trisphosphate (IP₃)-dependent signaling pathway. Intracellular IP₃ levels increased after IGF-1 stimulation in both the presence and absence of extracellular Ca²⁺. A different spatial distribution of IP₃ receptor isoforms in cardiomyocytes was found. Ryanodine did not prevent the IGF-1-induced increase of Ca²⁺/ levels but inhibited the basal and spontaneous Ca²⁺/i oscillations observed when cardiac myocytes were incubated in Ca²⁺/-containing resting media. Spatial analysis of fluorescence images of IGF-1-stimulated cardiomyocytes incubated in Ca²⁺/-containing resting media showed an early increase in Ca²⁺/i, initially localized in the nucleus. Calcium imaging suggested that part of the Ca²⁺/ released by stimulation with IGF-1 was initially contained in the perinuclear region. The IGF-1-induced increase on Ca²⁺/i levels was prevented by 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid-AM, thapsigargin, xestospongin C, 2-aminooxy dihydro borate, U-73122, pertussis toxin, and βARKet (a peptide inhibitor of Gβγ signaling). Pertussis toxin also prevented the IGF-1-dependent IP₃ mass increase. Genistein treatment largely decreased the IGF-1-induced changes in both Ca²⁺/i and IP₃, LY29402 (but not PD98059) also prevented the IGF-1-dependent Ca²⁺/i increase. Both pertussis toxin and U73122 prevented the IGF-1-dependent induction of both ERKs and protein kinase B. We conclude that IGF-1 increases Ca²⁺/ levels in cultured cardiac myocytes through a GIP₃-containing signaling pathway that involves participation of IP₃.

Insulin-like growth factor-1 (IGF-1) plays important roles in numerous physiological processes, ranging from normal growth and development during the early stages of embryogenesis to the regulation of specific functions in several tissues and organs in later stages of development (1). IGF-1 and its receptor (IGF-1R) are present in rat heart, consistent with IGF-1 regulating growth and hypertrophy in the developing heart in an autocrine or paracrine manner (2). IGF-1 induces cardiac hypertrophy in vitro and in vivo (3–5), protective and anti-apoptotic properties for this growth factor have also been demonstrated in different models of myocardial ischemia and infarction (6–9).

IGF-1R, which is closely related to the insulin receptor, is a protein-tyrosine kinase composed of two heterodimers linked by disulfide bonds (10). Following IGF-1 binding, IGF-1R becomes autophosphorylated on several tyrosine residues, allowing its interaction with phosphorytosine binding or Src homology 2 (SH2) domain-containing proteins, thereby transducing signals to downstream effectors (11). Through these effectors, IGF-1 activates two main signaling cascades, the Ras-Rafmitogen-activated protein kinase (MEK)-extracellular signal-regulated kinase (ERK) and the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB/Akt) pathways (12). Several recent studies have demonstrated that IGF-1R also activates heterotrimeric G proteins in some cell types. Luttrel et al. (13) first demonstrated in rat-1 fibroblasts that treatment with pertussis toxin (PTX) or transfection with a Gβγ scavenger (BARK-CT) blocked IGF-1-induced ERK activation (13). This study suggested the involvement of the Gβγ subunit of a PTX-sensitive G protein in the activation of ERK (13). Similar results have been obtained with human intestinal smooth muscle cells (14), 3T3-L1 mouse pre-adipose cells (15), and rat cerebellar granule neurons (16).

Conflicting observations on the roles of intracellular calcium (Ca²⁺) and inositol 1,4,5-trisphosphate (IP₃) in IGF-1 signaling have been reported. In BALB/c 3T3 cells, IGF-1 stimulates Ca²⁺ influx via IGF-1R by activating a Ca²⁺-permeable cation channel (17). The resulting Ca²⁺ influx produces oscillations in the concentration of free intracellular Ca²⁺ ([Ca²⁺][]) independently of phosphoinositide turnover (18). In contrast, in cultured bovine alveolar macrophages, nanomolar concentrations of
FIG. 1. IGF-1 induces the intracellular Ca\(^{2+}\) level increase in the nucleus and cytosol of cultured rat cardiomyocytes. Cells, maintained in Ca\(^{2+}\)-free resting media, were preloaded with Fluo3-AM and then stimulated with IGF-1 (1 nM). Using a fluorescence microscopy equipped with a CCD camera, serial Ca\(^{2+}\) images of Fluo3 fluorescence in a single cardiomyocyte were registered (A), relative total fluorescence (ratio of fluorescence difference, stimulated − basal \((F_s − F_b)\), to basal value \((F_b)\)) as a function of time of each image were calculated (B). Relative total fluorescence of Fluo3-AM-preloaded cardiomyocytes incubated in Ca\(^{2+}\)-containing resting media and stimulated with IGF-1 (1 nM) (C) and ROI analysis of fluorescence images of a cardiomyocyte nucleus and cytosol maintained in Ca\(^{2+}\)-free (D) and Ca\(^{2+}\) containing resting media (E) were measured.

IGF-1 stimulate after 30 s the accumulation of IP\(_3\) and inositol 1,3,4,5-tetrakisphosphate, and induce a rise in [Ca\(^{2+}\)]\(_i\) (19). In chondrocytes, IGF-1 induces Ca\(^{2+}\) release from the endoplasmic reticulum, which is partially blocked by phospholipase C (PLC) inhibitors and PTX (20). Short term exposure of neurons and neuronal cell lines to IGF-1 leads to direct activation of voltage-gated L-type Ca\(^{2+}\) channels (21–23), whereas in rat pinealocytes IGF-1 inhibits L-type currents (24). IGF-1 also modulates L-type Ca\(^{2+}\) channels in adult rat cardiac myocytes and in skeletal muscle of young and middle-aged rats, but not of aged rats (25, 26). In whole heart and in ferret papillary muscle, IGF-1 displays an acute positive inotropic effect, which is secondary to augmented myofilament responsiveness to Ca\(^{2+}\) (27). In rat ventricular papillary muscle cells, however, acute exposure to IGF-1 or insulin results in enhanced muscle contractility that is associated with [Ca\(^{2+}\)]\(_i\) transients (28).

In cardiac myocytes, IGF-1 activates multiple signaling pathways, including ERK-PKC, PKB/Pi3K, PLC-γ, and JAK-STAT (9, 29, 30, 32). However, the role of cytosolic Ca\(^{2+}\) as the second messenger in the complex IGF-1 signaling pathways present in cardiac myocytes has not been examined.

In the present study we show that, both in the presence or absence of extracellular Ca\(^{2+}\), addition of IGF-1 to cultured cardiac myocytes induced a rapid [Ca\(^{2+}\)] increase in both the nucleus and cytoplasm and also produced a rapid increase of IP\(_3\) levels. The presence of extracellular Ca\(^{2+}\) modified the kinetics of the [Ca\(^{2+}\)] increase, delaying an increase of cytosolic but not nuclear [Ca\(^{2+}\)]. The role of tyrosine kinase, G-protein, PI3K, PLC, and the IP\(_3\) receptor in the pathway leading to these calcium signals was established using specific inhibitors.

EXPERIMENTAL PROCEDURES

Materials—[\(^{3}H\)IP\(_3\)] was from PerkinElmer Life Sciences. Fluoro-3-acetoxyethyl ester (Fluo3-AM) was from Molecular Probes (Eugene, OR). Thapsigargin, xestospongin C, genistein, BAPTA-acetoxyethyl ester (BAPTA-AM), LY294002 (LY), PD98059 (PD), and SB203580 (SB) were from Calbiochem-Novabiochem Corp. (San Diego, CA). 2-Aminooxy diphenyl borate was from Aldrich. Polyclonal antibodies against type 1 and type 2 IP\(_3\) receptor were from Affinity BioReagents (Golden, CO) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Anti-type 3 IP\(_3\) receptor was kindly donated by G. A. Mignery (Loyola University, Chicago, IL). Polyclonal antibodies against phosphorylated (Ser\(^{73}\)) PKB, total PKB, phosphorylated (Thr\(^{308}/\text{Thr}^{322}\)) ERK, and total ERK were from Cell Signaling Technology Inc. (Beverly, MA). Dulbecco’s modified Eagle’s medium, medium 199, U-73122, ryanodine, nifedipine, PTX, IP\(_3\), and other biochemicals were purchased from Sigma unless stated otherwise. Human recombinant IGF-1 was donated by Dr. C. George-Nascimento (Austral Biologicals, San Ramon, CA).

Animals—Rats were bred in the Animal Breeding Facility from the Faculty of Chemical and Pharmaceutical Sciences, University of Chile (Santiago, Chile). We performed all studies with the approval of the institutional bioresearch committee at the Faculty of Chemical and Pharmaceutical Sciences, University of Chile, Santiago. This investigation conforms to the “Guide for the Care and Use of Laboratory Animals” published by the United States National Institutes of Health (31).

Culture of Cardiac Myocytes—Cardiac myocytes were prepared from hearts of 1-3-day-old Sprague-Dawley rats as described previously (29). For IP\(_3\) determination, cardiomyocytes were plated at a final density of 0.7 × 10\(^3\)mm\(^2\) on gelatin-precoated 60-mm dishes. For detection of Ca\(^{2+}\), cells were plated with a final density of 1.0 × 10\(^5\)mm\(^2\) on gelatin-precoated coverslips. Serum was withdrawn for 24 h before the
cells were treated further with agonist IGF-1 (1–100 nM) in serum-free medium (Dulbecco’s modified Eagle’s medium/medium/medium) at 37 °C. Cultured cardiomyocytes, assessed with an anti-β-myosin heavy chain antibody, were at least 95% pure.

Recombinant Adenoviruses—Adenoviral vectors (Ad) were propagated and purified as previously described (33). Transgenes (a gift from Dr. W. J. Koch, Duke University Medical Center, Durham, NC) were used: βARKct (Ad-βARKct) and an empty viral construct (Ad-EV). βARKct is a peptide inhibitor of Gβγ signaling (34). Cardiomyocytes were infected with adenoviral vectors at a multiplicity of infection of 300.

Determination of IP3 Mass—Cardiomyocytes were rinsed and preincubated for 20 min at room temperature in 58 mM NaCl, 4.7 mM KCl, 2.6 mM CaCl2, 1.2 mM MgSO4, 0.5 mM EDTA, 10 mM LiCl, 10 mM glucose, and 20 mM Hepes, pH 7.4. Cells were stimulated by fast (1 s) replacement of this solution by a solution containing IGF-1. At the times indicated, the reaction was stopped by rapid aspiration of the stimulating solution, addition of 0.8 mM 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 MES, and 15 mM EDTA. The neutralized extracts were frozen at −80 °C until required for IP3 determination. Measurements of IP3 mass were made by radioreceptor assay (35).

Briefly, a crude rat cerebellum membrane preparation was obtained after homogenization of tissue in 50 mM Tris-HCl, pH 7.7, containing 1 mM EDTA, 2 mM β-mercaptoethanol and centrifugation at 20,000 × g for 15 min. This procedure was repeated 3 times, suspending the final pellet in the same solution plus 0.3 M sucrose and freezing it at −80 °C until required for use. The rat cerebellar membrane preparation was calibrated for IP3 binding with 1.6 nM [3H]IP3 and 2–120 nM cold IP3, with sample analysis performed in a similar way but replacing cold IP3 with a portion of the neutralized supernatant. [3H]IP3 radioactivity, which remained bound to membranes, was measured in a Beckman LS-6000TA liquid scintillation spectrometer (Beckman Instruments Corp., Fullerton, CA). Protein was determined by the Lowry method (36).

Measurement of Intracellular Calcium—Cellular calcium images were obtained from neonatal cardiac myocytes preloaded with Fluo3-AM, using an inverted confocal microscope (Carl Zeiss Axiovert 135 M-LSM Microsystems) or a fluorescence microscope (Olympus Diaphot-TMD, Nikon Corporation) equipped with a cooled CCD camera and image acquisition system (Spectra Source MCD 600). Cardiac myocytes were washed three times with Ca2+-containing resting media (Kreb’s 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 Dulbecco’s modified Eagle’s medium/medium 199 culture medium, and loaded with 5.4 μM Fluo3-AM (coming from a stock in 20% pluronic acid, Me2SO) for 30 min at room temperature. After loading, cardiac myocytes were washed either with the Ca2+-buffer or with Ca2+-free resting media (145 mM NaCl, 5 mM KCl, 1.0 mM EGTA, 2 mM MgCl2, 10 mM Hepes-Na, 5.6 mM glucose, pH 7.4) and 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. IGF-1 was either added directly to the solution or the solution was fast (1 s) changed in the chamber. The fluorescent images were collected every 0.4–2.0 s for fast signals and analyzed frame by frame with the image data acquisition program (Spectra-Sourse) of the equipment. An objective lens PlanApo 60X (numerical aperture 1.4) was generally used. In most of the acquisitions, the image dimension was 512 × 120 pixels. Intracellular calcium 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 [Ca2+]i (37).

Digital Image Processing—Elimination of out-of-focus fluorescence was performed using both the “no neighbors” deconvolution algorithm and Castelmann’s (38) point spread function theoretical model, as described previously (39). For quantitation of fluorescence, the summed pixel fluorescence intensity was calculated from the section delimited by a contour. As a way of increasing efficiency of these data manipulations, action sequences were generated. To avoid interference in the fluorescence by possible IGF-1 effects on the cellular volume, the area of each fluorescent cell was determined by image analysis using adaptive contour and then creating a binary mask, which was compared with its bright-field image.

Western Blot Analysis—Cell lysates were matched for proteins (10–40 μg) and were separated by SDS-PAGE on 10% polyacrylamide gels and electrotransferred to nitrocellulose. Membranes were blocked with 5% nonfat milk powder in Tris-buffered saline (TBS) (pH 7.6) containing 0.1% (w/v) Tween 20 (TBST) for 60 min at room temperature. Primary antibodies were diluted 1:1,000 in blocking solution. Nitrocellulose membranes were incubated with primary antibodies overnight at 4 °C. After washing in TBST (3 × 10 min each), the blots were incubated for 2 h at room temperature with horseradish peroxidase-linked secondary antibody (1:5,000 in 1% (w/v) nonfat milk powder in TBST). The blots were washed again in TBST and the bands were detected using ECL with exposure to Kodak film for 0.5–30 min. Blots were quantified by scanning densitometry.

Subcellular Fractionation—Nuclear and cytosolic fractions from cultured cardiac myocytes were prepared as described by Schreiber et al. (40). Homogeneity of fractions was assessed by Western blot using α-actinin (marker for myofibril Z bands) (41), lamina-associated polypeptide 2 (marker for nuclear inner membrane) (42), and calnexatin (marker for sarcoplastic reticulum) (43).

Immunocytochemistry—Cardiomyocytes grown on coverslips were fixed in iced methanol, blocked in phosphate-buffered saline containing 1% bovine serum albumin for 30 min, and incubated with primary anti-IP3 receptor antibodies at 4 °C overnight. The cells were then washed 5-fold with phosphate-buffered saline/histidine buffer and incubated with the appropriate goat anti-rabbit secondary antibody for 1 h at room temperature. After washing three more times, the coverslips were mounted in Vectashield (Vector Laboratories, Inc.). The samples were evaluated in a scanning confocal microscope (Carl Zeiss Axiovert 135, LSM Microsystems) and documented through computerized images (44).

Expression of Results and Statistical Analysis—Data are represented as mean ± S.E. of the number of independent experiments indicated (n) or as examples of representative experiments performed on at least three separate occasions. Data were analyzed by analysis of variance and comparisons between groups were performed using a protected Tukey’s test. A value of p < 0.05 was set as the limit of statistical significance.
RESULTS

IGF-1-induced Intracellular Ca\(^{2+}\) Transients in Rat Cardiac Myocytes—Changes in intracellular Ca\(^{2+}\) concentration, [Ca\(^{2+}\)]\(_i\), were visualized in single cardiomyocytes preloaded with Fluo3-AM. Increases of relative fluorescence represent an increase of free cytosolic [Ca\(^{2+}\)]. Multiple region of interest (ROI) analysis of single cells revealed that similar kinetics and fluorescence intensities were detected disregarding the analyzed ROI window (data not shown). Quiescent cardiomyocytes, maintained in Ca\(^{2+}\)-containing resting solution, exhibited basal [Ca\(^{2+}\)] oscillations that were blocked by 30 min preincubation with ryanodine (50 \(\mu\)M) (data not shown). Incubation of cardiomyocytes in resting solution without Ca\(^{2+}\) also prevented [Ca\(^{2+}\)] oscillations (data not shown).

Addition of IGF-1 (1 nM) to cardiomyocytes maintained in Ca\(^{2+}\)-free resting solution induced a fast and transient (10–20 s) increase in [Ca\(^{2+}\)]\(_i\), with a maximum at 4–5 s after IGF-1 addition (Fig. 1, A and B). The lowest concentration of IGF-1 that induced [Ca\(^{2+}\)]\(_i\) transients was 0.01 nM. When cardiomyocytes were maintained in Ca\(^{2+}\)-containing resting solution, addition of IGF-1 induced a similar, although slower increase of [Ca\(^{2+}\)]\(_i\), reaching a maximum 40–50 s after IGF-1 addition (Fig. 1C). Cardiomyocytes maintained in Ca\(^{2+}\)-containing resting solution usually showed basal [Ca\(^{2+}\)] oscillations; these oscillations were not altered by addition of IGF-1. Following IGF-1 addition in Ca\(^{2+}\)-free resting solution, an increase of [Ca\(^{2+}\)] was observed both in the nucleus and cytoplasm (Fig. 1A). Analysis of ROI in the fluorescence images showed that both nuclear and cytosolic Ca\(^{2+}\) transient time courses were similar in IGF-1-stimulated cardiomyocytes maintained in the

Fig. 3. Effect of ryanodine and nifedipine in the IGF-1-induced Ca\(^{2+}\) transients in cultured rat cardiomyocytes. Fluo3-AM-preloaded cardiomyocytes, maintained in Ca\(^{2+}\)-free resting media, were preincubated with (A) culture media as control (B) ryanodine (20 \(\mu\)M) or (C) nifedipine (10 \(\mu\)M) for 30 min and then stimulated with IGF-1 (1 nM). Fluorescence images were registered and relative fluorescence was calculated. ROI analysis of fluorescence images of a IGF-1-stimulated cardiomyocyte nucleus and cytosol preincubated with (D) ryanodine or (E) nifedipine were obtained.
Ca\textsuperscript{2+}-free resting solution (Fig. 1D). However, when cardiomyocytes were treated with IGF-1 in Ca\textsuperscript{2+}-containing resting solution, nuclear [Ca\textsuperscript{2+}] increased faster than cytosolic [Ca\textsuperscript{2+}] (Fig. 1E). ROI analysis clearly showed that extracellular Ca\textsuperscript{2+} slowed down the cytosolic but not the nuclear increase in [Ca\textsuperscript{2+}] stimulated by IGF-1 (Fig. 1D and E). Rates of rise of the nuclear Ca\textsuperscript{2+} transients in the presence and absence of external Ca\textsuperscript{2+} were 32.3 ± 7.6 and 38.6 ± 8.1 (Δff per s) (n = 4), respectively. However, rates of rise of Ca\textsuperscript{2+} transients in the cytosol were 3.9 ± 1.4 and 20.1 ± 5.3 (Δff per s) (n = 10), respectively. In all cells studied, the IGF-1-induced increase in [Ca\textsuperscript{2+}] did not trigger regenerative waves through the cytosol.

IGF-1-induced Ca\textsuperscript{2+} Release from Ryanodine and Nifedipine-insensitive Intracellular Stores—The increase in [Ca\textsuperscript{2+}] in cardiomyocytes upon stimulation with IGF-1 in the absence of extracellular Ca\textsuperscript{2+} suggests involvement of Ca\textsuperscript{2+} release from internal stores. Resting Fluo3-AM preloaded cardiomyocytes usually displayed increased basal fluorescence in the perinuclear region (Fig. 2, A and B). After addition of IGF-1, the distribution of fluorescence changed and the increase in fluorescence was homogeneous in both the nucleus and cytoplasm (Fig. 2B, 2 s and 10 s). These observations suggest that IGF-1 induced Ca\textsuperscript{2+} release from perinuclear Ca\textsuperscript{2+} stores.

IGF-1-induced Ca\textsuperscript{2+} transients in cardiomyocytes maintained in Ca\textsuperscript{2+}-free resting media were not suppressed by ryanodine (Fig. 3B). However, ROI analysis of nuclear and cytosolic fluorescence revealed that in the presence of ryanodine the increase in cytosolic [Ca\textsuperscript{2+}] was slower than in the nucleus (Fig. 3D). Cardiomyocytes incubated in Ca\textsuperscript{2+}-containing resting solution displayed an analogous behavior. These results suggest that the IGF-1 stimulated fast increase in cytoplasmic Ca\textsuperscript{2+} was partly because of calcium release through ryanodine receptors because a fast component was inhibited after preincubation with 20 μM ryanodine.
Nifedipine did not modify IGF-1-induced Ca\[^{2+}\]/H\[^{100}\] transients in cardiomyocytes maintained in Ca\[^{2+}\]/H\[^{100}\] -free resting solution (Fig. 3C). ROI analysis of images revealed that both nuclear and cytosolic fluorescence increases presented similar kinetics in the presence or absence of nifedipine (Fig. 3C). These results suggest that voltage-dependent L-type Ca\[^{2+}\]/H\[^{100}\] channels are not involved in the IGF-1-induced Ca\[^{2+}\]/H\[^{100}\] transients in cardiomyocytes.

To further demonstrate the role of intracellular Ca\[^{2+}\] stores in the generation of IGF-1-induced Ca\[^{2+}\] transients, cardiomyocytes were preloaded with both BAPTA-AM and Fluo3-AM, and then stimulated with 1 nM IGF-1. BAPTA-AM inhibited the increase of fluorescence induced by IGF-1 and only a small decrease in fluorescence was apparent in these conditions (Fig. 4A). Treatment of Fluo3-AM-preloaded cardiomyocytes with thapsigargin, a sarco/endoplasmic reticulum Ca\[^{2+}\]/H\[^{110}\]-ATPase inhibitor, led to a slow increase of intracellular fluorescence because of Ca\[^{2+}\] loss from internal stores. Subsequent addition of IGF-1 (5 min) to these cells did not induce the characteristic increase in the fluorescence signal (Fig. 4B). Similar results were obtained when internal Ca\[^{2+}\] stores were depleted using caffeine, a ryanodine receptor activator (Fig. 4C).

When thapsigargin-pretreated cardiomyocytes were incubated with caffeine, no additional Ca\[^{2+}\] release was detected (Fig. 4D). Further treatment with IGF-1 did not increase fluorescence, an effect obtained by the addition of external Ca\[^{2+}\] (Fig. 4D). This Ca\[^{2+}\] influx elicited by calcium add back suggests the activation of a plasma membrane store-operated channel activity in neonatal rat cardiac myocytes. However, external Ca\[^{2+}\] addition to cardiac myocytes after IGF-1 treatment showed a low increase of [Ca\[^{2+}\]]\(_i\), indicating that IGF-1 poorly induced the capacitative calcium entry (Fig. 4E). These results indicate that in cardiomyocytes Ca\[^{2+}\] is released from internal stores, sensitive to both caffeine and thapsigargin, probably located at the perinuclear region, after IGF-1 stimulation.

**IGF-1 Induction of Ca\[^{2+}\] Transients Was Dependent on IP\(_3\) Receptors and IP\(_3\) Production—Xestospongin C and 2-aminoethoxy diphenyl borate, inhibitors of IP\(_3\)-mediated Ca\[^{2+}\] re-
leak, inhibited IGF-1-induced Ca^{2+} transients in cardiomyocytes (Fig. 5, A and B). Furthermore, U-73122, an inhibitor of agonist-induced PLC activation, blocked the increase in [Ca^{2+}], induced by IGF-1 (Fig. 5C). These results suggest that IGF-1-induced Ca^{2+} release from internal stores is a PLC-mediated/IP_{3}-dependent process.

Furthermore, IGF-1 induced a significant IP_{3} mass increase in cardiomyocytes. A dose-response study determined that concentrations of IGF-1 ≥ 1 nM induced a maximal increase of IP_{3} mass, with K_{0} = 0.3 nM (Fig. 6A). In cardiomyocytes incubated in a Ca^{2+}-free resting solution, a time course analysis showed that IGF-1 induced a significant IP_{3} mass increase only after 15 s, reaching maximal levels 30 s post-stimulus (Fig. 6B). The basal mass of IP_{3} was ~20 pmol/mg of protein; IGF-1 stimulation increased this value to 90 and 130 pmol/mg of protein (4.5- and 6.5-fold increase) for cardiomyocytes incubated in Ca^{2+}-free and Ca^{2+}-containing resting media, respectively (Fig. 6B). These results indicate that IGF-1-induced Ca^{2+} transient kinetics was associated with an IP_{3} mass increase.

Effect of Pertussis Toxin and Genistein on the IGF-1-induced Ca^{2+} Transients and IP_{3} Mass Increase—Several reports indicate that IGF-1 activates different intracellular signaling cascades through both protein-tyrosine kinase and G_{i} protein mechanisms (13–16). To assess both possibilities, Fluo3-AM-preloaded cardiomyocytes in Ca^{2+}-free resting media were pre-incubated for 45 min with genistein or PTX and then stimulated with IGF-1. As shown in Fig. 7, A and B, IGF-1-induced Ca^{2+} transients were largely inhibited by genistein, whereas PTX totally blocked these signals. Preincubation of cardiomyocytes with PTX also completely inhibited the IP_{3} mass increase induced by IGF-1, whereas genistein exerted only a partial inhibitory effect (Fig. 7C). These results suggest that the signaling cascade induced via the IGF-1 receptor requires the combined participation of both protein-tyrosine kinase and PTX-sensitive G protein to induce Ca^{2+} transients and to increase the mass of IP_{3}.

**Effect of a Peptide Inhibitor of G_{i}\beta\gamma Signaling on the IGF-1-induced Ca^{2+} Transients**—To further determine which subunits of trimeric G proteins are essential to the IGF-1-induced Ca^{2+} transients, myocytes were infected with Ad-\betaARKct (34, 45). Similar to PTX treatment, \betaARKct effectively abolished the IGF-1-induced Ca^{2+} transients (Fig. 8). This result indicates that G_{i}\beta\gamma subunits dissociated from G_{i} proteins are critical components of the IGF-1-induced Ca^{2+} transients in cardiac myocytes.

**PI3K but Not MEK1 Is Involved in the IGF-1-dependent Ca^{2+} Transients**—The two major routes for IGF-1 receptor signaling are the PI3K and the ERK pathways (21–23). To determine whether IGF-1-induced Ca^{2+} transients involve previous activation of PI3K or MEK/ERK signaling pathways, we used chemical inhibitors of PI3K (LY294002 or LY) and MEK1 (PD98059 or PD). Fluo3-AM-preloaded cardiomyocytes incubated in Ca^{2+}-free resting media were preincubated with ei-
other LY or PD and then treated with IGF-1. LY completely suppressed IGF-1-induced Ca\(^{2+}\) transients, but PD only slowed down the kinetics of the response (Fig. 9, A and B). These results suggest that while PI3K participates directly in the IGF-1-induced Ca\(^{2+}\) transient, the MEK/ERK pathway only modulates this signal.

To evaluate whether IGF-1 activation of ERK and PI3K pathways requires previous Ca\(^{2+}\) release, cultured cardiomyocytes were pretreated with PTX, BAPTA-AM, or U-73122, and then stimulated with IGF-1. PTX blocked IGF-1-stimulated phosphorylation of ERK1/2 but not of PKB (Fig. 10, A and B). These results are consistent with previous reports indicating that IGF-1-dependent ERK activation requires both tyrosine kinase activity and PTX-sensitive G protein, while the PI3K/PKB signaling pathway requires only tyrosine kinase activity (13–16). U-73122 but not BAPTA-AM inhibited IGF-1-dependent activation of both ERK1 and ERK2 (Fig. 10, A and B). BAPTA-AM and U-73122 did not modify IGF-1-dependent activation of PKB (Fig. 10B). Together, these results indicate that IGF-1-dependent activation of ERK but not of PKB required previous Ca\(^{2+}\) release.

**IP\(_3\) Receptor Isoforms Are Located Differentially within Cardiac Myocytes**—To explain the specificity and compartmentalization of the IP\(_3\)-induced Ca\(^{2+}\) transients, the presence of different IP\(_3\) receptor isoforms was investigated in cardiomyocytes by immunoblotting. Cardiomyocytes expressed type 1, type 2, and type 3 IP\(_3\) receptor isoforms (Fig. 11). Western blots of nuclear and cytosolic fractions revealed that type 1 and type 2 IP\(_3\) receptors were mainly located in the nuclear fraction, whereas type 2 IP\(_3\) receptor was located almost exclusively in the cytosolic fraction (Fig. 11).

The intracellular localization of IP\(_3\) receptor isoforms was monitored by immunofluorescence labeling and confocal microscopy. As shown in Fig. 11, fluorescence because of the type 1 IP\(_3\) receptor was seen primarily in the nuclear envelope and some cytosolic structures. The staining of type 1 IP\(_3\) receptor appears to be continuous around the nuclear envelope. On the other hand, immunoreactivity against type 2 IP\(_3\) receptor was located almost exclusively in cytoplasmic structures, with no presence in the nucleus (Fig. 11). Finally, the antibody against type 3 IP\(_3\) receptor shows a significant amount of internal nuclear labeling as a punctuate pattern. This fluorescence pattern was also observed throughout the cellular matrix. These results indicate that there is a different spatial distribution of IP\(_3\) receptor isoforms in cardiomyocytes.

**DISCUSSION**

**Summary of Main Findings**—In the present study, we show for the first time that IGF-1 induced a fast and transient increase in [Ca\(^{2+}\)], in cultured rat cardiomyocytes. This Ca\(^{2+}\) release was released from intracellular stores through an IP\(_3\)-dependent mechanism, essentially independent of ryanodine receptors or extracellular Ca\(^{2+}\) entry. Our results also indicate that the effect of IGF-1 on calcium release was dependent on both tyrosine kinase activity and \(\beta\gamma\) subunits of a PTX-sensitive G protein.

**IGF-1 Increased [Ca\(^{2+}\)], in Cultured Cardiac Myocytes through an IP\(_3\)-dependent Mechanism**—We described an IGF-1-dependent [Ca\(^{2+}\)], increase in cultured rat cardiomyocytes; this increase was not because of calcium entry through voltage-gated L-type Ca\(^{2+}\) channels, because the effect persisted even when cardiomyocytes were incubated in Ca\(^{2+}\)-free/EGTA-containing resting solution or in the presence of nifedipine. The inhibition of IGF-1-dependent [Ca\(^{2+}\)], increase by BAPTA-AM and thapsigargin indicate that Ca\(^{2+}\) was released from intracellular stores.

Confocal and epifluorescence microscopy images suggest that IGF-1 stimulates Ca\(^{2+}\) release from perinuclear regions, producing first a fast Ca\(^{2+}\) increase in the nucleus followed later by a cytosolic increase. Jaconi et al. (46) showed that...
adenosine-dependent [Ca\textsuperscript{2+}] oscillations in cardiac myocytes are elicited by Ca\textsuperscript{2+} release from the perinuclear region, which also produce a fast increase of nuclear Ca\textsuperscript{2+} levels.

Cardiomyocytes stimulated with IGF-1 in the presence of extracellular Ca\textsuperscript{2+} exhibited a slower increase in [Ca\textsuperscript{2+}]i than that observed in the absence of extracellular Ca\textsuperscript{2+}. ROI analysis of fluorescence images revealed that external Ca\textsuperscript{2+} slowed down only the cytosolic but not the nuclear Ca\textsuperscript{2+} increase. As discussed below, differences in the time course of the IP\textsubscript{3} mass increase induced by IGF-1 addition with and without external Ca\textsuperscript{2+} could explain the differences in Ca\textsuperscript{2+} transient kinetics seen in both conditions.

Cardiomyocytes pretreated with ryanodine (20 \textmu M) and stimulated with IGF-1 in the absence of extracellular Ca\textsuperscript{2+} exhibited a slower increase in [Ca\textsuperscript{2+}]i than that observed in the absence of ryanodine. ROI analysis of fluorescence images revealed that ryanodine, in analogy to the effect of extracellular Ca\textsuperscript{2+}, slowed down the cytosolic but not the nuclear Ca\textsuperscript{2+} increase. These results suggest that Ca\textsuperscript{2+} release through ryanodine receptors contributes to the fast cytosolic [Ca\textsuperscript{2+}]i increase but not to the nuclear Ca\textsuperscript{2+} increase produced by IGF-1 stimulation in the absence of extracellular Ca\textsuperscript{2+}. The ensemble of results suggests that at least two independent pathways for IGF-1-induced calcium release coexist in cardiac myocytes; release at the nuclear level is independent of both external calcium and ryanodine receptors. At the cytosolic level, on the other hand, a fast component of the calcium transient appears to be regulated by the presence of external calcium and also to partly depend on ryanodine receptor activation. A slow cytosolic component, not altered by ryanodine, also appears to be there.

The fact that different types of IP\textsubscript{3} receptors are expressed in cytosolic and perinuclear regions may explain the differences in calcium signaling kinetics between the two compartments. In fact, both affinity for IP\textsubscript{3} and calcium dependence for activation have been reported to differ between the different isoforms (47).

The effects of IP\textsubscript{3} inhibitors on both IGF-1-induced [Ca\textsuperscript{2+}] oscillations and IP\textsubscript{3} mass increase strongly suggest a role for IP\textsubscript{3}-regulated Ca\textsuperscript{2+} channels in eliciting these signals. The slower increase in IP\textsubscript{3} mass induced by IGF-1 in the presence of external Ca\textsuperscript{2+} could explain why the cytosolic Ca\textsuperscript{2+} increase was also slower in this condition. Different PLCs generate IP\textsubscript{3} in the heart (48–53). Some neurohumoral agonists, including...
acetylcholine, endothelin, catecholamines, and prostaglandins (48–50) activate a G protein-dependent phospholipase Cβ (PLC-β). In contrast, purines or angiotensin II stimulate a tyrosine kinase-dependent PLC-γ (51, 52). PLC-δ has also been described in heart (53). Although all PLC isoforms require Ca²⁺ for activity, the PLC-δ type has the highest Ca²⁺ sensitivity, suggesting that it may be physiologically regulated by Ca²⁺ (54). A differential regulation of cardiac PLC isoforms by both IGF-1 and Ca²⁺ could explain the different kinetics for the IP₃ mass increase observed in the presence or absence of external Ca²⁺. However, further studies are needed to clarify this point.

In cardiac muscle, some reports have also described the IP₃-dependent Ca²⁺ release. Thus, IP₃ is capable of inducing a slow release of calcium from vesicular preparations and of activating contraction in skinned ventricular rat muscle and chick heart preparations (55). Borgatta et al. (56) identified a low conductance, IP₃-sensitive, calcium release channel in sarcoplasmic reticulum vesicle preparations from canine heart. Consistently with the presence of an IP₃ signaling pathway in cardiac tissue, IP₃ receptors have also been described. Rat and ferret adult ventricular myocytes (57, 58) and rat adult atrial myocytes (59) express type 2 IP₃ receptors as their main isoforms. Both type 2 and type 3 IP₃ receptors were also identified in neonatal rat cardiomyocytes but with different intracellular localizations (46). Our results also described the presence of type 1 IP₃ receptor in neonatal rat cardiac myocytes. Western blot and immunocytochemical studies showed that type 1 and type 3 IP₃ receptors were mainly located in the nucleus, whereas type 2 IP₃ receptor was located almost exclusively in the cytosol. The participation of IP₃ receptors on IGF-1 action was established because both xestospongin C and 2-aminoethoxy diphenyl borate completely blocked the IGF-1-induced [Ca²⁺] increase. Moreover, differences in the cellular distribution of IP₃ receptor isoforms could explain in part the cellular compartmentalization of Ca²⁺ transients induced by IGF-1. The role of IP₃ receptors in cardiac myocytes is controversial, with suggestions that they play only a minor role during cardiac Ca²⁺ signaling or have a solely organelle-specific function (58). However, Lipp et al. (59) and Mackenzie et al. (60) showed that the type 2 IP₃ receptor regulates the arrhythmogenic potency of endothelin-1 in atrial myocytes and could modulate excitation-contraction coupling, respectively.

**Participation of Other Signaling Cascades in IGF-1 Stimulation of Cultured Cardiomyocytes**—The IGF-1-induced increases in Ca²⁺ transients and IP₃ mass were both totally suppressed by pretreatment of rat cardiomyocytes with PTX, suggesting the involvement of a PTX-sensitive G protein in these responses. The IGF-1-induced Ca²⁺ transients were also reduced by the expression of βARK-ct, indicating that Gβγ subunits from the G protein are involved. Additionally, LY294002, a PI3K inhibitor, completely blocked the IGF-1-induced Ca²⁺ transients, indicating the participation of PI3K in the IGF-1 induced Ca²⁺ signaling pathway. Bony et al. (61) have described that PI3K-γ activation is a crucial step in the purinergic regulation of rat cardiomyocyte spontaneous Ca²⁺ spiking, and LY294002 treatment of these cells prevented tyrosine phosphorylation and membrane translocation of PLC-γ, as well as IP₃ generation on ATP-stimulated cells. Hong et al. (62) showed that in IGF-1-induced muscle differentiation, using I9c2 rat cardiac myoblasts as a model, there was activation of PLC-γ 1 via both PI3K-dependent and tyrosine kinase-dependent mechanisms. Furthermore, PLC-γ 1 activation was independent of PKB (62).

As indicated above, IGF-1 binding to the IGF-1 receptor activates PI3K through the involvement of IRS proteins (12, 63) in a G protein-independent mechanism (14). However, the Gβγ subunit of trimeric G proteins can activate PI3K-γ by direct interaction with two domains of the catalytic p110 subunit (64). Using a pressure load hypertrophy model, Prasad et al. (65) demonstrated that PI3K was activated in mice in vivo by a Gβγ-dependent process. Therefore, IGF-1 could activate PI3K by a PTX-sensitive pathway through the Gβγ subunit of a G protein.

Recent reports in different cell types have proposed the involvement of the G protein but not of PI3K/PKB signaling pathways in IGF-1 receptor-dependent ERK activation (13–16). Our results also show that in cardiomyocytes IGF-1 receptor-dependent ERK activation required PTX-sensitive G protein. In contrast, IGF-1 receptor-dependent PKB activation was insensitive to PTX.

Pretreatment of rat cardiomyocytes with PD98059, a MEK inhibitor, did not block the IGF-1-induced Ca²⁺ transients, but modified its kinetics. Because cells were preincubated with PD98059 30 min before IGF-1 stimulation, this inhibitor could change basal ERK-dependent phosphorylation within the cells. This alteration could in turn change the observed Ca²⁺ transient kinetics. Moreover, the Ca²⁺ signaling inhibitor U73122, but not BAPTA, prevented IGF-1-dependent ERK activation. Taken together, these results suggest that the MEK-ERK signaling cascade was not necessary for IGF-1-dependent Ca²⁺ release, but intracellular Ca²⁺ increases may promote ERK phosphorylation. This observation agrees with previous findings in PC12 cells and chicken motor neurons; in these cells membrane depolarization activates the ERK signaling pathway through a Ca²⁺/calmodulin-dependent mechanism that involves p21ras activation (66).

In summary (see Fig. 12), our results suggest that IGF-1 induces Ca²⁺ release by a sequential mechanism involving its binding to the IGF-1 receptor, activation of a PTX-sensitive G protein followed by Gβγ subunit-dependent activation of PI3K. This kinase, through indirect activation of PLC, would be responsible in turn for the IP₃ mass increase; finally the subsequent activation of IP₃ receptors in the perinuclear region.
would result in Ca\(^{2+}\) release from intracellular stores to the nucleus and the cytoplasm. IGF-1 activation of MEK-ERK is not directly implicated in Ca\(^{2+}\) release in cardiac myocytes. However, the basal ERK phosphorylation state is somehow involved in modulating Ca\(^{2+}\) signaling kinetics.

**Hypertrophy and IGF-1-dependent Calcium Increase**—IGF-1 is a well known pro-hypertrophic hormone (3–5). Cardiac hypertrophy is an adaptive mechanism to enhance cardiac output in response to several cardiovascular disorders, including hypertension, myocardial infarction, vascular disease, and contractile abnormalities resulting from sarcomeric protein mutations (67). During the hypertrophic response, cardiac myocytes increase in size without undergoing cell division, assemble additional sarcomeres, and activate a fetal program of cardiac gene expression (68). Hypertrophic growth of cardiomyocytes is triggered by Ca\(^{2+}\), acting as an important second messenger for diverse signals including angiotensin II, endothelin-1, α-adrenergic agents, and mechanical stretching (69). The pro-hypertrophic action of IGF-1 has been associated with the MEK-ERK signaling pathway (70). The effects of IGF-1 on Ca\(^{2+}\) are open the possibility of studying Ca\(^{2+}\) as a new potential second messenger for Ca\(^{2+}\) signaling pathways.

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