Insulin-like Growth Factor-I Rapidly Activates Multiple Signal Transduction Pathways in Cultured Rat Cardiac Myocytes*

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In response to insulin-like growth factor-I (IGF-I), neonatal rat cardiac myocytes exhibit a hypertrophic response. The elucidation of the IGF-I signal transduction system in these cells remains unknown. We show here that cardiac myocytes present a single class of high affinity receptors (12,446 ± 3,669 binding sites/cell) with a dissociation constant of 0.36 ± 0.10 nM. Two different β-subunits of IGF-I receptor were detected, and their autophosphorylation was followed by increases in the phosphotyrosine content of extracellular signal-regulated kinases (ERKs), insulin receptor substrate 1, phosphatidylinositol 3-kinase, and Grb-2, growth factor receptor-binding protein 2, PLC-γ, phospholipase C-γ, Shc, Src homology/collagen; Raf, MEK-activating kinase; MEK, ERK-activating kinase; ERK, extracellular signal-regulated kinase; p90 RSK, p90 S6 kinase; TPA, 12-O-tetradecanoylphorbol-13-acetate; DMEM, Dulbecco’s modified Eagle’s medium; M199, medium 199; MBP, myelin basic protein; GST, glutathione S-transferase; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; DT-T, dithiothreitol, FPLC, fast protein liquid chromatography; MOPS, 4-morpholinepropanesulfonic acid.

1 The abbreviations used are: IGF-I, insulin-like growth factor I; IGF-IR, IGF-I receptor; IRS-1, insulin receptor substrate 1; SH2, Src homology 2; PI 3-kinase, phosphatidylinositol 3-kinase; Grb-2, growth factor receptor-binding protein 2; PLC-γ, phospholipase C-γ; Shc, Src homology/collagen; Raf, MEK-activating kinase; MEK, ERK-activating kinase; ERK, extracellular signal-regulated kinase; p90 RSK, p90 S6 kinase; TPA, 12-O-tetradecanoylphorbol-13-acetate; DMEM, Dulbecco’s modified Eagle’s medium; M199, medium 199; MBP, myelin basic protein; GST, glutathione S-transferase; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; DT-T, dithiothreitol, FPLC, fast protein liquid chromatography; MOPS, 4-morpholinepropanesulfonic acid.

Left ventricular hypertrophy is both an important cardiovascular risk factor epidemiologically associated with cardiac failure and a frequent pathology of major significance in cardiovascular medicine. The myocardium is composed of many different cell types with cardiac myocytes contributing the greatest protein mass. In response to hormonal (polypeptide}

growth factors, endothelin, angiotensin II) and mechanical stimuli (1–6), the myocardium adapts by hypertrophy of individual myocytes. Because these cells are terminally differentiated cells that have lost the ability to proliferate, cardiac growth during hypertrophy results primarily from an increase in cellular protein content, with little or no change in cardiac myocyte number (7). The central features of the myocardial hypertrophic response are increases in the mass, volume, and contractile protein content of the myocytes, the induction of contractile protein gene expression, and the expression of embryonic genes and proto-oncogenes (7, 8).

Among growth factors, insulin-like growth factor-I (IGF-I)1 is significant because it promotes hypertrophy by growth and differentiation in many types of cells (9–11); it also mediates the anabolic and cardiovascular actions of growth hormone in vivo (12). IGF-I and its receptor (IGF-IR) are present on the neonatal rat myocardium, consistent with IGF-I regulating growth and hypertrophy of cardiac myocytes in the developing heart in an autocrine or paracrine manner (13). IGF-I acts directly on cultured neonatal cardiomyocytes to induce myocyte hypertrophy, which leads to increases in mRNA expression and synthesis of contractile proteins (14). The administration of recombinant IGF-I enhances ventricular hypertrophy and function during the onset of experimental heart failure in the rat (15, 16). There is also clear clinical evidence for a role of IGF-I in the initiation and development of left ventricular hypertrophy. Patients with dilated cardiomyopathy and heart failure treated with recombinant growth hormone showed increased myocardial mass and contractile performance accompanied by a doubled serum IGF-I concentration (17, 18). Moreover, cardiac hypertrophy develops in acromegalic patients with concomitantly increased plasma concentrations of both growth hormone and IGF-I (19). Increased plasma IGF-I concentrations have been reported in patients with hypertension and cardiac hypertrophy (20). The hypertension induces significant increases in cardiac IGF-I mRNA and protein in parallel with the onset and early development of experimental cardiac hypertrophy; this is followed by a normalization of the IGF-I mRNA once the hypertrophic response is established (21). A
very recent report has established that the overexpression of IGF-I in cardiac myocytes leads to cardiomyopathy mediated by an increased number of cells in the heart (22).

The mechanism by which IGF-I exerts its growth effect on cardiac myocytes is still unknown. The IGF-IR is an $\alpha\beta^2$ heterotetrameric protein with ligand-stimulated tyrosine kinase activity (23). Binding of IGF-I to its receptor induces receptor autophosphorylation in the intracellular kinase domain of the $\beta$-subunit and results in activation of the intrinsic tyrosine kinase activity of the IGF-IR (24, 25). The predominant substrate of the IGF-IR is IRS-1, a docking protein that has multiple tyrosines in YMMX or related motifs known to associate with proteins containing SH2 domains. Phosphorylated IRS-1 regulates the activity of certain SH2 domain-containing proteins such as phosphatidylinositol 3-kinase (PI 3-kinase) (26, 27). IRS-1 also associates with other SH2 domain-containing proteins involved in growth factor signaling pathways, including Grb-2, Nck, and Syp (23). Other phosphorytrosine substrates of the IGF-I signaling pathway are PLC-γ and Shc (23); and direct association of Shc with activated IGF-IR has also been detected (28). Phosphorylated Shc associates with Grb-2 and subsequently, through a Grb-2-SOS (Son of Sevenless) complex, activates Ras and Raf-1, an intermediate in the Ras-ERK signaling pathway (29). In the ensuing phosphorylation cascade, activation of ERKs occurs after phosphorylation by the mixed function threonine-tyrosine kinase MEK. Some of these kinases, as well as protein kinase C and RSK, can regulate the activities of a diverse array of cellular and nuclear proteins, including transcription factors.

The signaling pathways of other hypertrophic agonists (endothelin and acidic fibroblast growth factor) may converge at or above the level of the ERK cascade in cardiac myocytes (30). The elucidation of the IGF-I signal transduction system, and especially the degree of involvement of the IGF-IR and the ERK cascade during the cardiac hypertrophic process, is of considerable interest and constitutes the aim of this work.

EXPERIMENTAL PROCEDURES

Animals—Harlan Sprague Dawley rats were bred in the Animal Breeding Facilities from the National Heart and Lung Institute (London, U.K.), NIDDK (Bethesda, MD), or the Faculty of Chemical and Pharmaceutical Sciences, University of Chile (Santiago).

Materials—[3H]PIATP, [3H]Phenylalanine, and [methyl-3H]thymidine were from Amersham International (Bucks, U.K.), and [125I]-IGF-I was from NEN Life Science Products. 12-Tetradecanoylphorbol-13-acetate (TPA), Dulbecco’s modified Eagle’s medium (DMEM), medium 199 (M199), n-octyl $\beta$-D-glucopyranoside, protease inhibitors, protein A-Sepharose, bovine myelin basic protein (MBP), and other biochemicals were purchased from Sigma unless stated otherwise. Culture dishes (Primaria) were from Falcon, and heat-inactivated fetal calf serum and horse serum were from Sera-Lab. Other tissue culture products were from Life Technologies, Inc. Glutathione-S-transferase was from Pharmacia Biotech Inc. Affinity-purified antibodies raised against COOH-terminal peptide sequences of c-Raf (CTLTTSPLRPVF) or A-Raf (CLLSAARLVP), and the corresponding peptides were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) as were the antibodies raised against IGF-IR $\beta$-subunit, tyrosine-phosphorylated proteins (anti-phosphotyrosine antibody clone 4G10), PLC-γ1, and RSK. The polyclonal anti-IGF-IR $\beta$-subunit antibody was raised against the COOH-terminal peptide sequence of the $\beta$-subunit of IGF-IR (AHMNGKRKNERALPFLPQST). We have also assessed that this antibody does not cross-react with insulin receptor $\beta$-subunit using different cell lines that express different numbers of insulin receptor and IGF-IR (31, 32).

Enhanced chemiluminescence (ECL) immunoblotting detection reagents, autoradiographic film, and prestained molecular mass standards were purchased from Amersham International (Bucks, U.K.) and were from Bio-Rad. Human recombinant IGF-1 was donated by Drs. P. Valenzuela and C. George-Nascimento (Chiron Corp.) or was purchased from Boehringer-Mannheim GmbH (Mannheim, Germany). IRS-1 antibody was a gift from Dr. J. Pierce (NICI, NIH). Clones for Glutathione S-transferase (GST) fusion proteins and anti-murine ERK antibody were donated by Prof. C. J. Marshall (Chester Bently Laboratories, Institute of Cancer Research, London, U.K.). Recombinant murine ERK2 and MEK1 were expressed as GST fusion proteins in Escherichia coli and were purified by glutathione-Sepharose affinity chromatography as described previously (29). For the assay of MEK phosphorylation by the immunoprecipitated protein kinases, recombinant MEK1 was modified by site-directed mutagenesis to produce GST-MEK1 (R97/A291/A385), a catalytically inactive form of MEK1 in which a Lys residue essential for kinase activity is mutated (K97R) and which also lacks ERK consensus phosphorylation sequences (T291A and T385A). Recombinant GST-MEK2 was stored at $-20^\circ$C in 50% (v/v) glycerol (final protein concentration 25 mg/ml), whereas recombinant MEK1 protein was stored at $-80^\circ$C in 5% (v/v) glycerol (final protein concentration 10 mg/ml).

Primary Culture—Neonatal ventricular myocytes were prepared from hearts of 1-3-day-old Harlan Sprague Dawley rats as described previously (33). Briefly, ventricles were trisected, pooled, and myocytes dissociated in a solution of collagenase and pancreatic. After enzymatic dissociation, the cells were selectively enriched for cardiac myocytes by being preplated in DMEM/M199 (4:1) containing 10% (v/v) horse serum, 5% (v/v) heated-inactivated fetal calf serum, penicillin, and streptomycin (100 units/ml). The myocytes, plated at a final density of 1.0–1.4 $\times$ 106 cells (0–60 min) or TPA (1 $\mu$g for 5–30 min) in serum-free medium (DMEM/M199) at 37 $^\circ$C.

IGF-I Binding Assay—IGF-I binding to ventricular cardiac myocytes was quantified using monoidoniated $^{125}$I-IGF-I. Confluent cardiac myocytes in 12-well culture plates were washed with 1 ml of binding buffer (100 mM Hepes, pH 7.4, 120 mM NaCl, 1 mM EDTA, 5 mg/ml bovine serum albumin) and incubated in binding buffer containing 25,000 cpm (approximately 20 pm of $^{125}$I-IGF-I and increasing amounts of unlabeled IGF-I (0–1,000 ng/ml) for 5 h at 4 $^\circ$C. The cells were washed with cold PBS and solubilized in 0.2 M NaOH before being counted with a $\gamma$-counter (GammaTrac 1290, TM Analytical, Brandon, FL).

IGF-I-stimulated Cardiac Myocyte Tyrosine Phosphorylations—Tyrosine phosphorylation of cellular proteins was monitored by immunoblotting with an anti-phosphotyrosine antibody. Confluent cardiac myocytes were seeded in 60-mm dishes were exposed to IGF-I (100–1,000 ng/ml) for 0–60 min in serum-free medium at 37 $^\circ$C. Upon completion of the exposure protocol, the medium was removed by aspiration and the cells washed twice with cold Ca$^{2+}$/Mg$^{2+}$-free Dulbecco’s PBS. Myocytes were scraped into 150 ml of cold lysis buffer (20 mM Heps, pH 7.4, 100 mM NaCl, 4 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 10 mM EDTA, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 10 mM PMSF, 3 mM aprotinin, 1% (v/v) Triton X-100, 2 $\mu$g/ml leupeptin, and 2 $\mu$g/ml aprotinin). The protein content of the lysate was determined by Bradford’s method (34), and equal amounts of protein were separated by SDS-PAGE on 9% gels. Proteins were then transferred electrophoretically to a nitrocellulose membrane (0.45 pm). The membranes were blocked with 3% (w/v) bovine serum albumin in PBST (80 mM Na$_2$HPO$_4$, 20 mM NaH$_2$PO$_4$, 100 mM NaCl, 0.05% Tween-20, pH 7.4) for 1 h at room temperature. Tyrosine-phosphorylated proteins were probed with a monoclonal anti-phosphotyrosine antibody and detected with horseradish peroxidase-conjugated anti-mouse IgG. The blots were then developed by ECL.
overnight at 4 °C. Then, 50 μl of protein A-Sepharose was added to monoclonal anti-phosphotyrosine antibody immunoprecipitated with a monoclonal anti-phosphotyrosine antibody overnight at 4 °C. Then, 50 μl of 10% protein A-Sepharose in 50 mM Tris-HCl, pH 7.4, was divided equally. Antibodies that recognized c-Raf or A-Raf (3A-Sepharose immunoprecipitates of c-Raf and A-Raf were assayed for their activation of recombinant GST-MEK1 (36) using a coupled assay in which GST-MEK1 activates GST-ERK2. The activity of GST-ERK2 is that amount that catalyzed the activation of sufficient GST-ERK2 to transfer 1 fmol of phosphate from \( ^{32}P \text{ATP} \) to MBP phosphorylation buffer (50 mM Tris, 0.1 mM EGTA, pH 8.0, containing 0.4 mg/ml MBP, 50 μM ATP, 12.5 mM magnesium acetate, and 0.5 mM N-ethyl β-glyceroxypyridine) that had been supplemented with 80 μM unlabeled IGF-I and represented 3.4 ± 0.1% (n = 4) of the total input radioactivity. Each value is the mean of duplicate determinations in one representative experiment. Similar profiles were obtained with four independent experiments. Inset, Scatchard plot for IGF-I binding to cultured cardiac myocytes.

**FIG. 1. IGF-I binding assay in cultured neonatal cardiac myocytes.** Confluent cardiac myocytes were incubated with [125I]IGF-I and the indicated amounts of unlabeled IGF-I as described under “Experimental Procedures.” Maximal binding was determined in the absence of unlabeled IGF-I and represented 3.4 ± 0.1% (n = 4) of the total input radioactivity. Each value is the mean of duplicate determinations in one representative experiment. Similar profiles were obtained with four independent experiments. Inset, Scatchard plot for IGF-I binding to cultured cardiac myocytes.
and ERK2 activities changed in parallel with activities measured by the direct assay. Agonist-stimulated phosphorylation of endogenous ERK2 was also determined from the shift in electrophoretic mobility as described (38).

**p90 RSK Activity—**RSK activity was measured by an immune complex kinase assay using an S6 peptide (RRRLSSLRRA) as a substrate as described previously (39). The lysates were prepared from cardiomyocytes grown in 60-mm dishes. After stimulation with IGF-I (10 nM), the cells were removed by scraping and lysed with lysis buffer (10 mM potassium phosphate, pH 7.4, 1 mM EDTA, 5 mM EGTA, 10 mM MgCl₂, 50 mM β-glycerophosphate, 1 mM Na₃VO₄, 2 mM DTT, 40 μg/ml PMSF, 10 μM okadaic acid, 0.8 μg/ml leupeptin, 10 μM n-nitrophenyl phosphate, and 10 μg/ml aprotinin). Equal amounts of lysate proteins (300 μg) were diluted with 10 volumes of RIPA buffer (10 mM Tris, 150 mM NaCl, 1% (v/v) Triton X-100, 1% (w/v) deoxycholic acid, and 0.1% (w/v) SDS, pH 7.2) and were incubated with 2 μl of anti-S6 kinase antibody for 2 h at 4 °C. Then protein A-Sepharose was added, and the immunoprecipitates were washed with lysis buffer without p-nitrophenyl phosphate. Ten μl of immunoprecipitate was incubated with 12.5 μl of 2 × kinase buffer (50 mM MOPS, 120 mM β-glycerophosphate, 10 mM EDTA, 30 mM MgCl₂, 60 μM p-nitrophenyl phosphate, 2 mM DTT, pH 7.2) containing 2 mM Na₃VO₄, 2 μM protein kinase A inhibitor, 12.5 μM of γ-[³²P]ATP, and 12.5 μl of 5 μM S6 peptide (RRRLSSLRRA) at 30 °C for 15 min. To stop the reaction, samples were spotted onto Whatman P-81 paper and washed five times (5 min each) with 180 mM H₃PO₄ and once with 95% ethanol. Radioactivity was determined in a β-counter.

**Thymidine Incorporation—**Confluent cardiac myocytes in 12-well plates were grown to quiescence in serum-free DMEM/M199 for 24 h. IGF-I (0–100 nM) in fresh serum-free DMEM/M199 was added, and the cells were incubated for an additional 18 h. [methyl-³H]Thymidine (1 μCi/well) was then added and incubation continued for a further 6 h. Cells were rinsed twice with ice-cold PBS, twice with ice-cold 5% trichloracetic acid, and twice with ethanol. The cells were dissolved in 0.3 ml of 1 M NaOH; the resulting solutions were neutralized with 0.3 ml of 1 M HCl and counted in a liquid scintillation counter.

**Phenylalanine Incorporation—**Confluent cells were grown to quiescence in serum-free medium for 24 h. Cardiac myocytes were stimulated with IGF-I (0–100 nM) in fresh serum-free medium for 24 h in the presence of [³H]phenylalanine (5 μCi/well). The cells were washed with ice-cold PBS, and 10% trichloracetic acid was added at 4 °C for 60 min to precipitate the proteins. The precipitates were washed twice with ethanol and dissolved in 1 M NaOH; the resulting solutions were neutralized with 1 M HCl and counted in a liquid scintillation counter.

Expression of IGF-IRs on Cultured Neonatal Cardiac Myocytes—Binding of IGF-I to neonatal cardiac myocytes was saturable and specific (Fig. 1). The Scatchard plot was curvilinear, and the analysis (40) suggested the presence of two classes of binding sites (Fig. 1, inset). Cultured cardiac myocytes had high affinity binding sites of 12,446 ± 6,669 binding sites/cell with a dissociation constant for IGF-I of 0.36 ± 0.10 nM (n = 4). Low affinity binding sites (Kd = 27 ± 11 nM), whose number was estimated to be 77,911 ± 7,683 binding sites/cell, were also detected in neonatal cardiac myocytes. Binding studies with ¹²⁵I-IGF-I(N-3), an IGF-I analog lacking the three NH₂-termini-
confirmed the identity of PLC-$\gamma$-1 and IRS-1 and that similar amounts of PLC-$\gamma$-1 immunoprecipitates were present in all samples. The positions of PLC-$\gamma$-1 and IRS-1 are indicated by arrows. The result shown is representative of two independent experiments.

IGF-IR Autophosphorylation and Phosphorylation of Endogenous Substrates—As shown in Fig. 2 (A and B upper), incubation of cardiac myocytes with IGF-I resulted in increases in the phosphotyrosine content of several proteins with apparent molecular masses of 42, 44, 96, 100, and 185 kDa, corresponding to the two ERK isoenzymes, the two $\beta$-subunits of IGF-IR and IRS-1. IGF-IR autophosphorylation was much greater in neonatal cardiac myocytes exposed to IGF-I concentrations above 5 nM; two separate bands were observed at low levels of receptor autophosphorylation. In contrast, receptor autophosphorylation was undetectable in cells incubated without IGF-I. Membranes shown in Fig. 2 B, upper, were stripped and rebotted with a polyclonal antibody directed against the $\beta$-subunit of IGF-IR to confirm both the presence of two isoforms of the $\beta$-subunit of IGF-IR and that similar amounts of IGF-IR protein were present in all samples (Fig. 2B, lower). Fig. 2C shows the time course of stimulation of cultured cardiac myocytes by 10 nM IGF-I; phosphorylation of the IGF-IR $\beta$-subunit was maximal within 1 min. The IRS-1 pathway is activated following IGF-IR phosphorylation (42). We evaluated IRS-1 activation by incubating cells with IGF-I (0–100 nM) for 1 min at 37 °C. Exposure to concentrations of IGF-I above 10 nM was accompanied by phosphorylation of the expected 185-kDa band (Fig. 2A); phosphorylation was apparent 1 min after stimulation with 10 nM IGF-I (Fig. 2C). When membranes were stripped and rebotted with a polyclonal antibody directed against IRS-1, we confirmed the presence of IRS-1 and that similar amounts of IRS-1 protein were present in all samples (results not shown).

Tyrosine Phosphorylation of PLC-$\gamma$-1 and Activation of PI 3-Kinase by IGF-I—Fig. 3A shows the enhancement of tyrosine phosphorylation of PLC-$\gamma$-1 in cells stimulated with IGF-I. Maximum stimulation (5-fold) occurred 2 min after the addition of IGF-I (Fig. 3A). PLC-$\gamma$-1 coprecipitated with a 185-kDa phosphotyrosine protein. When membranes were stripped and rebotted with a polyclonal antibody directed against PLC-$\gamma$-1, we confirmed the identity of PLC-$\gamma$-1 and that similar amounts of PLC-$\gamma$-1 protein were present in all samples (Fig. 3B). Since the 185-kDa phosphorylated protein that coprecipitated with PLC-$\gamma$-1 may correspond to IRS-1, the membrane was rebotted with different anti-IRS-1 polyclonal antibodies (catalog numbers 06-248 and 06-524 from Upstate Biotechnology Inc., Lake Placid, NY, and sc-559 from Santa Cruz Biotechnology, Santa Cruz, CA). However, the presence of IRS-1 in the immunoprecipitates was not detected. A similar result was obtained when IRS-1 was first immunoprecipitated, and the membrane was blotted with an anti-PLC-$\gamma$. PI 3-kinase activity was increased in cardiac myocytes incubated with 10 nM IGF-I for 5 min (Fig. 4A); this increase was maximal (2.5-fold) after 5 min of incubation with 30 nM IGF-I (Fig. 4B).

IGF-I Activates c-Raf Much More Than A-Raf in Cultured Neonatal Cardiac Myocytes—c-Raf is activated after exposure of neonatal cardiomycocytes to agonists acting through protein tyrosine kinase and G-protein receptors (36). Both c-Raf and A-Raf from neonatal cardiac myocytes form a stable complex with kinase inactive GST-MEK1 (R97/A291/A385) (36). Fig. 5A shows that IGF-I (10 nM) activated c-Raf through its ability to activate GST-MEK1 in a coupled assay in vitro. Maximal activation occurred within 3–10 min and returned to basal levels after 30 min. We confirmed that the amounts of immunoprecipitated c-Raf did not vary greatly (data not shown). There was no detectable activity in c-Raf immunoprecipitated from serum-starved ventricular myocytes exposed to serum-free medium. Although A-Raf was present in cultured ventricular myocytes, its activation by IGF-I was much less than c-Raf (closed circles, Fig. 5A). The amounts of A-Raf immunoprecipitates from the cells did not vary (data not shown).

Activity of the immunoprecipitated c-Raf was also studied by the phosphorylation of GST-MEK1 (R97/A291/A385), a recombinant mutant MEK1 unable to undergo autophosphorylation and retrophosphorylation by endogenous ERK (43). This assay showed that c-Raf is activated by IGF-I to a much greater extent than A-Raf.
confirmed the rapid and transient activation of immunoprecipitated c-Raf (Fig. 5B). The maximum increase in c-Raf activity was 400% of the control value and the EC₅₀ for IGF-I was approximately 0.1 nM (Fig. 5C). In cultured cardiac myocytes, TPA activates different isoenzymes of protein kinase C and causes sustained activation of MEK and ERK (33). Activation of c-Raf (15-fold) and A-Raf (3.3-fold) was attained after a 3-min exposure to TPA (1 μM). c-Raf was 29-fold more activated by TPA than by IGF-I (Fig. 5C).

Activation of MEK by IGF-I in Cultured Neonatal Cardiac Myocytes—Crude extracts of unstimulated cultured cardiac myocytes poorly activate exogenous inactive recombinant GST-ERK2. In contrast, IGF-I-treated cultured cardiac myocytes substantially stimulated MEK activity (results not shown). Supernatant fractions of cultured neonatal ventricular myocytes were subjected to FPLC on a Mono Q column, and fractions were assayed for their ability to phosphorylate and activate GST-ERK2. Extracts of cells exposed to 10 nM IGF-I for 5 min showed two peaks of MEK activity (KK1 and KK2) eluted at 70 and 130 mM NaCl (Fig. 6A), although the second peak was smaller than the first. The combined stimulation of MEK activities approached 10-fold. A similar MEK activity profile was obtained after exposure to 1 μM TPA for 5 min (results not shown). We have demonstrated previously that endogenous ERK activity does not interfere with the assay of GST-ERK2 activity (30).

Activation of ERK by IGF-I in Crude Extracts of Cultured Neonatal Cardiac Myocytes—To examine whether ERKs are activated by IGF-I, we next measured ERK activity by an in-gel ERK assay. There were only small changes in the intensity of the ERK2 band after the exposure of myocytes to IGF-I between 1 and 3 min, without any change in ERK1 band (Fig. 7A). The maximal intensities of ERK1 (12-fold) and ERK2 (8.3-fold) were attained after a 5-min exposure to IGF-I. Thereafter, activities declined rapidly returning to control values within 30 min (Fig. 7A). The time course for the effects of IGF-I (10 nM) on ERK activity is shown in Fig. 7B. The effect of IGF-I on the time course of ligand-stimulated phosphorylation of ERK2 in cultured cardiac myocytes was determined from the electrophoretic mobility shift of endogenous ERK2. IGF-I provoked a transient increase in ERK2 phosphorylation which followed a time course similar to that determined by the in-gel assay technique (data not shown). The dependence of ERK activation on the concentration of IGF-I was also characterized by in-gel
assays (Fig. 7, panels C and D). The EC_{50} values for the stimulation of ERK1 and ERK2 in cultured cardiac myocytes by IGF-I were 0.10 and 0.12 nM, respectively. When cardiac myocytes were exposed for 5 min to a saturating concentration of IGF-I (10 nM), ERK1 and ERK2 activities were stimulated to 35 and 58%, respectively, of the TPA-induced activity (Fig. 7D).

Exposure of cardiac myocytes to increasing concentrations of IGF-I for 5 min also caused phosphorylation of ERK2, as demonstrated by delayed mobility on SDS-PAGE (data not shown). Separation by FPLC of IGF-I-activated Isoforms of ERK in Cultured Neonatal Cardiac Myocytes—IGF-I stimulates ERK activity in cultured ventricular myocytes; this can be assayed directly in supernatants with MBP as substrate. FPLC of supernatant fractions from IGF-I-treated cells on the Mono Q (Fig. 6A) revealed two major peaks of MBP kinase activity eluting at 160 (peak K1) and 220 (peak K2) mM NaCl, respectively. The mean stimulation of each peak fraction was 3-fold.

Identification of ERK Isoenzymes Activated by IGF-I—Fractions from peaks K1 and K2 from Mono Q chromatography of extracts from cardiac myocytes incubated with 10 nM IGF-I for 5 min were pooled, concentrated, and analyzed by in situ phosphorylation of MBP gels. Two proteins of 42 and 44 kDa were identified in fractions corresponding to peak K1 and K2, respectively (Fig. 6B).

FPLC of supernatant fractions from IGF-I-treated cells on the Mono Q column with elution with a linear NaCl gradient (0–0.5 M, dashed line) as described under “Experimental Procedures.” Fractions (0.5 ml) were collected and assayed for MEK (○) or ERK (○). The open symbols represent the profiles of control cell extract activities. The two major peaks for MEK are labeled K1 and K2, and the two major peaks for ERK are labeled K1 and K2. Similar elution profiles were obtained with three separate preparations of cardiac myocytes. Panel B, samples of the fractions corresponding to peaks K1 and K2 (in panel A) from cardiac myocytes stimulated with 10 nM IGF-I for 5 min at 37 °C were pooled, concentrated, and examined by the in-gel MBP phosphorylation assay as described under “Experimental Procedures.” The numbers to the left of the panel refer to the molecular masses (kDa) of marker proteins. The positions of the isotypes of ERK, ERK1 (44 kDa) and ERK2 (42 kDa), are indicated by the arrows. The result presented is representative of three separate experiments.

FIG. 6. IGF-I activates MEK and ERK activity in cultured cardiac myocytes. Four 60-mm dishes of cardiac myocytes were treated with 10 nM IGF-I (solid symbols) for 5 min at 37 °C. Panel A, extracts were prepared and the supernatants combined and subjected to FPLC using a Mono Q column with elution with a linear NaCl gradient (0–0.5 M) as described under “Experimental Procedures.” Fractions (0.5 ml) were collected and assayed for MEK (○) or ERK (○). The open symbols represent the profiles of control cell extract activities. The two major peaks for MEK are labeled K1 and K2, and the two major peaks for ERK are labeled K1 and K2. Similar elution profiles were obtained with three separate preparations of cardiac myocytes. Panel B, samples of the fractions corresponding to peaks K1 and K2 (in panel A) from cardiac myocytes stimulated with 10 nM IGF-I for 5 min at 37 °C were pooled, concentrated, and examined by the in-gel MBP phosphorylation assay as described under “Experimental Procedures.” The numbers to the left of the panel refer to the molecular masses (kDa) of marker proteins. The positions of the isotypes of ERK, ERK1 (44 kDa) and ERK2 (42 kDa), are indicated by the arrows. The result presented is representative of three separate experiments.

FIG. 7. Time course for the activation of ERK by IGF-I and dose-dependent effect in cultured cardiac myocytes. Cardiac myocytes in serum-free medium were stimulated with 10 nM IGF-I or 1 μM TPA for the times indicated at 37 °C (panels A and B) or were treated for 5 min at 37 °C with increasing concentrations of IGF-I or 1 μM TPA (panels C and D). Soluble fractions were prepared, and SDS-PAGE sample buffer was added. Samples were subjected to SDS-PAGE in 10% gel containing 0.2 mg/ml MBP. Panels A and C show a representative result of phosphorylation of MBP assayed in situ as described under “Experimental Procedures.” The arrows to the right of the panels indicate the positions of ERK2 and ERK1. Panel B and D graphs show results of ERK1 (○) and ERK2 (●) activation from autoradiograms quantified by laser-scanning densitometry of gels depicted on panels A and C, respectively. Results (means ± S.E., n = five to eight separate observations) are expressed relative to ERK activities in extracts from control cells or to ERK activities in extracts from cells treated with 1 μM TPA for 5 min (maximal activation).

Stimulation of p90 RSK Activity by IGF-I in Cultured Cardiac Myocytes—p90 RSK is activated by ERK1 and ERK2 both in vivo and in vitro (44). The time course of activation of the p90 phosphorylation of MBP gels. Two proteins of 42 and 44 kDa were identified in fractions corresponding to peak K1 and K2, respectively (Fig. 6B).
RSK by IGF-I (10 nM) in cultured cardiac myocytes is depicted in Fig. 8. p90 RSK activity reached a peak at 10 min and then decreased slowly.

**Stimulation of Phenylalanine and Thymidine Incorporation by IGF-I in Cultured Cardiac Myocytes**—After a 24-h incubation of myocytes with 10 nM IGF-I, incorporation of [3H]phenylalanine was increased 1.6-fold over control levels (Fig. 9A). No IGF-I-dependent stimulation of [3H]thymidine incorporation was observed in cultured cardiac myocytes (Fig. 9B).

**DISCUSSION**

The development of cardiac hypertrophy constitutes a compensatory adaptation of cardiac myocytes to increased hemodynamic stress or to loss of contractile myocytes. In addition to an overall increase in protein and RNA content, there are alterations at the level of gene expression which distinguish the hypertrophic process from normal maturational growth (7). The mechanisms by which the mechanical stress is sensed by cardiac myocytes and growth-related signals are activated and integrated to regulate gene expression programs selectively during the hypertrophic process are largely unknown. The hypothesis is that growth factors (such as IGF-I) are produced by cardiac non-muscle cells or by the myocytes themselves in response to mechanical stress and that these factors, through specific cell surface receptors and intracellular signaling cascades, regulate protein synthesis and transcription of genes of the contractile apparatus, as well as others genes involved in cell growth. In cultured neonatal cardiac myocytes, IGF-I induces an early and sustained expression of the muscle-specific genes for troponin I and myosin light chain-2 with myocyte size almost doubling after 48 h of treatment with IGF-I (14). An increase in left ventricular IGF-I mRNA and its protein has been described in pressure overload hypertrophy in various models of hypertension; this suggests that IGF-I may be an important mediator of an adaptive hypertrophic response (21, 45, 46). However, the results of overexpression of IGF-I in transgenic mice have been contradictory (22, 47). For example, when overexpression of IGF-I was restricted to the heart, IGF-I stimulated an increase in ventricular muscle cell number in vivo. Surprisingly, cardiac myocyte cellular hypertrophy was not enhanced (22). Studies with IGF-I knockout mice have shown that lack of IGF-I does not prevent the development of pressure overload hypertrophy, suggesting that IGF-I alone is not the sole precondition for the hypertrophic phenotype (48).

Our results showed that IGF-I binding was specific with a single class of high affinity sites ($K_d = 0.36$ nM) in neonatal cardiac myocytes (Fig. 1) in agreement with an earlier work (14). Although there are no comparative studies between receptors for insulin and IGF-I in neonatal cardiac myocytes, our results show that there appear to be fewer insulin receptors than IGF-IRs (data not shown). A previous study showed that specific binding for insulin and IGF-I/100 mg of rat cardiac tissue heart reached 52 and 20%, respectively, with $K_d$ values of 0.25 nM for insulin receptors and of 0.40 nM for IGF-IR for its own ligand (49), indicating that insulin receptors are more abundant in non-myocyte cell types.

It is well known that IGF-I stimulates tyrosine phosphorylation of several proteins in various cell types (23, 50, 51). We detected several tyrosine-phosphorylated proteins with apparent molecular mass of 42, 44, 95, 105, and 180 kDa, whose tyrosine phosphorylation in response to IGF-I increased rapidly (Fig. 2). By immunoblotting, these proteins were identified as p42 Erk2, p44 Erk1, IGF-IR β-subunit, and IRS-1 (Figs. 2 and 7, and data not shown) (52, 53). The results also showed
that the autophosphorylation of IGF-IR β-subunit as well as phosphorylation of IRS-1, both on tyrosines, was IGF-I concentration dependent, although with a different sensitivity. IGF-IR β-subunit autophosphorylation became evident with 5 nM IGF-I, whereas for IRS-1 phosphorylation was observed after 10 nM (Fig. 2A). The presence of two isoforms for IGF-IR β-subunits with molecular masses of 95 and 105 kDa (Fig. 2B) was also detected in neonatal cardiac myocytes; similar isoforms have been reported earlier (23). Although it remains unknown whether each subunit isoform may have a particular signal transduction system and if their expression changes during the myocyte development of cardiac myocytes, the higher and lower molecular masses may correspond to a fetal and adult forms, respectively (54, 55). Other hypertrophic stimuli (stretch, angiotensin II) also stimulate protein tyrosine phosphorylation in cardiac myocytes (56–58), and it has been hypothesized that cardiac hypertrophy might be associated with altered tyrosine phosphorylation of certain proteins (i.e. a 120-kDa cytosolic protein) in the heart (59). Although in cardiac myocytes there is no evidence of cross-talk between angiotensin II and IGF-I, this interaction has been demonstrated at multiple levels in vascular smooth muscle cells (60). Both angiotensin II and thrombin caused rapid tyrosine phosphorylation of the IGF-IR β-subunit and of IRS-1 in this cellular type (61). IRS and Shc are considered the two major IGF-I signaling pathways. IRS-1 is an adapter protein phosphorylated on multiple tyrosine residues upon receptor stimulation, providing multiple sites of interactions for proteins with SH2 domains such as p85 PI 3-kinase, Nck, Grb-2, and PTP1B (23, 62). PLC-γ1 also contains SH2 domains that recognize and bind to the phosphorylated tyrosine residues of the receptor tyrosine kinases (63). In contrast with other RTKs, it is not well established whether PLC-γ1 associates with IGF-IR and becomes activated by tyrosine phosphorylation upon receptor stimulation. We demonstrated that IGF-I induces a rapid tyrosine phosphorylation of PLC-γ1 reaching 3.5-fold (Fig. 3). Although we have detected coprecipitation of a 185-kDa phosphorylated protein with PLC-γ1 which might correspond to IRS-1, our results suggest that both proteins are not associated after IGF-IR activation, and further studies will be required to identify the phosphorylated protein that coprecipitates with PLC-γ1. PI 3-kinase activation in IGF-I signal transduction may play an important role because this enzyme mediates the activation of other protein kinases such as protein kinase C, S6 kinase, and serine-threonine kinase Akt as well as the regulation of cytoskeletal function (63–65). Our results also show that its activation was IGF-I concentration-dependent and that it was activated by tyrosine phosphorylation. IGF-I also causes rapid translocation of two Shc isoforms (46 and 52 kDa) to the membrane fraction with a maximum increase in membrane (6–11-fold) after 30 s with 10 nM IGF-I.² It has been shown that Shc, like IRS-1, associates with Grb-2, upon tyrosine phosphorylation, and subsequently activates the p21 Ras-ERK pathway via a Grb-2-SOS complex (23). Our results stress that IGF-I has multiple signal transduction pathways through different adapter proteins in cardiac myocytes.

We and others have previously proposed that activation of the p21 Ras-ERK pathway may be involved in the growth response of cardiac myocytes to hypertrophic agonists (66–69). In contrast, others have reported that ERK activation is not sufficient for G-protein receptor-mediated induction of cardiac cell growth and gene expression and is apparently not required for transcriptional activation of the atrial natriuretic factor gene (70).

Diverse hypertrophic agonists converge to produce cardiac hypertrophy following a sequential activation of Raf → MEK → ERK. The upstream event in the ERK cascade involves the activation of Raf kinases. We have presented evidence that exposure of cardiac myocytes to IGF-I rapidly activated c-Raf. A-Raf was activated much less than c-Raf in neonatal cardiac myocytes. This finding is similar to that reported previously for acidic fibroblast growth factor in cardiac myocytes but differs from that produced by TPA (36). The reason for this differential activation of Raf isoforms by IGF-I is unclear; and its functional effectors, in addition to Ras and Src, are yet to be identified (71, 72). The differential activation of both Raf isoforms may also reflect different requirements for effectors or the interaction with other proteins like 14-3-3 proteins (73). We have also demonstrated that IGF-I activated two peaks of MEK (Fig. 6) which were capable of phosphorylating exogenously added recombinant GST-ERK2. Both peaks may correspond to the MEK1 and MEK2 isoforms activated by acidic fibroblast growth factor and endothelin-1 (30). The two isoforms of ERK (ERK1 and ERK2) were coordinately activated by IGF-I (Fig. 7). The maximum extent of phosphorylation of ERK2 elicited by IGF-I corresponded to 44% of the total ERK2 pool (results not shown). This response is similar to that seen with other hypertrophic agonists (endothelin-1, phenylephrine, acidic fibroblast growth factor) (33, 36–38). Nonhypertrophic agents such as bradykinin, carbachol, and ATP do, however, also activate ERK in cardiac myocytes (70, 74). In PC-12 cells the duration of the activation of ERK1 and ERK2 is critical in determining whether the cells differentiate (prolonged activation) or proliferate (transient activation) (75, 76). In neonatal cardiac myocytes in culture activation of ERKs induced by mechanical stretch and angiotensin II is accompanied by sustained activation of p90 RSK (37, 58). We have shown here that IGF-I causes activation of p90 RSK in cardiac myocytes. Last, we have shown that IGF-I stimulates [3H]phenylalanine incorporation into cardiac myocyte proteins without changes in the incorporation of [3H]thymidine into DNA in neonatal cardiac myocytes. This agrees with previous reports showing the hypertrophic action of IGF-I in vitro (14). Our preliminary results using different inhibitors of the IGF-I signal transduction pathway (i.e. PD 098059, an inhibitor of MEK) suggest that IGF-I-induced cardiac hypertrophy may be mediated by the ERK cascade.

We concluded that IGF-I activates multiple signal transduction pathways that involve tyrosine phosphorylation of IRS-1, PI 3-kinase, and PLC-γ1 and the activation of the ERK cascade and p90 RSK in neonatal cardiac myocytes in culture. The use of both selective inhibitors of signal transduction pathways as well as the transient transfection of cardiac myocytes with dominant-negative forms and antisense oligodeoxynucleotide approach for the different components of signal transduction mechanism of IGF-I will allow us to establish their contribution to cardiac myocyte hypertrophy.

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