Extracellular Regulated Kinase, but Not Protein Kinase C, Is an Antiapoptotic Signal of Insulin-like Growth Factor-1 on Cultured Cardiac Myocytes

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This study aims to elucidate the signaling pathway for insulin-like growth factor-1 (IGF-1) in cultured neonatal rat cardiomyocytes and particularly the role of IGF-1 in cardiac apoptosis. IGF-1 stimulated polyphosphoinositide turnover, translocation of protein kinase C (PKC) isoforms (α, ε, and δ) from the soluble to the particulate fraction, activation of phospholipid-dependent and Ca^{2+}-, phospholipid-dependent PKC, and activation of the extracellular-regulated kinase (ERK). IGF-1 attenuated sorbitol-induced cardiomyocyte viability and nuclear DNA fragmentation. These antiapoptotic effects of IGF-1 were blocked by PD-098059 (an MEK inhibitor) but not by bisindolylmaleimide I (BIM, a specific PKC inhibitor). The ERK pathway may therefore be an important component in the mechanism whereby IGF-1 exerts its antiapoptotic effect on the cardiomyocyte.

Key Words: **insulin-like growth factor-1; protein kinases; signal transduction; apoptosis; cardiac myocytes.**

Abbreviations used: BIM, bisindolylmaleimide I; BK, bradykinin; cPKC, classical PKC; DAG, 1,2-diacylglycerol; DO, 1,2-diolein; DMEM, Dulbecco's modified Eagle's medium; ET-1, endothelin-1; ERK, extracellular signal-regulated kinase; IGF-I, insulin-like growth factor-I; IGF-IR, IGF-I receptor; InsPs, inositol phosphates; MAPK, mitogen-activated protein kinase; M199, medium 199; MBP, myelin basic protein; MEK, ERK activating kinase; nPKC, novel PKC; PD, PD-098059; PdtInsP2, polyphosphoinositides; PI3-K, phosphatidylinositol 3-kinase; PLC, phospholipase C; PKC, protein kinase C; PS, phosphatidylserine; Raf, MEK activating kinase; PMA, 12-*O*-tetradecanoylphorbol-13-acetate.

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Insulin-like growth factor-1 (IGF-1) regulates several pleiotropic cellular responses and mediates the anabolic and cardiovascular effects of growth hormone *in vivo* (1, 2). Although IGF-1 controls apoptosis and promotes hypertrophy by growth and differentiation in many types of cells (3), we know little about its mechanism of action on cardiac myocytes where IGF-1 may act in an autocrine or paracrine manner (4).

Cardiac remodeling in response to mechanical and chemical stimuli classically implies the hypertrophy of cardiomyocytes and proliferation of non-muscle cells (5, 6). Whether the heart responds to an increase in workload by both hypertrophy and hyperplasia is, however, still controversial (7). Apoptosis occurs in the myocardium in a variety of pathological situations (8–12). It has been recently proposed that remodeling of heart requires an apoptotic phase followed, or paralleled by, cell growth (10).

Clinical and experimental studies have shown that IGF-1 participates in the initiation and development of left ventricular hypertrophy (13–16). The selective expression of IGF-1 in cardiac myocytes leads to a physiological, then pathological, cardiac hypertrophy in transgenic mice (17). IGF-1 also preserves ischemic myocardium from reperfusion injury (18) and protects against cardiac necrosis and inhibition of reperfusion-induced apoptosis of cardiac tissue (19). We have recently shown that IGF-1 prevents cardiomyocyte apoptosis induced by hyperosmotic stress (20). Because IGF-1 might be a critical factor for the function and survival of cardiomyocytes, the elucidation of the molecular mechanisms are of practical interest.

The mitogen-activated protein kinase (MAPK) family is important in signal transduction and is activated by growth factors and cellular stresses. Among the MAPK family, the extracellular signal-regulated ki-

nases (ERKs), c-Jun NH_2-terminal kinases (JNKs) and p38 MAPKs, have been well characterized (reviewed in Ref. 21). In the terminally-differentiated cardiomyocyte, some hypertrophic agonists such as endothelin-1, bradykinin and α_1-adrenergic agonists promote an activation of the ERK cascade (21). This activation is mediated at least in part through stimulation of membrane phospholipid hydrolysis, and activation of the diacylglycerol-sensitive isoforms of protein kinase C (PKC) (21).

IGF-1 activates multiple signal transduction pathways in cardiac myocytes (22–25) and some of these (such ERK, phosphatidylinositol 3-kinase-Akt and JAK-STAT3) may be relevant to the hypertrophic and apoptotic responses of the heart (26). It is unclear, however, whether the ERK and PKC signal pathways are involved in the antiapoptotic actions of IGF-1 on cardiac myocytes. We have examined the participation of ERK and PKC on the regulation of apoptosis process in cultured cardiomyocytes.

MATERIALS AND METHODS

Materials. Dulbecco's modified Eagle's medium (DMEM), medium 199 (M199), protease inhibitors, phosphatidylserine (PS), diolein (DO), sorbitol (SOR), proteinase K, RNAse, agarose, genistein and other biochemicals were purchased from Sigma (St. Louis, MO) unless stated otherwise. [γ-^{32}P]ATP and [^{3}H]myoinositol were from NEN Life Science Products (Boston, MA). Bisindolylmaleimide-I (BIM) and PD-098059 (PD) were from Calbiochem (La Jolla, CA). AG1x8 ion-exchange resin (formate form) and protein assay reagents were from Bio-Rad Lab. (Hercules, CA). Phosphocellulose paper P81 was from Whatman (Maidstone, Kent, UK). Culture dishes were from Nalgene NUNC International (Naperville, IL) and heat-inactivated fetal calf serum (FCS) and other tissue culture products were from Life Technologies Inc. (Gaithersburg, MD). Affinity-purified antibodies raised against PKC-α, PKC-β, PKC-δ, PKC-ε and PKC-ζ, and the corresponding peptides and [Ser^{25}]-PKC (19–31) were from Gibco BRL Co. (Gaithersburg, MD). Anti-murine ERK antibody, which recognize both phosphorylated and unphosphorylated ERK-2 was given by Professor C. J. Marshall (Chester Beatty Laboratories, Institute of Cancer Research, London, UK). Apoptosis detection system was from Promega (Woods Hollow Road, Madison, WI). ECL immunoblotting detection reagents, autoradiographic film and pre-stained molecular mass standard proteins were from Amersham International. Human recombinant IGF-1 was donated by Dr. C. George-Nascimento (Chiron Corp., CA).

Culture and treatment of cardiac myocytes. Neonatal ventricular myocytes were prepared from hearts of 1-3 day-old Sprague–Dawley rats (Animal Breeding Facility from the Faculty of Chemical and Pharmaceutical Sciences, University of Chile) as described previously (22). The myocytes, plated at a final density of 1.0–1.4 $\times$ 10^3/mm^2 on gelatin-precoated 35- or 60-mm dishes, respectively, were confluent and spontaneously beating after 18 h. Serum was withdrawn for 24 h before the cells were further treated with IGF-1 (0–100 nM for 0–60 min) in serum-free medium (DMEM-M199) at 37°C.

Agonist-stimulated hydrolysis of polyphosphoinositide (PdtInsP2) in cultured cardiomyocytes. Myocytes (1.4 $\times$ 10^5 cells/mm^2, 35-mm dishes) were incubated with serum-free medium (2 mL) containing 5 μCi of [^{3}H]myoinositol for 24 h. They were then exposed to IGF-1 in serum-free medium containing 1 mM LiCl. The incubation was terminated by the removal of medium and addition of 0.8 M $HClO_4$ (0.5 mL). Each plate was scraped and washed with a further 0.5 mL of 0.8 M $HClO_4$. The $HClO_4$ extracts were combined and precipitated protein was removed by centrifugation at 10,000*g* for 10 min at 4°C; the supernatant fractions were neutralized with 5 M KOH/0.5 M Tris base and were recentrifuged. The resulting supernatants were applied to columns of AG1x8 ion-exchange resin (formate form). Pooled inositol mono-, bis- and tris-phosphates were purified essentially as described in (27).

Preparation of the soluble and particulate fractions of cardiomyocytes. Cells were washed three times in 1 mL of ice-cold PBS and lysed in 150 μL of buffer A (12.5 mM Tris–HCl, 2.5 mM EGTA, 1 mM EDTA, 5 mM dithiothreitol, 100 mM NaF, 300 μM PMSF, 120 μM pepstatin A, 10 μM trans-epoxysuccinyl-L-leucylamido(4-guanidino)-butane, 200 μM leupeptin, pH 7.4) containing 0.05% (w/v) digitonin (28). Extracts were incubated for 5 min at 4°C. For the determination of subcellular distributions, extracts were centrifuged at 10,000*g* for 15 min at 4°C. The soluble fraction was retained. The particulate fraction was washed with 150 μL of buffer A, centrifuged and finally resuspended in the same volume of buffer A containing 1% (v/v) Triton X-100. Protein concentrations were determined by the Bradford method (29).

PKC activities. PKC activity was determined by measurement of the incorporation of ^{32}P from [γ-^{32}P]ATP into [Ser^{25}]-PKC (19–30) (30). Samples (5 μL of cell fraction, containing 3-5 μg of protein) were incubated in a final volume of 25 μL at 30°C in a shaking bath for 10 min. The reaction mixture contained: 1 μM [Ser^{25}]-PKC (19–31), 10 mM $MgCl_2$, 20 mM Tris–HCl (pH 7.5) and 24 μM ATP (0.25 μCi [γ-^{32}P]ATP), 100 μM $CaCl_2$, 125 μg/mL phosphatidylserine (PS) and 50 μg/mL 1,2-diolein (DO) for the assessment of cPKC (classical PKC). Ca^{2+} was omitted in this reaction mixture for the measuring of nPKC (novel PKC). Lipids were added to the reaction mixture as freshly-sonicated dispersions. Control assays for assessment of the activities of Ca^{2+}- and phospholipid- independent kinases were routinely performed; in these $CaCl_2$, PS and DO were replaced by 2 mM EGTA. All assays were performed in duplicate. PKC activity was measured under conditions in which the enzyme activity is a linear function of enzyme concentration and incubation time. Activity is expressed as U/mg protein. One unit (U) is that amount of enzyme which catalyzes the incorporation of 1 nmol of ^{32}P into [Ser^{25}]-PKC (19–31) per minute at 30°C (pH 7.5). Calcium- and phospholipid-dependent PKC activity was defined as the difference between the ^{32}P incorporation in the presence and absence of PS/DO and Ca^{2+}. Phospholipid-dependent PKC activity was defined as the difference between the ^{32}P incorporation in the presence and absence of PS/DO. After incubation, 20 μL of the reaction mixture was adsorbed onto P81 phosphocellulose paper filters (1 $\times$ 2 cm). Filters were washed twice for 15 min each in 75 mM H_3PO_4. Filters were dried and radioactivity was measured by liquid scintillation spectrometry.

Immunowesternblot analysis. Soluble fractions from myocytes were heated at 95°C with 0.33 vol. of SDS–PAGE sample buffer while particulate fractions were solubilized by heating in a volume of SDS–PAGE sample buffer equal to the volume of the original extract. Proteins (20–90 μg) were separated by SDS–PAGE on a 10% polyacrylamide gel and were transferred electrophoretically to nitrocellulose. Nonspecific binding sites were blocked with 5% (w/v) nonfat milk powder in PBS (pH 7.5) containing 0.05% (v/v) Tween 20 (PBST) for 60 min at room temperature. Primary antibodies were diluted in blocking solution (1:200 for anti-PKC isoenzymes and 1:100 for anti-ERK). Nitrocellulose was incubated with primary antibodies overnight at 4°C. After washing in PBST (3 times for 10 min each), nitrocellulose was incubated for 1.5 h at room temperature with horseradish peroxidase-linked secondary antibody (1:4000 in 1% (w/v) nonfat milk powder in PBST). After repeating the washing procedure described above, bound antibody was detected by ECL with exposure to Hyperfilm for 0.5–30 min. Immunoblots were quantified by scanning densitometry.

Measurement of ERK phosphorylation state. IGF-1-stimulated phosphorylation of endogenous ERK2 was determined from the shift in electrophoretic mobility as described (31), using an anti-murine ERK antibody which recognize both phosphorylated and unphosphorylated ERK-2.

Induction and prevention of cardiomyocytes apoptosis. Cardiomyocytes cultured in DMEM-M199 supplemented with 5% FBS and 10% FCS, were washed extensively with DMEM-M199 without serum. Then BIM (100 nM) or PD (100 μM) was added and incubated for 30 min at 37°C before incubation for 1 h with IGF-1 (100 nM). The cells were washed 3 times with sterile PBS and exposed for 24 h with or without 0.3 M sorbitol, IGF-1 and the inhibitors. Cells cultured in DMEM-M199 containing 5% FBS and 10% FCS served as control.

Cell viability. The number of viable cardiomyocytes was determined with trypan blue exclusion (20). Briefly, cells were rinsed once with PBS and the resuspended with trypsin and EDTA. The cells were immediately stained with 0.5% trypan blue, and the number of viable and nonviable cardiomyocytes was determined.

Detection of DNA ladder formation in apoptotic cultured cardiomyocytes. For the detection of DNA fragmentation, cardiomyocytes were washed 3 times with cold PBS and sedimented by centrifugation. Cellular DNA was prepared by scraping the cells into a 1 mL of lysis buffer consisting of 0.8 mM EDTA (pH 8.0), 8 mM Tris–HCl (pH 8.0) and 4% SDS. The DNA was extracted with an equal volume of phenol–chloroform–isoamyl alcohol (25:24:1) followed by centrifugation at 12,000*g* for 15 min at 4°C. The resulting DNA was incubated with proteinase K (50 μg/mL, Sigma) for 1 h at 50°C to facilitate membrane and protein disruption. DNA was reextracted from supernatants with an equal volume of phenol–chloroform–isoamyl alcohol (25:24:1). DNA was precipitated from the upper aqueous phase using 0.1 vol of 3 M sodium acetate with 2.5 vol of ice-cold ethanol and left at −20°C for 60 min before centrifugation. Pellets were resuspended in 200 μl TE buffer, followed by a 60-min incubation with DNase-free RNase A (2 mg/mL, Sigma) at 37°C. Samples were reextracted, and DNA was precipitated as described above. Pellets were resuspended in TE buffer, and DNA concentrations were quantified by measuring absorbance at 260 nm. DNA samples were analyzed by electrophoresis on 2% agarose, and visualized by staining with a solution containing 0.2 μg/mL of ethidium bromide.

Detection of apoptotic cardiomyocytes by the TUNEL method. Apoptotic cardiomyocytes were detected by the dideoxynucleotide transferase (TdT)-mediated X-dUTP nick end labeling (TUNEL) method using as *in situ* detection kit (Promega) according to the manufacturer's recommended protocol and as described in (20).

Expression of results and statistical methods. Results are expressed either as means ± SEM for the number of independent experiments indicated (n) or as examples of representative experiments performed on at least two or three separate occasions. Time-course and dose-dependent analysis were performed using ANOVA, and comparisons between groups were performed using a protected Tukey's t test.

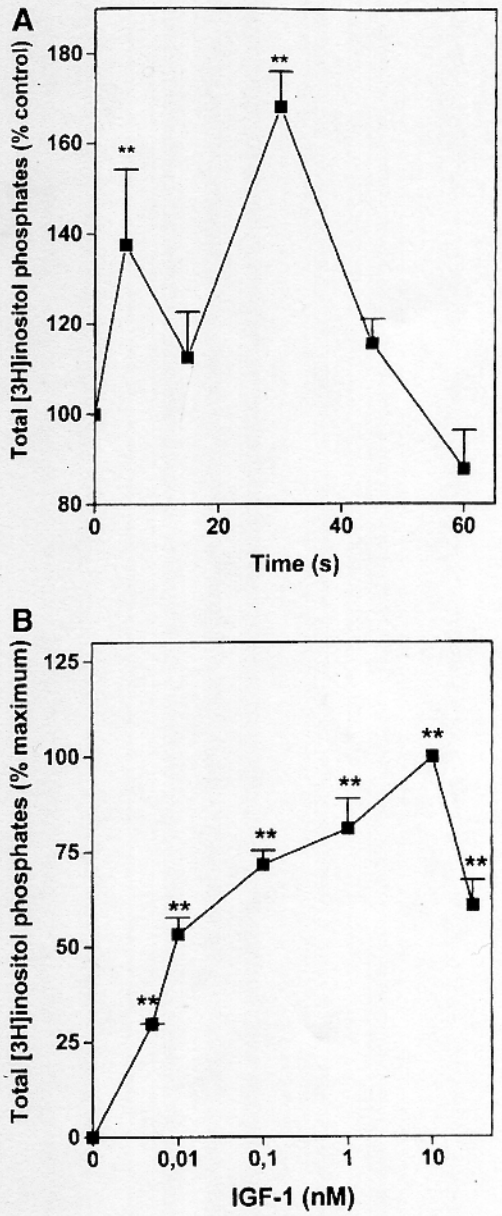

FIG. 1. Stimulation of phosphoinositide turnover by IGF-1 in cultured cardiomyocytes. Confluent cardiac myocytes were prelabeled with [^{3}H]inositol for 24 h, exposed to 10 nM IGF-1 for the indicated times (A) or to various concentrations of IGF-1 for 30 s (B). Phospho[^{3}H]inositide hydrolysis was measured as described under Materials and Methods. Each value is the mean ± SEM from four independent experiments. $^{**}P < 0.01$ vs control.

RESULTS

Effect of IGF-1 on phosphoinositide hydrolysis in cardiomyocytes. The effect of IGF-1 on PdtInsP2 hydrolysis is shown in Figs. 1A and 1B. Changes in PdtInsP2 hydrolysis by IGF-1 (10 nM) were biphasic (Fig. 1A) and rapid (maximum of 170% of control by 30 s). After 60 s, the content of inositol phosphates had returned to basal values. The effect of IGF-1 was concentration-dependent, with a EC_{50} value of 0.01 nM (Fig. 1B).

Effect of IGF-1 on PKC in cardiomyocytes. Figures 2A and 2B depict the changes in the distribution of cPKC and nPKC activities in particulate and soluble fractions after exposure of cardiomyocytes to 10 nM IGF-1. The activities of the particulate forms increased sharply reaching maximum values (twofold above control values) at 2–5 min, and then decrease to reach basal levels after 15 min. In contrast, activity in the soluble fraction increased to a maximum at 15 min

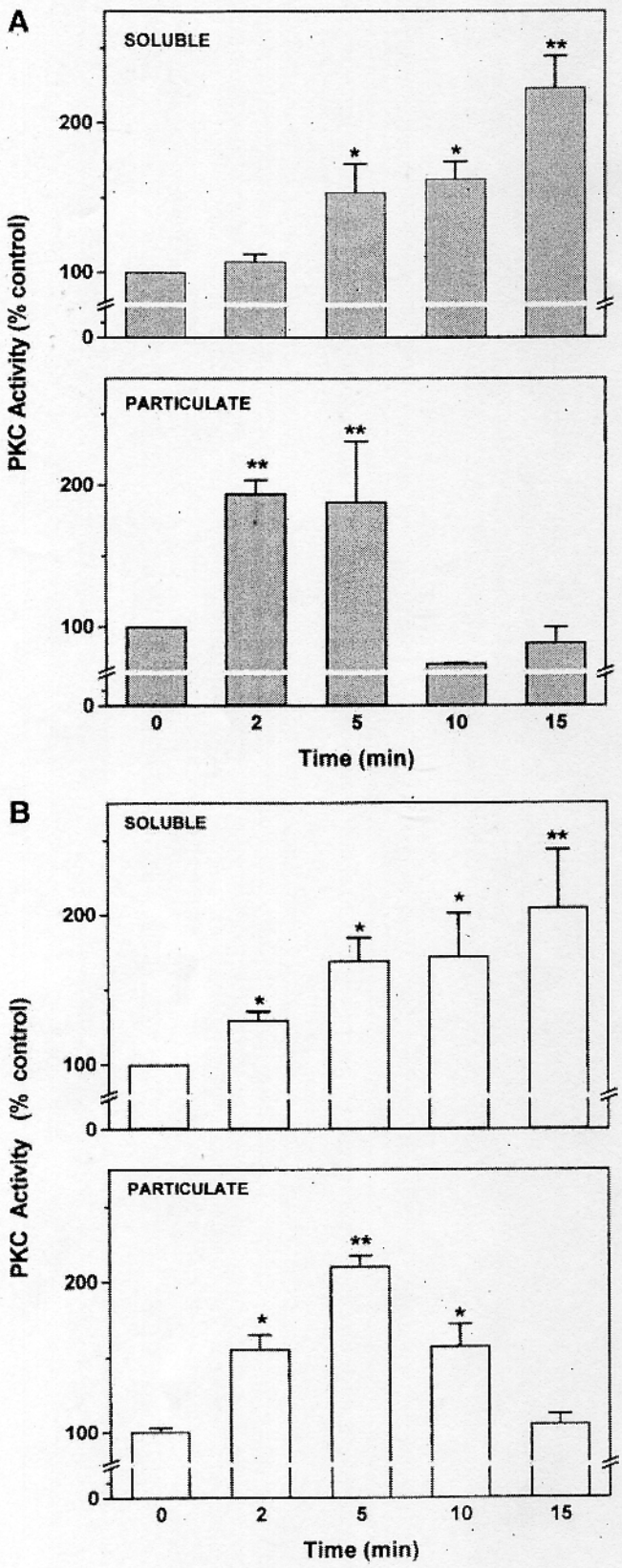

FIG. 2. Stimulation of the activities of PKC activities by IGF-1 in cultured cardiomyocytes. Cells were stimulated with 10 nM IGF-1 for the times indicated, and soluble (A and B, upper) and particulate (A and B, bottom) fractions were prepared as described under Materials and Methods. Kinase activity with [Ser^{25}]-PKC (19–31) as substrate was assayed in the absence or presence of PS, DO and Ca^{2+} (A for classical PKCs, cPKC) or in the absence or presence of PS and DO (B for novel PKCs, nPKC) both in the soluble and particulate fractions as described under Materials and Methods. Results are means ± SEM of four separate experiments. The specific activities at time zero of nPKC were 143 (soluble) and 292 (particulate) and of cPKC were 150 (soluble) and 300 (particulate), respectively; all values are units per mg protein. $^*P < 0.05$ and $^{**}P < 0.01$ vs time zero.

(200% of control), decreasing after 60 min (data not shown). We used immunoblot analysis with specific polyclonal antibodies to distinguish the different PKC isoforms and study the effect of IGF-1 on their subcellular redistribution in neonatal cardiomyocytes. We were able to detect PKCs α, δ, ε, and ζ in lysates of cardiomyocytes and their molecular masses of PKCs α, δ, ε, and ζ were 80, 76, 96, and 78 kDa, respectively. We observed rapid translocation of PKCs α (Fig. 3A), δ (Fig. 3B), and ε (Fig. 3C), but not ζ (data not shown), from the soluble to the particulate fraction in cardiomyocytes exposed to IGF-1. Translocation of isoenzymes PKC-α, PKC-δ and PKC-ϵ was almost completely reversed after 30, 15, and 5 min, respectively.

Effect of IGF-1 on activity of ERK in cardiomyocytes. ERK is primarily cytosolic in unstimulated cells. During exposure to 10 nM IGF-1 for 5 min, ERK is translocated from the cytoplasm to the nucleus (data not shown). This effect was blocked by preincubation with the protein-tyrosine kinase inhibitor genistein. IGF-1 stimulates ERK-1 and ERK-2 tyrosine phosphorylation (data not shown). As depicted in Fig. 4, ERK-2 was phosphorylated following activation of IGF-1R, its mobility on SDS–PAGE was slightly decreased. Phosphorylation of ERK-2 (Fig. 4A) was maximal within 5 min, reaching approximately 40% of that induced by 1 μM PMA (the best known stimulator of ERK). The anti-ERK2 antiserum used in this study cross-reacted better with ERK-2 than with ERK-1. The EC_{50} value for the phosphorylation of ERK-2 by IGF-I was approximately 0.05 nM (Fig. 4B).

Effect of PKC and ERK inhibitors on the preventive action by IGF-1 in hyperosmotic stress-induced cardiomyocyte apoptosis. We first examined whether sorbitol induced apoptosis of cardiomyocytes. As shown in Fig. 5A, when cultured cardiomyocytes were exposed to 0.3 M sorbitol for 24 h, their viability decreased significantly. This effect was partially prevented by IGF-1 (Fig. 5A). By 12 h myocytes exhibited shriveled, constricted shape and many floating, round cells were observed. There were few adherent cells remaining at 24 h. In contrast, myocytes incubated in DME-M199 containing serum or IGF-1 were confluent, well spread with flattened morphology and beat rapidly. To deter-

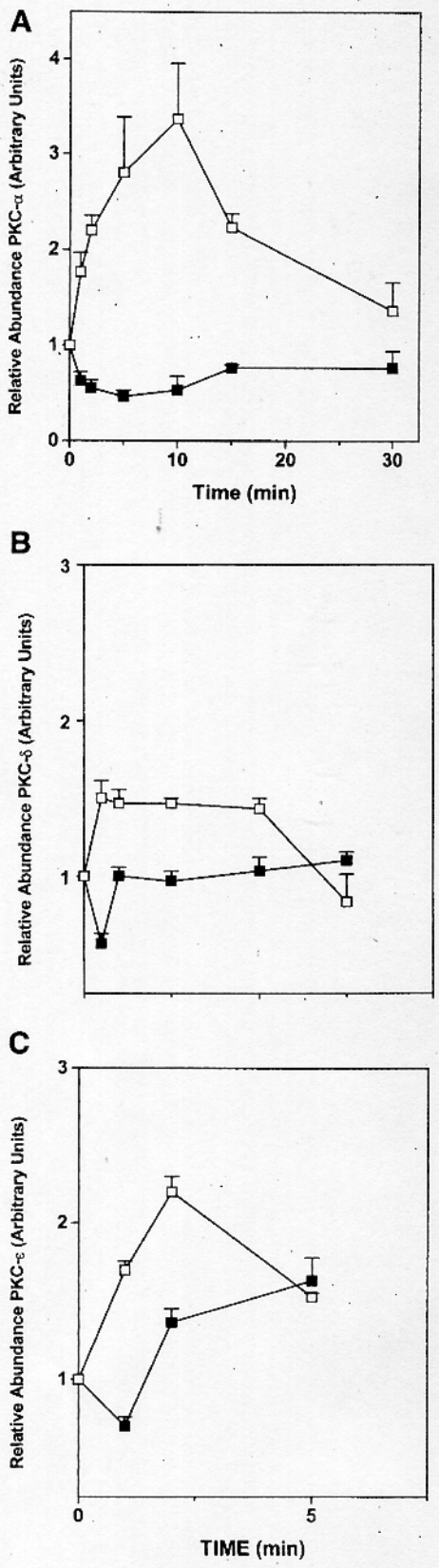

FIG. 3. Stimulation of the translocation of PKC isoforms from the soluble to the particulate fraction by IGF- 1 in cultured cardiomyocytes. Cells were exposed to 10 nM IGF-1 for the times indicated. Soluble and particulate fractions were prepared as described under Materials and Methods. For SDS–PAGE, protein loading was 40 μg per lane for soluble fractions and 90 μg per lane for particulate fractions. After SDS–PAGE and transfer of protein to nitrocellulose, immunoblotting with antiserum against specific PKC isoforms was performed as described under Materials and Methods with ECL detection. Immunoblots of denatured soluble and particulate fractions were scanned by laser densitometry. Immunoreactive PKC-α (A), PKC-δ (B) and PKC-ϵ (C) present in the soluble (solid square) and particulate (open square) fractions of cardiomyocytes exposed to IGF-1 for the times shown is expressed (mean ± SEM, n = three independent preparations of myocytes) of a relative abundance of the zero time control.

mine whether the above morphological changes were associated to apoptosis, nuclear DNA fragmentation was assessed using the TUNEL method and DNA gel electrophoresis (Figs. 5B and 5C). After incubation with 0.3 M sorbitol for 24 h, the number of TUNEL-positive cardiomyocytes was increased from 4.6 to 57% (Fig. 5B). Intranucleosomal DNA fragmentation in sorbitol-treated cells on 24 h was confirmed by the consistent observation of characteristic DNA ladders on agarose gels (Fig. 5C). Serum deprivation alone did not induce apoptosis in the time examined. Taken together, these results indicate that exposure of cardiac myocytes to 0.3 M sorbitol for 24 h was associated with the development of an apoptotic process. IGF-1 (100 nM) significantly prevented this sorbitol-induced cardiomyocyte apoptosis (Figs. 5B and 5C). Finally we examined the role of PKC and ERK on cardiac myocyte apoptosis using selective and specific chemical inhibitors. PD-098059 inhibited MEK and IGF-1 dependent-activation of ERKs while BIM abolished activation of both cPKC and nPKC by IGF-1 in cardiac myocytes (data not shown). When the ERK and PKC signaling pathways were blocked by pre-treatment with PD-098059 or BIM for 1 h, antiapoptotic action of IGF-1 on cardiomyocyte was only and completely antagonized by PD-098059 (Figs. 5A–5C). In contrast, PKC does not involve in the antiapoptotic effect of IGF-1 in cardiac myocytes. BIM did not change either the number of viable cells (Fig. 5A) or the number of TUNEL-positive cardiomyocytes when they were pre-exposed to IGF-1 before their treatment with sorbitol (Fig. 5B).

DISCUSSION

We have showed here that IGF-I causes rapid hydrolysis of PdtInsP2, a differential activation of PKC isoenzymes and stimulation of ERK activity in cultured rat cardiac myocytes. The exposure of these cells to sorbitol (hyperosmotic stress) induced apoptosis which was prevented by IGF-1. The antiapoptotic effect of IGF-1 on hyperosmotic stress-induced DNA fragmentation and cardiomyocyte survival were mediated by ERK, but not by PKC, in cultured cardiac myocytes.

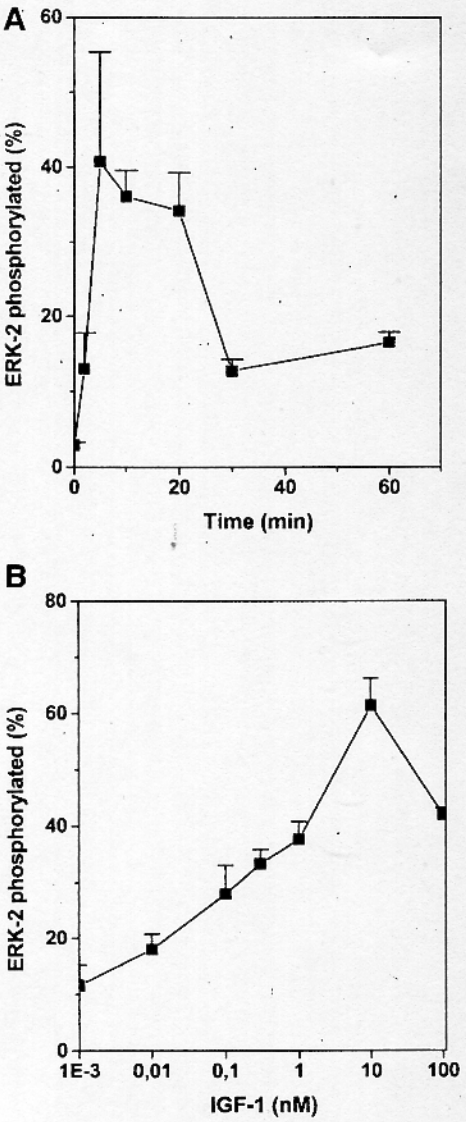

FIG. 4. Time course and dose dependence for phosphorylation of ERK-2 in cultured cardiomyocytes exposed to IGF-1. Cardiac myocytes in serum-free medium were stimulated with 10 nM IGF-1 or 1 μM PMA (positive control) for the times indicated at 37°C (A) or were treated for 5 min at 37°C with increasing concentrations of IGF-I or 1 μM PMA (B). Denatured soluble fractions were prepared and 20 μg protein/lane was subjected to SDS–PAGE and immunoblot analysis as described under Materials and Methods. A and B show results of ERK-2 phosphorylation obtained by laser-scanning densitometry of the immunoblots. Results (means ± SEM, n = 4–5 separate experiments) are expressed as percentage of ERK activity in extracts from cells treated with 1 μM PMA for 5 min (maximal activation).

Apoptosis is a form of cell death induced by a variety of stresses, including hyperosmotic shock (20). Apoptosis of cardiac myocytes may contribute to arrhythmias, progressive pump failure and cardiac remodeling. Mechanisms responsible for the death of cardiac tissue cells in many heart diseases have been of increasing interest in recent years (10). Because cardiac myocytes are terminally differentiated and have lost the ability to proliferate (6), the modulation of cardiac myocyte death through suppression of apoptotic signaling pathways represents a potential strategy to the development of cardioprotective agents. Evidences have shown that IGF-1 is a critical factor for the survival of cultured cardiac myocytes exposed to different apoptotic stimuli (20, 23). The relative importance of both intracardiac systems (such as renin-angiotensin, adrenergic and growth factors) and mechanical stimuli in the growth and apoptosis of myocardial cells remains unquantified (33–35). One hypothesis is that mechanical stimuli may be primary for the production of different hypertrophic and apoptotic agonists in cardiac tissue (36, 37). Although angiotensin II and ET-1 are synthesized in stretched cardiac myocytes *in vitro* (36), there is no evidence that the same is true for IGF-1 (an hypertrophic and antiapoptotic agent = Increased IGF-1 gene expression is, however, associated with the phenotypic adaptation and hypertrophy found in rabbit skeletal muscle subjected to stretch (38). Clinical and experimental evidence suggests that IGF-1 induces cardiac hypertrophy and attenuates cardiac apoptosis *in vivo* and *in vitro* (13–16, 20, 39, 40). Cardiomyocytes contain functional IGF-1 receptors (13, 22), the abundance of which may be instrumental in the growth-promoting effects of IGF-1 (41). We have previously reported that IGF-1 activates multiple signal transduction pathways in cultured cardiomyocytes, including the sequential activation of the Raf-MEK-ERK cascade (22). We have showed here that IGF-1 stimulates PdtInsP2 turnover in cardiomyocytes in agreement with ref. (42), and that this is concentration- and time-dependent. The accumulation of InsPs was transient and biphasic—contrasting with the effects of other G protein-coupled receptor agonists in cardiac myocytes (43). This differential effect may be explained by the selective activation of PLCs. In heart, the most abundant is PLC-γ, followed by PLC-β and PLC-δ (44). We have previously shown that, in cardiomyocytes, an early event after IGF-1R activation is an increased phosphorylation of PLC-γ, and that this may be responsible for the IGF-1-induced PdtInsP2 hydrolysis (22). Activation of the PdtInsP2 cycle results, through stimulation of PLC, in increased amounts of Ca^{2+} and DAG, both potent activators of PKC.

Some studies have shown a role of PKC in the induction of cardiac hypertrophy (reviewed in 6 and 45) and in the protection of ischemia-reperfusion-induced apoptosis (46). There are conflicting observations, however, about the involvement of PKC in apoptosis depending on cell type (47). There are no studies on the relationship between PKC and IGF-1 in cardiomyocytes and only a few in adipocytes (48) and 3T3 cells (49). In our system, activation of IGF-1R by IGF-1 leads to the translocation of PKCs α, δ, and ε and to an increase in the activity of both cPKC and nPKC subfamilies. Because our assays do not measure atypical

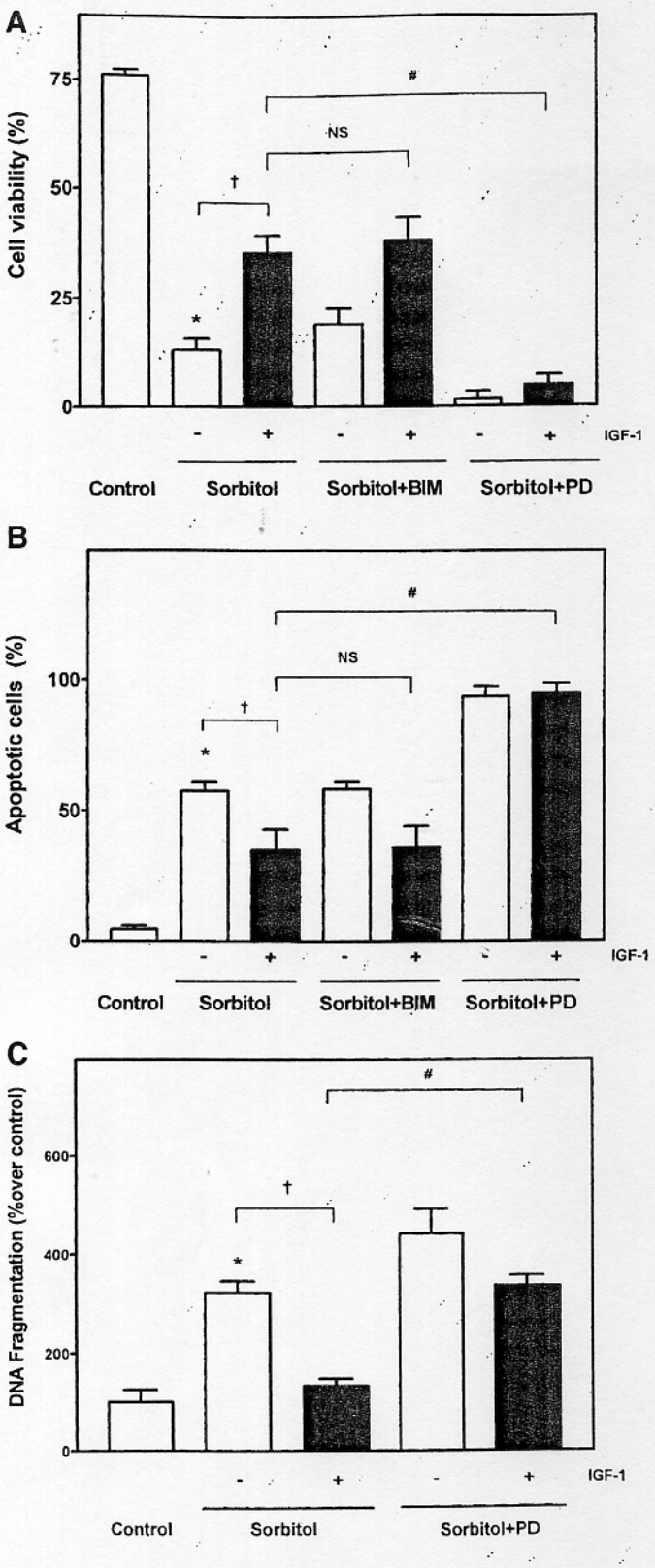

FIG. 5. Effect of PKC and MEK inhibitors on the antiapoptotic action of IGF-1 in cultured cardiomyocytes. Cells were preincubated sequentially in medium with or without 100 nM BIM or 100 μM PD-09850 for 30 min and then with 100 nM IGF-1 for 1 h. After, cells were washed with PBS and the medium was replaced by 0.3 M sorbitol in the presence or absence of inhibitors and IGF-1. Control cells were incubated in medium containing 5% FBS and 10% FCS. After 24 h of exposure, cell viability was determined by trypan blue exclusion (A) or nuclear DNA fragmentation was assessed using DNA gel electrophoresis (B) or by the TUNEL method (C) as described under Materials and Methods. The number of apoptotic cells was determined and expressed as a percentage of the total myocyte population. Results are means ± SEM of, at least, three independent experiments. *$P < 0.05$ vs control, †$P < 0.05$ vs sorbitol, #$P < 0.05$ vs sorbitol + IGF-1.

PKCs, such as PKC-ζ (50), we cannot discount the possibility that this isoenzyme might be activated by IGF-1 in cardiomyocytes. A role for this isoenzyme has been proposed for the IGF-1-induced mitogenic signaling cascade in brown adipocytes (48) and Standaert *et al.* have suggested that insulin activates PKC-ζ through PI3K, and that PKC-ζ may act as a downstream effector of PI3K in rat adipocytes (50). An understanding of the role of individual PKC isoenzymes has been hindered by the lack of isoenzyme-selective chemical inhibitors. Assessment of the contribution of each PKC isoenzyme in IGF-1 signal transduction in cardiomyocytes awaits the availability of new more specific inhibitors. Our results showed that PKC does not mediated the antiapoptotic action of IGF-1 in cardiac myocytes. Future research will be need it to investigate the role of PKC in cardiac myocyte hypertrophy induced by IGF-1.

We also showed that IGF-1 activates ERKs, stimulates its translocation to the nucleus, and could initiate the activation of nuclear proto-oncogenes in cardiomyocytes. The ERK cascade is activated in these cells by different stimuli (fibroblast growth factors, ET-1, α_1-adrenergic agonists, phorbol esters, cardiotrophin, and by stretching) (51–54). We showed here that ERK protected cardiomyocytes from sorbitol-induced apoptosis—in agreement with other recent reports which suggest that survival of cardiac myocyte depends on this signaling pathway (53, 55). Parrizas *et al.* have also showed that IGF-I receptor activation prevented apoptosis via the ERK pathway in neurons (56). Previous studies have indicated that the juxtaglomerular region of the cytoplasmic domain of the IGF-1 receptor β-subunit binds both Shc and the various isoforms of the IRS-1 family of proteins. Both IRS proteins and Shc proteins contain SH2 domains that bind to the activated receptor. After binding to the receptor, these proteins become phosphorylated on tyrosine residues by the IGF-1 receptor. Grb2, an adapter protein, binds both Shc and IRS via its SH2 domain and via its Sh3 domain to mSOS. MSOS is a guanine nucleotide-exchange protein that loads GTP onto the small G protein Ras, and thereby activates the Ras/Raf/MAP kinase pathway. Tyrosine phosphorylated IRS proteins bind to the regulatory subunit (p85) of PI3K via its SH2 domain and p85 bind the catalytic subunit of PI3K

via its SH3 domain. ERK and PI3K have been identified as playing important role in IGF-1R-induced cellular proliferation and apoptosis. Using cells that are do not express IRS-1 or IRS-2 (32D cells), Dews *et al.* have established that that in the absence IRS molecules, IGF-1R activation of the ERK pathway persisted (57). They also demonstrated that a quartet of serine residues, located at 1280-1283 in the C-terminal domain, along with tyr^{950} of the IGF-1R are critical for maximal activation of ERK pathway by IGF-1 (57). 14-3-3 proteins have been proposed as obvious candidates to signal ERK pathways because certain 14-3-4 isoforms activate Raf and bind to this region (58).

In conclusion, we have shown that the exposure of cultured neonatal rat cardiomyocytes to IGF-I causes rapid hydrolysis of PdtInsP2, a differential activation of PKC isoenzymes and activation and phosphorylation of ERK activity accompanied by subcellular redistribution. The antiapoptotic effect of IGF-1 on hyperosmotic stress-induced DNA fragmentation and decreasing in cardiomyocyte survival were mediated by ERK in cardiac myocytes. However, PKC activation did not mediate the antiapoptotic effect of IGF-1 in our experimental model of programmed cell death. These data complement our previous finding that activation of IGF-1 receptors stimulates multiple signaling pathways in cardiac myocytes and also suggest that the ERK, but not PKC, pathway may therefore be an important antiapoptotic component in the mechanism whereby IGF-1 exerts its effects.

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