Calmodulin Kinases II and IV and Calcineurin Are Involved in Leukemia Inhibitory Factor–Induced Cardiac Hypertrophy in Rats

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Abstract—We recently reported that leukemia inhibitory factor (LIF) enhances \( \text{Ca}^{2+} \), through an increase in L-type \( \text{Ca}^{2+} \) current \( (I_{\text{Ca,L}}) \) in adult cardiomyocytes. The aim of this study was to investigate whether LIF activates \( \text{Ca}^{2+} \)-dependent signaling molecules, such as calcineurin and calmodulin kinases II and IV (CaMKII and CaMKIV), and, if so, whether these \( \text{Ca}^{2+} \)-mediated signaling events contribute to LIF-mediated cardiac hypertrophy. We first confirmed that LIF increased \( I_{\text{Ca,L}} \) and \[ \text{Ca}^{2+} \], in primary cultured rat neonatal cardiomyocytes. Calcineurin, CaMKII, and CaMKIV activities increased at 2 minutes and peaked by 1.6-, 2.2-, and 2.2-fold, respectively, at 15 minutes. Nicardipine or verapamil fully inhibited these activities. Autophosphorylation of CaMKII was also observed to parallel the timing of CaMKII activity, and this phosphorylation was blocked by nicardipine, verapamil, or EGTA. LIF treatment led to a 3-fold increase in nuclear factor of activated T cell–luciferase activity. To confirm that inositol triphosphate (IP₃)-induced \( \text{Ca}^{2+} \) release from sarcoplasmic reticulum was not involved in this process, IP₃ content and phosphorylation of phospholipase C\( \gamma \) were investigated. LIF did not increase IP₃ content or phosphorylate phospholipase C\( \gamma \). KN62 (an inhibitor of CaMKII and CaMKIV) attenuated c-fos, brain natriuretic peptide, \( \alpha \)-skeletal actin, and atrial natriuretic peptide expression. KN62 suppressed the LIF-induced increase in \[^{3}H\]phenylalanine uptake and cell size. Cyclosporin A and FK506 slightly attenuated brain natriuretic peptide but did not affect c-fos or atrial natriuretic peptide expression. Cyclosporin A significantly reduced the LIF-induced increase in \[^{3}H\]phenylalanine uptake. These findings indicated that LIF activated CaMKII, CaMKIV, and calcineurin through an increase in \( I_{\text{Ca,L}} \) and \[ \text{Ca}^{2+} \], and that CaMKII, CaMKIV, and calcineurin are critically involved in LIF-induced cardiac hypertrophy. (Circ Res. 2000;87:937-945.)

Key Words: leukemia inhibitory factor ■ calcium ■ calmodulin-dependent kinase ■ calcineurin ■ cardiac hypertrophy

Leukemia inhibitory factor (LIF), a member of the interleukin-6 family of cytokines, has a potent hypertrophic effect on cardiomyocytes. However, the underlying molecular mechanisms that couple hypertrophic signals initiated at the cell membrane receptor to the reprogramming of cardiomyocyte gene expression remain poorly understood. We and others have demonstrated that the Janus kinase and signal transducers and activators of transcription pathway, mitogen-activated protein kinase (MAPK) pathway, and phosphatidylinositol 3 (PI3) kinase pathway were present downstream of gp130 in cardiomyocytes.1–4 We recently reported that LIF enhanced L-type \( \text{Ca}^{2+} \) current by 42% and \[ \text{Ca}^{2+} \], transient by 63% in cardiomyocytes.5 Interestingly, LIF-induced increases in L-type \( \text{Ca}^{2+} \) current and \[ \text{Ca}^{2+} \], transient took a unique time course, which gradually increased from 2 minutes and peaked at 15 minutes. Moreover, although the precise mechanism remains unknown, these increases were not mediated by protein kinase A (PKA) or protein kinase C (PKC) pathways.

There is a growing body of evidence to suggest that \( \text{Ca}^{2+} \) signaling plays an important role in the pathogenesis of cardiac hypertrophy and heart failure. Recently, the \( \text{Ca}^{2+} \)/calmodulin–dependent protein phosphatase calcineurin has attracted attention as a new signal transducer of hypertrophic stimuli in vitro and in vivo. Calcineurin dephosphorylates the nuclear factor of activated T cells (NFAT)-3 transcription factor, which is then translocated into the nucleus. Recently, Molkentin et al6 reported that NFAT-3, GATA-4, and calcineurin synergistically activate a marker gene of cardiac hypertrophy. In cultured cardiomyocytes, cyclosporin A (CsA), an inhibitor of calcineurin activity, inhibited angiotensin II–induced and phenylephrine-induced cardiac hypertrophy. Cardiac overexpression of the constitutively active form of calcineurin and NFAT-3 caused marked hypertrophy.
and heart failure in transgenic mice, which was blocked by CsA. Sussman et al.\(^7\) reported that CsA and FK506 prevent cardiac hypertrophy attributable to genetic perturbations in contractile proteins in hypertrophic cardiomyopathy model mice. However, they also reported that these blockers did not block hypertrophy attributable to overexpression of the constitutively active form of retinoic acid receptor.\(^7\) Moreover, the effects of CsA and FK506 remain controversial in pressure-overloaded cardiac hypertrophy models.\(^8\)–\(^12\) Thus, it remains to be determined whether the calcineurin pathway is universally implicated in cardiac hypertrophy in response to all kinds of hypertrophic stimuli.

Calmodulin-dependent kinase II (CaMKII) is an intracellular enzyme that was discovered several years ago in the brain. CaMKII is known to have several isoforms (α to δ), and the gene products are additionally divided into splicing variants.\(^13\) All α and β isoforms are found only in the brain, with the exception of the γ and δ isoforms, which have been detected in rat heart.\(^14\),\(^15\) CaMKII has also been implicated in the transduction of hypertrophic signals in cultured cardiomyocytes.\(^16\)–\(^18\) Expression of the 8B isoform of CaMKII specifically activates atrial natriuretic peptide (ANP) expression without inducing cellular hypertrophy.\(^19\)

Recently, Lu et al.\(^20\) reported that hypertrophic growth of cardiomyocytes in response to phenylephrine and serum is accompanied by muscle enhancer factor 2 (MEF2) activation through a posttranslational mechanism mediated by CaMKI, CaMKIV, and p38 MAPK, and these CaMks stimulate MEF2 activity by dissociating class II histone deacetylase from the DNA-binding domain. They concluded that these CaMks and p38 MAPK–dependent activation of MEF2 were endpoints for the hypertrophic stimuli in cardiomyocytes.

These findings suggested that calcium-regulated signaling molecules play a central regulatory role in coordinating the activities of multiple hypertrophic signaling pathways. On the basis of these findings, we investigated whether LIF activates the calcineurin, CaMKII, and CaMKIV pathways via an increase in 
\[\text{Ca}^{2+}\] transient in rat cardiomyocytes, and if so, whether this calcium-regulated signaling is involved in the hypertrophic response induced by LIF.

### Materials and Methods

#### Cell Culture

Primary cultures of cardiomyocytes were prepared from the ventricles of 1-day-old neonatal Wistar rats as described.\(^1\) Cells were stimulated with LIF 1000 U/mL.

#### Perforated Patch-Clamp Recording

Perforated patch-clamp recording was performed using gramicidin, as described in the online data supplement.

#### Measurement of the \([\text{Ca}^{2+}]\) Transient

The \([\text{Ca}^{2+}]\) transient was monitored by use of the fluorescent calcium indicator Fluo-4, as described in the online data supplement.

#### Measurement of CaMKII and CaMKIV Activity

Cells were collected in an assay dilution buffer and lysed by sonication. Anti-CaMKII (Santa Cruz) or CaMKIV (Transduction Laboratory) antibody and protein A Sepharose beads were added to the lysates and incubated overnight at 4°C. CaMKII activity was assayed with CaMKII assay kits (Upstate Biotechnology Inc) using a peptide substrate (KKALRQRQETVDAL) according to the manufacturer’s instructions. CaMKIV activity was assayed using a peptide substrate (KSDGGVKKRKSSSS).

#### Measurement of Calcineurin Activity

Calcineurin activity was determined by the protocol of Shibasaki and McKeon,\(^21\) with minor modifications. Cells were lysed in 400 μL of lysis buffer and freeze-thawed. After removing of cell debris, lysate was incubated in the assay buffer and \([\text{3}^\text{P}]\)-labeled RII peptide for 30 minutes at 30°C. The released \([\text{3}^\text{P}]\)-phosphate in 500 μL of supernatant was determined by Cherenkov’s method. Because the phosphatase activity using this assay buffer represents the mixed activity of PP2B and PP2C, we obtained the PP2B (calcineurin) activity by subtracting PP2C activity from the mixture activity. The PP2C activity was measured by a similar method using the same buffer by chelating Ca\(^{2+}\) with 5 mmol/L EGTA.

#### Immunoprecipitation (IP)-Western Blot Analysis

Antibody to phospholipase C (PLC)-γ\(_1\) was purchased from Santa Cruz Bioslabs. Monoclonal antibody to phosphotyrosine (4G10) was purchased from Chemicon International Inc. IP Western blot was performed as described.\(^1\)

#### Transfection and Luciferase Assay

Transient transfection was performed using Effectene transfection reagent (Qiagen). Within 24 hours after plating, cells were incubated with a transfection mixture with 0.24 μg of NFAT-luciferase reporter plasmids, 0.08 μg of plasmid encoding the constitutively active form of MEK1 (Stratagene), and 0.08 μg of pRL-SV40 (Promega) as an internal control plasmid. Total cell lysates were collected 6 hour after LIF stimulation, and luciferase activity was measured by Dual Luciferase reporter assay system (Promega).

#### Radioimmunoassay of Inositol Trisphosphate

Radioimmunooassay kit for inositol trisphosphate (IP\(_3\)) was purchased from Amersham Pharmacia. IP\(_3\) content was measured according to the manufacturer’s instructions.

#### Incorporation of \([\text{3}^\text{H}]\)Phenylalanine

The effects of LIF on \([\text{3}^\text{H}]\)phenylalanine uptake were determined as described.\(^1\) After 24 hours of serum depletion, cells were pretreated with or without KN62 or CsA, and then the cells were stimulated with LIF. Each data point was the mean of 5 separate experiments.

#### Immunofluorescence Microscopy and Cell Sizing Protocol

Cells grown on glass coverslips were permeabilized in cold methanol (1:1, −20°C) for 10 minutes and air dried. Immunofluorescence staining and measurement of the size (cell area, perimeter) were performed as described previously.\(^2\)

#### RNA Extraction and Northern Blot Analysis

Northern blots were performed as described.\(^2\) Rat ANP and brain natriuretic peptide (BNP) cDNA was obtained by reverse transcriptase–polymerase chain reaction from the heart RNA and cloned into the pCR II plasmid. Rat α-skeletal actin cDNA was provided by Hiroshi Ito (Tokyo Medical and Dental University, Tokyo, Japan). Rat GAPDH cDNA was used as an internal control.

#### Statistical Analysis

Values are presented as mean±SD. Statistical significance among mean values was evaluated with an ANOVA. Student’s \(t\) test was used when 2 values were compared. Differences were considered to be significant when \(P<0.05\).

An expanded Materials and Methods section can be found in an online data supplement available at http://www.circresaha.org.
Results

LIF Increased $I_{Ca,L}$ and $[Ca^{2+}]_i$ in Neonatal Rat Cardiomyocytes

Because our previous study used cultured adult rat cardiomyocytes, we first confirmed whether LIF also increases $I_{Ca,L}$ in neonatal rat ventricular myocytes. The points in the figure are peak inward current amplitudes at 0 mV every 30 seconds. Currents were elicited by the train protocol (described in the online data supplement available at http://www.circresaha.org). The solid bar on the top indicates the time of exposure to LIF (1000 U/mL). Current amplitude reached a maximum level at 10 to 15 minutes. The inset shows representative current traces before (a) and after (b) application of LIF at 15 minutes. B, Representative traces of Fluo-4 fluorescent intensity before (baseline, left) and after (right) 15 minutes of exposure to LIF (1000 U/mL). C, Percent changes in amplitude of the $[Ca^{2+}]_i$ with or without exposure to LIF. Values are mean±SEM of 10 experiments. ★P<0.05; ★★P<0.01 vs baseline.

Figure 1. LIF increased $I_{Ca,L}$ and $[Ca^{2+}]_i$ in neonatal rat cardiomyocytes. A, Time course of the effect of LIF on $I_{Ca,L}$ in neonatal rat ventricular myocytes. The points in the figure are peak inward current amplitudes at 0 mV every 30 seconds. Currents were elicited by the train protocol (described in the online data supplement available at http://www.circresaha.org). The solid bar on the top indicates the time of exposure to LIF (1000 U/mL). Current amplitude reached a maximum level at 10 to 15 minutes. The inset shows representative current traces before (a) and after (b) application of LIF at 15 minutes. B, Representative traces of Fluo-4 fluorescent intensity before (baseline, left) and after (right) 15 minutes of exposure to LIF (1000 U/mL). C, Percent changes in amplitude of the $[Ca^{2+}]_i$ with or without exposure to LIF. Values are mean±SEM of 10 experiments. ★P<0.05; ★★P<0.01 vs baseline.

LIF Activates the CaMKII and CaMKIV Pathways in Cardiomyocytes

To determine whether the LIF-induced increase in $[Ca^{2+}]_i$ transient can augment CaMKII and CaMKIV activities in cardiomyocytes, we initially measured the CaMKII activity in LIF-stimulated cardiomyocytes (Figure 2A). CaMKII activity increased from 2 minutes and peaked at 30 minutes, which paralleled the timing of the L-type $Ca^{2+}$ current and $[Ca^{2+}]_i$ transient. Experiments were repeated 7 times, and the results were reproducible in each experiment. ★P<0.01. B, Autophosphorylation of CaMKII in LIF-stimulated cardiomyocytes. After cardiomyocytes were stimulated with LIF, Western blotting was performed to detect phosphoCaMKII, as described in Materials and Methods. LIF augmented phospho-CaMKII from 5 minutes, and phosphorylation peaked at 15 minutes.

Figure 2. Effect of LIF on CaMKII activity and autophosphorylation of CaMKII in cardiomyocytes. A, Cardiomyocytes were stimulated with LIF for the indicated times. CaMKII was immunoprecipitated, and its activity was assayed using a synthetic peptide as a substrate. CaMKII activity was increased by 2.2-fold at 15 minutes, which paralleled the timing of the L-type $Ca^{2+}$ current and $[Ca^{2+}]_i$ transient. Experiments were repeated 7 times, and the results were reproducible in each experiment. ★P<0.01. B, Autophosphorylation of CaMKII in LIF-stimulated cardiomyocytes. After cardiomyocytes were stimulated with LIF, Western blotting was performed to detect phosphoCaMKII, as described in Materials and Methods. LIF augmented phospho-CaMKII from 5 minutes, and phosphorylation peaked at 15 minutes.

LIF Activates the CaMKII and CaMKIV Pathways in Cardiomyocytes

To determine whether the LIF-induced increase in $[Ca^{2+}]_i$ transient can augment CaMKII and CaMKIV activities in cardiomyocytes, we initially measured the CaMKII activity in LIF-stimulated cardiomyocytes (Figure 2A). CaMKII activity increased from 2 minutes, peaked by 2.2-fold at 15 minutes, and decreased thereafter.

Once CaMKII is activated by the $Ca^{2+}$/calmodulin complex, CaMKII is known to autophosphorylate and does not need calmodulin for its activity. To confirm that LIF really activates the CaMKII, we performed IP Western blot analysis to detect the phosphorylation of CaMKII with anti-phosphoCaMKII antibody (Figure 2B). LIF augmented phosphorylation of CaMKII from 2 minutes, peaked at 15 minutes, and then decreased thereafter.

Next, we measured the CaMKIV activity in LIF-stimulated cells (Figure 3). LIF increased CaMKIV from 2 minutes and
peaked by 2.2-fold at 15 minutes. These findings indicated that LIF activated CaMKII and CaMKIV in cardiomyocytes.

**LIF Activates Calcineurin and NFAT-3 Luciferase Activity in Cardiomyocytes**

We measured the calcineurin activity in LIF-stimulated cardiomyocytes (Figure 4). The calcineurin activity increased at 2 minutes, peaked by 1.6-fold at 15 minutes, and decreased thereafter. The time course of the activation of calcineurin corresponded to that of CaMKII and CaMKIV. It is well known that cytosolic NFAT is dephosphorylated by calcineurin and is translocated to the nucleus. NFAT proteins form cooperative DNA-binding complexes with dimers of the activator protein-1 (AP-1) (Fos/Jun) family at composite NFAT:AP-1 DNA elements that have been identified in multiple NFAT-regulated genes, including interleukin-2. To additionally confirm whether LIF activates calcineurin in cardiomyocytes, we examined NFAT transcriptional activity using an NFAT:AP-1 luciferase plasmid derived from interleukin-2 gene promoter. To investigate the activation of the Ca²⁺/calcineurin–NFAT pathway elicited by LIF independent of AP-1 activation, we cotransfected an active form of MEKK1, which activates c-Jun N-terminal kinase cascades, to constitutively activate AP-1 activity. In unstimulated cells, FK506-inhibitable NFAT-luciferase activity was observed. LIF increased this NFAT-luciferase activity by 3-fold, and this increase was completely inhibited by CsA (Figure 4B). These results suggested that LIF can activate calcineurin, which is in turn sufficient for dephosphorylation and activation of NFAT in cardiomyocytes.

**L-Type Ca²⁺ Channel Mediates the LIF-Induced Activation of CaMKII, CaMKIV, and Calcineurin**

To confirm that the LIF-induced increase in CaMKII, CaMKIV, and calcineurin activities were caused by Ca²⁺-induced Ca²⁺ release via an L-type Ca²⁺ current, we preincubated the cells with 10⁻⁶ mol/L nicardipine or 10⁻⁶ mol/L verapamil, stimulated with LIF, and measured CaMKII and CaMKIV activities (Figures 5A through 5C). KN62 (CaMKII and CaMKIV inhibitor) and CsA were used as a control. KN62 and CsA inhibited the LIF-induced increase in CaMKII and CaMKIV and CN activities, respectively. LIF-induced increase in CaMKII, CaMKIV, and calcineurin activity was almost completely inhibited by nicardipine or verapamil. These results were reproducible in 3 separate experiments. We also observed the effect of nicardipine, verapamil, or EGTA (1 mmol/L) on LIF-induced autophosphorylation of CaMKII. Nicardipine, verapamil, and EGTA fully inhibited this phosphorylation (Figure 5C). We additionally examined whether preincubation of nicardipine, verapamil, and EGTA can inhibit LIF-induced autophosphorylation of CaMKII (Figure 5D). These reagents strongly inhibited the autophosphorylation of CaMKII. These findings indicated that the L-type Ca²⁺ currents mediate LIF-induced activation of CaMKII, CaMKIV, and calcineurin.

Next, we examined whether sarcoplasmic reticulum Ca²⁺ store was involved in the LIF-induced activation of CaMKIV in cardiomyocytes. We preincubated the cells with thapsigargin (1 μm/L) for 8 hours, stimulated with LIF, and measured CaMKIV activity. Thapsigargin completely inhibited LIF-induced increase in CaMKIV activity, and its level was lower than that of the control (data not shown), indicating that the sarcoplasmic reticulum Ca²⁺ store was involved in LIF-induced activation of CaMKIV. Taken together, these findings indicated that Ca²⁺-induced Ca²⁺ release might be involved in activation of these kinases and phosphatase.
Nicardipine and Verapamil Inhibited LIF-Induced Increase in \[^{3}\text{H}\]Phenylalanine Uptake

To confirm that a LIF-induced increase in L-type Ca\(^{2+}\) current was involved in LIF-induced cardiac hypertrophy, we performed \[^{3}\text{H}\]phenylalanine uptake experiments to test whether nicardipine or verapamil can inhibit LIF-induced hypertrophy (Figure 5E). The LIF-induced increase in \[^{3}\text{H}\]phenylalanine uptake was significantly attenuated by preincubation of nicardipine and verapamil.

LIF-Induced Activation of CaMKII, CaMKIV, and Calcineurin Was Independent of PLC/IP\(_3\) Pathway

To date, there is no evidence to suggest that the gp130 receptor mediates its signal through the PLC/IP\(_3\) pathway. To confirm that LIF-induced activation of Ca\(^{2+}\)-dependent pathway was not mediated by the PLC/IP\(_3\) pathway, we performed IP Western blot analysis to detect the tyrosine phosphorylation of PLC-\(\gamma_1\). Platelet-derived growth factor (PDGF) was used as a positive control. PDGF strongly phosphorylated PLC-\(\gamma_1\), but LIF did not phosphorylate PLC-\(\gamma_1\) (Figure 6A). We also confirmed that LIF did not increase IP\(_3\) content in cardiomyocytes (Figure 6B). Together, these results indicated that LIF-induced activation of CaMKII, CaMKIV, and calcineurin was mediated not by the PLC/IP\(_3\) pathway but by L-type Ca\(^{2+}\) current-induced Ca\(^{2+}\) release.

Role of CaMKII and CaMKIV in LIF-Induced Cardiac Hypertrophy

To determine whether LIF-induced activation of CaMKII and CaMKIV plays an important role in mediating cardiac hypertrophy, we investigated the effect of KN62 on the LIF-induced increase in \[^{3}\text{H}\]phenylalanine uptake and cell size and induction of hypertrophic marker gene expression. LIF induced c-fos (30 minutes), BNP (1 hour), skeletal \(\alpha\)-actin (24 hours), and ANP (24 hours). KN62 slightly decreased LIF-induced expression of c-fos, BNP, \(\alpha\)-skeletal actin, and ANP (Figure 7A). LIF caused a 43% increase in \[^{3}\text{H}\]phenylalanine uptake compared with control. KN-62 dose-dependently inhibited the LIF-induced \[^{3}\text{H}\]phenylalanine uptake by 78.5%, whereas KN-62 at this concentration had no effect on the baseline \[^{3}\text{H}\]phenylalanine uptake (Figure 7B). LIF caused a 48% and 27% increase in cell area and perimeter compared with the control cells, respectively. KN-62 (1 \(\mu\)mol/L) significantly decreased the LIF-induced increase in cell area and perimeter by 46% and 57%, respectively, whereas KN-62 alone did not significantly attenuate the cell size (Figures 7C and 7D). The results were fully reproducible.
and indicated that the LIF-induced increase in cardiac hypertrophy was partially mediated by CaMKII or CaMKIV.

Role of Calcineurin in LIF-Induced Cardiac Hypertrophy

To investigate the role of calcineurin in LIF-induced cardiac hypertrophy, we also performed the same assay using FK506 or CsA. Northern blotting revealed that both FK506 and CsA did not affect the LIF-induced induction of c-fos, BNP (1 hour), or ANP (Figure 8A) expression. Because a previous report found that the promoter sequences of BNP contained NFAT-binding sequences and that calcineurin plays an important role in BNP expression, we additionally analyzed the effect of CsA on LIF-induced induction of the BNP gene at 30 minutes; however, it attenuated BNP expression from 2 to 24 hours (Figure 8B). CsA inhibited the LIF-induced increase in [Ca\textsuperscript{2+}] in cardiomyocytes. These findings indicated that calcineurin might be involved in LIF-induced cardiac hypertrophy.

Discussion

In cardiomyocytes, extracellular Ca\textsuperscript{2+} entering through L-type Ca\textsuperscript{2+} channels during depolarization triggers additional elevation of intracellular Ca\textsuperscript{2+} by stimulating Ca\textsuperscript{2+} release from the sarcoplasmic reticulum (Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release) and induces muscle contraction. \(\beta\)-Adrenergic stimulation of cardiomyocytes increases L-type Ca\textsuperscript{2+} current \((I_{Ca,L})\) and augments muscle contraction. In neuronal cells, transient changes in the concentration of intracellular Ca\textsuperscript{2+} attributable to membrane depolarization and activation of voltage-dependent L-type Ca\textsuperscript{2+} channels can lead to various physiological responses, including neurotransmitter release, modulation of synaptic transmission, and changes in gene expression.\(^{22}\) However, it is not known whether increased intracellular Ca\textsuperscript{2+} concentration through L-type Ca\textsuperscript{2+} channels can cause Ca\textsuperscript{2+}-sensitive signal transduction, leading to gene expression and protein synthesis in cardiomyocytes. Stimulation with a Ca\textsuperscript{2+}-channel agonist induces cardiac hypertrophy, and, conversely, blockade of \(I_{Ca,L}\) prevents cardiac hypertrophy, suggested that Ca\textsuperscript{2+} entry through L-type Ca\textsuperscript{2+} channels plays a critical role in cardiac hypertrophy.\(^{23}\)

Several lines of evidence suggest that the activity of voltage-dependent L-type Ca\textsuperscript{2+} channels is regulated by various second messengers, including PKA, PKC, CaMKII,\(^{24}\) and tyrosine kinases. In a previous study, we reported that LIF augmented [Ca\textsuperscript{2+}], via an increase in L-type Ca\textsuperscript{2+} in adult rat cardiomyocytes. It is worth noting that an LIF-induced increase in [Ca\textsuperscript{2+}], and L-type Ca\textsuperscript{2+} was observed from 2 minutes and peaked at 15 minutes and was therefore a slower activation process than that mediated by PKA or PKC. We confirmed that the PKA, PKC, and CaMKII pathways were not involved in this activation, which suggested that this slow increase was caused by another mechanism. In the present study, we demonstrated that LIF augmented \(I_{Ca,L}\) and [Ca\textsuperscript{2+}], in neonatal rat cardiomyocytes. The time course of the activation of CaMKII, CaMKIV, and calcineurin was slow and in accordance with that of the increase in [Ca\textsuperscript{2+}], and L-type Ca\textsuperscript{2+}. We also confirmed that the LIF-induced activation of CaMKII, CaMKIV, and calcineurin was completely abrogated by blocking of L-type Ca\textsuperscript{2+} with nicardipine or verapamil, and that LIF does not increase IP\textsubscript{3}, in cardiomyocytes. IP\textsubscript{3}-induced Ca\textsuperscript{2+} release plays an important role in intracellular Ca\textsuperscript{2+} signaling in a wide variety of cell types.\(^{25}\)

Activation of PLC\(\beta\) is mediated by the \(\alpha\) or \(\beta\gamma\) subunit of the heterotrimeric G proteins, whereas the \(\gamma\)-type enzymes are activated by phosphorylation with receptor- or nonreceptor-type protein tyrosine kinases, such as the Src family proteins. The present study also demonstrated that LIF did not phosphorylate PLC\(\gamma\) in cardiomyocytes. These findings suggested that IP\textsubscript{3}-induced Ca\textsuperscript{2+} release was not involved in augmentation of [Ca\textsuperscript{2+}], transient.

A previous report implicated calmodulin and CaMKII kinases in transduction of hypertrophic signals in cultured cardiomyocytes. Gruver et al.\(^{26}\) reported that targeted developmental overexpression of calmodulin induces proliferative and hypertrophic growth of cardiomyocytes in transgenic mice. Ramirez et al.\(^{27}\) showed that the CaMKII inhibitors M7 and KN93 prevent myocardial hypertrophy and upregulation of ANP in response to phenylephrine, which indicates that CaMKII activation is an essential step in phenylephrine-mediated hypertrophy.\(^{28}\) Abraham et al.\(^{29}\) reported that angiotensin II, vasopressin, and PDGF increased CaMKII activity

![Image](942 Circulation Research November 10, 2000)

Figure 6. LIF did not increase IP\textsubscript{3} or phosphorylate PLC-\(\gamma\) in cardiomyocytes. A, To confirm that the LIF-induced increase in [Ca\textsuperscript{2+}] was not mediated by PLC, the tyrosine phosphorylation of PLC-\(\gamma\) was measured. PDGF was used as a positive control. LIF did not increase the tyrosine phosphorylation of PLC-\(\gamma\) in cardiomyocytes. B, Cells were stimulated with endothelin-1 (ET-1: 10\textsuperscript{-9} mol/L) for 5 minutes or with LIF for 15 minutes and lysed. IP\textsubscript{3} content was assayed by radioimmunoprecipitation assay. LIF did not increase IP\textsubscript{3} content in cardiomyocytes. *P<0.01 vs control.
by 4.6-, 2-, and 1.7-fold, respectively, in rat vascular smooth muscle cells. In this study, we determined that CaMKII was activated by LIF via an L-type Ca\(^{2+}\) current and that inhibition of CaMKII by KN62 partially prevented LIF-induced protein synthesis, induction of immediate early gene, and upregulation of fetal gene expression. These findings indicated that CaMKII is critically involved in LIF-induced cardiac hypertrophy.

### Figure 7

Role of CaMKII and CaMKIV in LIF-induced cardiac hypertrophy. A, Effect of KN62 (an inhibitor of CaMKII and CaMKIV) on LIF-induced hypertrophic marker gene expression in cardiomyocytes. c-fos, BNP, \(\alpha\)-skeletal actin, and ANP expression was attenuated by KN62. B, Effect of KN62 on LIF-induced increase in \([^{3}H]\)phenylalanine uptake. KN62 dose-dependently decreased the LIF-induced increase in \([^{3}H]\)phenylalanine uptake, whereas the maximal dose of KN62 (10 \(\mu\)mol/L) did not affect \([^{3}H]\)phenylalanine uptake. C and D, Effect of KN62 on LIF-induced increase in cell area and perimeter. KN62 decreased the LIF-induced increase in cell area and perimeter, whereas KN62 had no effect. Results are mean±SEM. * \(P<0.01\) vs control; ** \(P<0.05\) vs control; # \(P<0.01\) vs LIF alone.

### Figure 8

Role of calcineurin in LIF-induced cardiac hypertrophy. A, Effect of CsA and FK506 on LIF-induced hypertrophic marker gene expression in cardiomyocytes. c-fos (30 minutes), BNP (1 hour), and ANP (24 hours) expression was unaffected by CsA or FK506. B, Time course of the effect of CsA on BNP expression in LIF-stimulated cardiomyocytes. CsA did not affect BNP expression at 30 minutes, but significantly attenuated BNP from 2 to 24 hours. C, Effect of CsA on LIF-induced increase in \([^{3}H]\)phenylalanine uptake. D and E, Effect of CsA on LIF-induced increase in cell area and perimeter. Results are mean±SEM. * \(P<0.01\) vs control; ** \(P<0.05\) vs control; # \(P<0.01\) vs LIF alone. NS indicates not significant vs LIF alone.
Lu et al. recently suggested the new idea that MEF2 is an endpoint for the hypertrophic stimuli in cardiomyocytes and that MEF2 mediates synergetic transcriptional response to the CaMKs and p38 MAPK signaling pathways by signal-dependent dissociation of histone deacetylase. They proposed a model for the regulation of MEF2 by CaMKI and CaMKIV. Hypertrophic signals that activate CaMKI, CaMKIV, and p38 MAPK lead to MEF2 activation by a different mechanism. Some stimuli, such as phenylephrine, may activate both pathways. Association of histone deacetylase 4/5 with the DNA-binding domain of MEF2 represses MEF2 transcriptional activity. CaMKI and CaMKIV activates MEF2 by preventing association of histone deacetylase 4/5 with MEF2. p38 MAPK stimulates MEF2 by direct phosphorylation of the transcription activation domain. Together, the CaMKs and p38 MAPK pathways synergize to activate MEF2. The present study demonstrated that LIF activated CaMKIV in cardiomyocytes and KN62 inhibited LIF-induced cardiac hypertrophy. Because KN62 can inhibit both CaMKI and CaMKIV, the inhibitory effect of KN62 might be an additive effect of the inhibition of the 2 kinases. We have already confirmed that LIF activated p38 MAPK in cardiomyocytes (data not shown). Our findings indicated that the CaMK–p38 MAPK–MEF2 theory might also play an important role in gp130-mediated cardiac hypertrophy, although this theory is still controversial.

Since Molkentin et al. reported that this calcineurin-NFAT3 pathway was critical to the induction of cardiac hypertrophy, several studies of this pathway have been reported. Sussman et al. reported that inhibition of this pathway by CsA or FK506 could prevent pressure overload–induced in vivo cardiac hypertrophy produced by aortic banding. Luo et al., Ding et al., and Zhang et al. independently reported that these calcineurin inhibitors did not inhibit pressure overload–induced cardiac hypertrophy, and Meguro et al. reported that CsA attenuates pressure overload hypertrophy in mice while enhancing susceptibility to decompensation and heart failure. Moreover, Force et al. reported that a human transplanted heart shows long-term cardiac hypertrophy despite the use of these compounds. CsA and FK506 are well known to cause hypertension in vivo and have several side effects. Thus, it is difficult to conclude that inhibition of this pathway in vivo is sufficient to prevent cardiac hypertrophy. In the present study, we have shown that LIF activated calcineurin in cardiomyocytes and that inhibition of this pathway by CsA or FK506 partially blocked protein synthesis and slightly attenuated BNP expression but did not affect c-fos and ANP expression. On the basis of these findings, we suspect that the calcineurin pathway might well be involved in LIF-induced cardiac hypertrophy.

In conclusion, we have demonstrated for the first time, to our knowledge, that LIF activates CaMKII, CaMKIV, and calcineurin by an increase in Ik,Ca in cardiomyocytes and that CaMKII, CaMKIV, and calcineurin contributed significantly to LIF-induced cardiac hypertrophy in vitro. The importance of the calcineurin/NFAT3 and CaMKIV-MEF2 pathways in cardiac hypertrophy is still controversial, and additional investigation is needed to clarify their role.

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