The Nuclear \( \delta_\beta \) Isoform of Ca\(^{2+} \)/Calmodulin-dependent Protein Kinase II Regulates Atrial Natriuretic Factor Gene Expression in Ventricular Myocytes*

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Cultured neonatal ventricular myocytes display features of myocardial hypertrophy including increased cell size, myofilament organization, and reexpression of the embryonic gene for atrial natriuretic factor (ANF). KN-93, an inhibitor of multifunctional Ca\(^{2+} \)/calmodulin-dependent protein kinase (CaM kinase II), blocked the induction of these responses by the \( \alpha_1 \)-adrenergic receptor agonist phenylephrine, whereas its inactive analog KN-92 did not. To directly determine whether CaM kinase II could regulate ANF gene expression, we transiently expressed each of three isoforms of CaM kinase II (\( \alpha \), \( \delta \), and \( \gamma \)) along with an ANF promoter/luciferase reporter gene. The \( \delta \) isoform markedly increased luciferase gene expression, whereas comparable levels of the \( \delta_\beta \) and \( \alpha \) isoforms were ineffective. Expression of \( \delta_\beta \)-CaM kinase II also potentiated phenylephrine-mediated ANF gene expression, and this effect was blocked by KN-93 but not by KN-92. The ability of \( \delta_\beta \)-CaM kinase II to transactivate a truncated ANF promoter, containing a serum response element (SRE) required for phenylephrine-inducible gene expression, was lost when this SRE was mutated. The \( \delta_\beta \) isoform of CaM kinase II has been shown to exhibit nuclear localization. Coexpression of the non-nuclear \( \delta_c \) or \( \alpha \) isoforms, which can form multimers with the \( \delta_\beta \) isoform, prevented the nuclear localization of \( \delta_\beta \)-CaM kinase II and also blocked its effects on ANF reporter gene and protein expression. In addition, a chimeric \( \alpha \)/CaM kinase II which contains the nuclear localization signal of the \( \delta_\beta \) isoform was able to induce ANF reporter gene expression, albeit to a lesser extent than \( \delta_\beta \)-CaM kinase II. These data are the first to assign a function to the \( \delta_\beta \) isoform of CaM kinase II and to link its nuclear localization to subsequent activation of cardiac gene expression.

A role for Ca\(^{2+} \) signaling in myocardial hypertrophy is suggested by several lines of evidence. Overexpression of calmodulin, an intracellular Ca\(^{2+} \)-binding protein, results in myocardial hypertrophy in transgenic mice (1). In cultured cardiac myocytes, elevated extracellular Ca\(^{2+} \) (2) or stimulation with a Ca\(^{2+} \)-channel agonist (3) is associated with increased expression of atrial natriuretic factor (ANF), an established indicator of ventricular hypertrophy. Electrical pacing also elevates intracellular Ca\(^{2+} \), and cardiac myocytes respond with increased expression of ANF as well as myosin light chain-2, another marker of hypertrophy (4). Conversely, blockade of depolarization-induced Ca\(^{2+} \) entry prevents specific increases in gene expression associated with cardiomyocyte hypertrophy (3–5). In addition, both pacing and \( \alpha_1 \)-AdR-induced ANF expression in cultured myocytes can be blocked by the calmodulin antagonist W-7 (3, 4), further implicating Ca\(^{2+} \) as a mediator of cardiac gene expression in the hypertrophic response.

The multifunctional Ca\(^{2+} \)/calmodulin-dependent protein kinase, also referred to as CaM kinase II, is a well-known effector of the actions of Ca\(^{2+} \) and calmodulin. This protein kinase has been shown to phosphorylate myocardial proteins involved in Ca\(^{2+} \) transport, including the ryanodine receptor, the sarcoplasmic reticular Ca\(^{2+} \)-ATPase, and phospholamban (6–8), and to modulate l-type Ca\(^{2+} \) channels and excitation-contraction coupling in cardiac myocytes (9–11). CaM kinase II functions as a multimer consisting of 8–12 subunits, which range in size from 51–60 kDa and are derived from one of four (\( \alpha \), \( \beta \), \( \gamma \), and \( \delta \)) isoforms (reviewed in Refs. 12 and 13). The \( \alpha \) isoform, the best characterized CaM kinase II, is found primarily in brain and has been shown to regulate neuronal function including memory formation and neurite outgrowth (12, 14, 15). A constitutively activated form of \( \alpha \)/CaM kinase II has been shown to induce transcriptional activation of the c-fos promoter through the serum response element (SRE) in mesangial cells (16). The \( \delta_\beta \) and \( \delta_\gamma \) isoforms of CaM kinase II were recently cloned from rat heart, and \( \delta_\beta \) was determined to be the predominant isoform of CaM kinase II in the heart (17, 18). The \( \delta_\alpha \) and \( \delta_\gamma \)-CaM kinase II isoforms differ only by an 11-amino acid sequence in their variable region. Interestingly, this sequence contains a nuclear localization signal which is absent from the \( \delta_\beta \) and previously described \( \alpha \) isoform of CaM kinase II (19). At present, no specific function has been attributed to the \( \delta \) isoform of

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1 The abbreviations used are: ANF, atrial natriuretic factor; CaM kinase II, multifunctional Ca\(^{2+} \)/calmodulin-dependent protein kinase; \( \alpha_1 \)-AdR, \( \alpha_1 \)-adrenergic receptor; PE, phenylephrine; KN-93, (2-[N-(2-hydroxyethyl)-N-(4-methoxybenzenesulfonyl)]amino-N-(4-chlorocinnamyl)-N-methylbenzylamine); KN-92, (2-[N-(4-methoxybenzenesulfonyl)]-amino-N-(4-chlorocinnamyl)-N-methylbenzylamine); SRE, serum response element; SRP, serum response factor; HA, hemagglutinin; RSV, Rous sarcoma virus.
CaM kinase II.

In light of the evidence implicating CaM kinase in cardiac hypertrophy and in regulating c-fos gene expression we hypothesized that CaM kinase II might contribute to the increased gene expression seen in a cell model of cardiac hypertrophy. The studies reported here demonstrate that in cardiac myocytes transient expression of the \( \delta_B \) but not other isoforms of CaM kinase II leads to transcriptional activation of ANF gene expression, mediated in part through an SRE, and associated with the localization of CaM kinase II to the nucleus.

**MATERIALS AND METHODS**

**Cell Culture Procedure**—Neonatal ventricular myocytes from 1–3-day-old Sprague-Dawley rats were plated at a density of 4 × 10^6/cm^2 on 60-mm gelatin-coated tissue culture dishes or 25-mm glass coverslips and maintained in 4:1 Dulbecco’s modified Eagle’s medium/medium 199 (Life Technologies, Inc.) containing 10% fetal calf serum, and antibiotics (100 units/ml penicillin and 100 \( \mu \)g/ml streptomycin) (20).

**Plasmid Constructs**—A 638-base pair fragment of the rat ANF promoter (ANF638) (21), a 134-base pair fragment of the rat ANF promoter (ANF134) or a mutant 134-base pair (ANF134/C114) (22), or the Rous sarcoma virus (RSV) long term repeat (23) fused to firefly luciferase cDNA were used as reporter genes. The cDNAs for wild-type and hemagglutinin (HA)-tagged \( \alpha_B \), \( \alpha_C \), and \( \delta_B \)-CaM kinases and for the chimeric \( \alpha_B\delta_B \)-CaM kinase were cloned into the SmaII site of the cytomegalovirus promoter (18, 19, 24).

**Transient Transfection Assays**—Ventricular myocytes, cultured overnight, were washed and incubated in serum-containing medium 2–4 h prior to transfections. Cells were cotransfected for 18 h with a total of 8–12 \( \mu \)g of DNA (ANF/luciferase reporter gene along with vector alone or vector encoding various CaM kinase II isoforms) using a modified calcium-phosphate technique (23, 25). Unless otherwise mentioned, the reporter gene is the 638-base pair ANF promoter-luciferase described previously (21). Myocytes were washed and incubated for an additional 48 h in serum-free medium. Luciferase activity in cell lysates was measured and normalized to total protein as described (23, 25).

**Immunofluorescence Studies**—Immunofluorescence studies were performed on myocytes cultured on laminin-coated chamber slides for 24 h in serum-containing medium and subsequently serum-starved for 36–48 h (26). Cells were fixed with 3% paraformaldehyde, permeabilized with 0.3% Triton X-100, and blocked with 1% bovine serum albumin. To examine endogenous ANF protein immunoreactivity, cells were incubated with a polyclonal anti-ANF antibody (Peninsula Labs, Belmont, CA) followed by a fluorescein isothiocyanate-conjugated goat anti-rabbit secondary antibody. Cells were stained with rhodamine-conjugated phalloidin to examine myofilament organization. To study the localization of different CaM kinase II isoforms, myocytes were transfected with vector alone or vector encoding HA-tagged \( \delta_B \)-CaM kinase II plus or minus other isoforms as described above and stained with a mouse anti-HA antibody (12CA5; Boehringer Mannheim) followed by a rhodamine-conjugated goat anti-mouse antibody. For examining changes in ANF expression in myocytes transfected with CaM kinase II, cells were cotransfected with an RSV/luciferase gene together with CaM kinase II. Transfected cells were identified by immunostaining with a rabbit anti-luciferase antibody (Cortex Biochem) followed by a fluorescein isothiocyanate-conjugated goat anti-rabbit antibody. ANF protein was detected using a mouse anti-ANF antibody (provided by Dr. C. Glembotski, San Diego State University) and a rhodamine-conjugated, goat anti-mouse antibody. Immunofluorescence analysis was performed by confocal microscopy using a Plan-neofluar 40× objective (Zeiss).

**Western Analysis of CaM Kinase II Expression**—Myocytes were transfected as described above with 2–6 \( \mu \)g of vector encoding either HA-tagged \( \alpha_B \), \( \beta_B \), or \( \delta_B \)-CaM kinase II. After 48 h cells were lysed in TBS containing 3 mM EDTA, 3 mM EGTA, and 0.5% Nonidet P-40. Cell lysates were subjected to Western blotting with a monoclonal anti-ANF antibody (12CA5; Boehringer Mannheim) and ECL (Amersham) chemiluminescent detection.

**RESULTS**

Activation of \( \alpha_B \)-AdR in cultured ventricular myocytes leads to increases in cell size, myofilament organization, and ANF expression. To determine whether CaM kinase II is a mediator of these hypertrophic responses, we examined the effect of KN-93, an inhibitor of CaM kinase II, and KN-92, its structurally similar but inactive analog (27, 28). \( \alpha_B \)-AdR activation with phenylephrine (PE) led to a marked increase in the percent of cells staining positive for ANF protein (Fig. 1A), ANF-
luciferase expression (Fig. 1B), and myofilament organization (Fig. 1C). These responses were all significantly attenuated by pretreatment with KN-93 but not with equimolar concentrations of KN-92, suggesting a requirement for CaM kinase II in α₁-AdrR-induced myocardial hypertrophy.

To determine which isoforms of CaM kinase II might be involved in the hypertrophic response, we transiently expressed the α, δβ, and δc isoforms of CaM kinase II and examined ANF-luciferase reporter gene expression. Expression of δβ-CaM kinase II in neonatal ventricular myocytes increased ANF reporter gene expression 3–4 fold whereas the δc (Fig. 2A) and α isoforms (data not shown) were ineffective. All three isoforms were expressed in ventricular myocytes, as demonstrated by transfecting cells with the HA-tagged δβ, δc, or α-CaM kinase II expression vectors and comparing protein levels by Western blotting using an anti-HA antibody (Fig. 2B).

Since the δβ-CaM kinase II appeared to be expressed at a somewhat higher level than δc-CaM kinase II (using 2.4 μg of DNA), the concentration of the δc expression vector was increased 3-fold. Under these conditions δc-CaM kinase II was expressed at levels equivalent to those of δβ-CaM kinase II (Fig. 2B), but still failed to increase ANF-luciferase gene expression (Fig. 2A). Similar results were obtained with increasing amounts of the α isoform of CaM kinase II (data not shown). These findings suggest that the activation of cardiac gene expression by CaM kinase II is specific for the δc isoform.

The ability of α₁-AdrR agonist to synergize with the expressed δβ-CaM kinase II was then examined. Myocytes were cotransfected with either empty vector (SRα) or δβ-CaM kinase II along with the ANF reporter gene, as above. Cells were subsequently treated with a range of concentrations of PE (0–100 μM), and a dose-response curve for ANF-luciferase expression was generated (Fig. 3A). Overexpression of δβ-CaM kinase II significantly increased the maximum response to PE (Fig. 3A). To demonstrate that the potentiation was mediated by CaM kinase II, myocytes were pretreated with KN-93 or KN-92 for 1.5 h and then challenged with PE. KN-93 treatment inhibited the response to PE by ~60%, as shown earlier, and markedly attenuated the synergistic effect of CaM kinase II on the response to PE whereas KN-92 was ineffective (Fig. 3B). These data support the notion that δβ-CaM kinase II can serve as a mediator of the hypertrophic response elicited by α₁-AdrR activation.

CaM kinase II is known to phosphorylate and activate SRF which binds to the SRE in target genes (29). Sprenkle et al. (22) characterized a truncated ANF promoter (ANF134) sufficient for PE-inducible gene expression and demonstrated that an SRE at position −114 bound SRF and was required to mediate responses to PE (22). Because the contribution of this SRE to ANF expression is minimized in the ANF638 promoter, which binds only SRF and not SRF associated proteins (23), we used this promoter to test the requirement for an SRE in CaM kinase II action. Overexpression of δβ-CaM kinase II increased PE-stimulated ANF-luciferase expression from the ANF134-luciferase reporter. In contrast the same promoter containing a disrupted SRF/SRE binding site (ANF134/C114-luciferase; Ref. 22) was not further activated by δβ-CaM kinase II (Fig. 4).

The δβ isoform of CaM kinase II contains a nuclear localization signal that is absent in both the δc and α isoforms. Accordingly heterologously expressed δβ-CaM kinase II localizes...
to the nucleus in fibroblasts and cardiac myocytes whereas δC does not (19). The CaM kinase II holoenzyme can be formed from either homomultimeric or heteromultimeric association of subunits (12, 13). We asked whether δγ-CaM kinase II could be prevented from entering the nucleus of ventricular myocytes, as shown for fibroblasts (18), by coexpression of the non-nuclear (δC or α) isoforms of CaM kinase II. Myocytes were transfected with the vector encoding hemagglutinin (HA)-tagged δγ-CaM kinase II, either alone or along with the δC- or α-CaM kinase II expression vector. Localization of HA-tagged δγ-CaM kinase II was subsequently determined and scored as either nuclear or cytosolic. Approximately 82% of myocytes expressing HA-tagged δγ-CaM kinase II showed nuclear HA staining (Fig. 5) while the remaining cells showed cytosolic staining. In contrast, when the δC or α isoform of CaM kinase II was coexpressed with HA-tagged δγ-CaM kinase II fewer than 3% of the cells showed nuclear HA staining (Fig. 5), with the majority of the cells showing cytosolic staining. These observations indicate that localization of δγ-CaM kinase II to the nucleus can be prevented by coexpression of non-nuclear isoforms of CaM kinase II in ventricular myocytes.

Using this approach to alter the localization of δγ-CaM kinase II, we then asked whether nuclear localization of CaM kinase II correlated with its ability to induce transcriptional activation of the ANF gene. Myocytes were cotransfected with the ANF reporter gene along with the δγ-CaM kinase II expression vector alone or with a 2-fold higher concentration of the vector encoding the δC- or α-CaM kinase II isoform. Loss of nuclear localization of δγ-CaM kinase II (Fig. 5) was associated with its inability to activate ANF reporter gene expression (Fig. 6A), linking cellular localization of this enzyme to its functional effects.

In addition to examining reporter gene activity, we used immunofluorescence to detect ANF staining in cells transfected with the δγ-CaM kinase II expression vector alone or together with that encoding either δC- or α-CaM kinase II. To identify transfected cells, an RSV/luciferase reporter gene was coexpressed; cells which stained positive for luciferase were then scored for ANF immunoreactivity using a mouse anti-ANF antibody. The percent of cells exhibiting ANF immunoreactivity was 5 times higher in cells transfected with the δγ-CaM kinase II expression vector than in cells transfected with vector alone (Fig. 6B). However, no increase in ANF staining was observed in cells transfected with δγ-CaM kinase II together with either the δC- or α-CaM kinase II expression vector. These data complement the ANF reporter gene experiments in demonstrating at the single cell level that nuclear localization of δγ-CaM kinase II is required to induce ANF gene expression. Finally, as an additional means of testing the hypothesis that nuclear localization of CaM kinase II is associated with its ability to stimulate ANF gene expression, we transfected cells with cDNA encoding a chimeric form of α-CaM kinase II containing the nuclear localization signal of the δC isoform (αδC). This chimeric kinase significantly increased ANF reporter gene expression, although to a lesser degree than δγ-CaM kinase II (Table I), further demonstrating a correlation between the nuclear localization of CaM kinase II and induction of ANF gene expression.

**DISCUSSION**

Protein kinases play an important role in regulating gene expression. However, information concerning the involvement of CaM kinase II and its various isoforms in gene expression is limited. Most published studies have focused on the α isoform of CaM kinase II and have demonstrated positive regulation of gene expression through a CAAT/enhancer binding protein.
Fig. 6. Effects of non-nuclear CaM kinase II isoforms on δ B CaM kinase-mediated ANF reporter gene and endogenous protein expression. A, myocytes were cotransfected with 3 μg of the ANF reporter gene along with 2.4 μg of δ B-CaM kinase II plus or minus 4.8 μg of δ C- or α- CaM kinase II. The data are expressed as fold stimulation by the CaM kinase II expression vector relative to vector alone and are the mean ± S.E. of 11–27 determinations. B, myocytes were transfected as described above except 2 μg of the RSV/luciferase expression vector was used instead of the ANF reporter. Transfected cells (luciferase-positive) were identified, and ANF protein expression was detected in these cells by staining with a monoclonal anti-ANF antibody and a rhodamine-conjugated secondary antibody. Cells with perinuclear ANF staining were scored as positive. The percent of cells expressing ANF is calculated relative to that seen with δ B-CaM kinase II alone (100%). The data are the mean of duplicate coverslips from a single experiment, in which 117–175 cells were analyzed per experimental condition.

Table I
Activation of ANF gene expression by different isoforms of CaM kinase II

|          | SRα | δ B | δ B + δ C | δ B + α |
|----------|-----|-----|-----------|---------|
| Mean     | 1.0 | 9.4a| 4.7b      | 2.1     |
| S.E.     | 0.05| 1.2 | 0.8       | 0.3     |

a p < 0.001.
b p < 0.01.

(C/EBP) (30, 31). This isoform has also been shown to play a role in activating the c-fos gene through the c-fos SRE, and perhaps also through an adjacent AP-1-like sequence (16, 29, 32). The ability of the γ isoform of CaM kinase II to regulate interleukin-2 gene expression is another published example of transcriptional regulation by CaM kinase II (33). Nothing is known regarding the regulatory effects of the δ isoform of CaM kinase II on gene expression.

Earlier studies suggested a role for CaM kinase II in cardiac gene expression associated with hypertrophy (3–5). Studies presented here using KN-93 demonstrated that both ANF gene expression and myofilament organization induced by PE could be blocked, indicating a role for CaM kinase II in mediating hypertrophic responses in this cell system. The discovery that the predominant CaM kinase II isoform in the heart is the δ B isoform and that it contains a nuclear localization signal (18, 19) prompted us to examine the role of this CaM kinase II isoform in cardiac gene expression. As shown here expression of δ B-CaM kinase II leads to induction of an ANF reporter gene and to an increase in endogenous ANF protein. This indicates that CaM kinase II is sufficient to induce at least one of the well characterized responses associated with hypertrophy. In studies where cells transfected with CaM kinase II were identified, no increase in myofilament organization was observed (data not shown). Thus while myofilament organization appears to be CaM kinase-dependent, additional signals may be necessary for expression of this morphological change.

That expression of wild-type (rather than constitutively activated) CaM kinase II can induce ANF expression suggests that the enzyme has activity at basal Ca 2+ concentrations. This is not surprising since it has been shown that there is a significant degree of CaM kinase II activation levels in unstimulated cardiac myocytes (9). Furthermore, overexpression of wild-type α-CaM kinase II in neuronal cells can elicit neurite outgrowth, even at resting Ca 2+ levels (15). The observation that stimulation with PE gives an enhanced response when δ B-CaM kinase II is expressed suggests that α 1-AdrR activation leads to further activation of this enzyme. This synergistic interaction further supports a regulatory role for CaM kinase II in mediating the effect of α 1-AdrR stimulation on ANF gene expression.

In contrast to the effects of δ B-CaM kinase, neither wild-type δ C- nor α-CaM kinase II induced ANF gene expression. These functional differences indicate that the effects of CaM kinase II on cardiac gene expression are isoform-specific. The basis for the differential effects of the CaM kinase II isoforms on gene expression is not known. However, comparison of the sequences of the isoforms suggests that the specificity does not correlate with differences in the catalytic properties of the enzymes but could be attributed to differences in cellular localization since the δ, and α lack a nuclear localization signal found in the δ B isoform.

More direct evidence for a correlation between cellular localization and function comes from experiments in which nuclear localization of δ B-CaM kinase II was altered by coexpression of δ C or α isoforms. It has been shown in fibroblasts that a nuclear form of CaM kinase II can be excluded from the nucleus when coexpressed with non-nuclear forms (19). However, no functional correlate of this altered cellular localization has been shown. We show here that in ventricular myocytes δ B-CaM kinase II can also be prevented from localizing to the nucleus when coexpressed with either the δ C- or α isoforms. Under these conditions, δ B-CaM kinase II does not activate the ANF reporter gene or endogenous ANF protein expression. Further evidence for the importance of cellular localization comes from studies using a chimeric α-CaM kinase II containing the δ B nuclear localization signal (αδ B) and showing that it significantly increases ANF gene expression. The finding that CaM kinase II can activate the ANF reporter gene only when targeted to the nucleus demonstrates the correlation of localization with the functional effect of CaM kinase II.

2 M. T. Ramirez and J. H. Brown, unpublished data.
The signaling pathways through which CaM kinase II induces ANF gene expression remain to be elucidated. In PC12 cells, activation of CaM kinase II by depolarization leads to phosphorylation of the nuclear transcription factor SRF at Ser-103, which increases the ability of the SRF to associate with the SRE in the c-fos promoter and possibly to transactivate this gene (29, 34). Expression of constitutively activated α-CaM kinase II transactivates the c-fos promoter through both SRE and AP-1-like sequences located in the 638-base pair ANF promoter (22). The SRE at position –114 has been shown to bind to SRF, and mutation of this site in the truncated ANF promoter (ANF134) disrupts SRF binding and PE-inducible ANF expression (22). We demonstrate here that this site is also required for δB-CaM kinase II transactivates the ANF134-luciferase gene expression. Thus, δB-CaM kinase II can regulate ANF gene expression at least in part through the proximal SRE in the ANF promoter.

While further studies are required to define the mechanism of transcriptional activation by CaM kinase II in this system, our findings show for the first time that the δ isoform of CaM kinase II, which is the predominate isoform in the heart, can correlate the nuclear localization of transcriptional activation by CaM kinase II in this system, at least in part through the proximal SRE in the ANF promoter. The signaling pathways through which CaM kinase II induces ANF gene expression at least in part through the proximal SRE in the ANF promoter.

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