Calcineurin and Calcium/Calmodulin-dependent Protein Kinase Activate Distinct Metabolic Gene Regulatory Programs in Cardiac Muscle*  

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To learn more about the targets of Cn (Cn) and calcium/calmodulin-dependent protein kinase in cardiac muscle, we investigated their actions in cultured cardiac myocytes and the hearts of mice in vivo. Adenoviral-mediated expression of constitutively active forms of either pathway induced expression of peroxisome proliferator-activated receptor γ coactivator 1a, a transcriptional coactivator involved in the control of multiple cellular energy metabolic pathways in cardiac myocytes. Transcriptional profiling studies demonstrated that Cn and calcium/calmodulin-dependent protein kinase activate distinct but overlapping metabolic gene regulatory programs. Expression of the nuclear receptor, peroxisome proliferator-activated receptor α, was markedly increased by Cn, but not calcium/calmodulin-dependent protein kinase, providing one mechanism whereby cellular fatty acid utilization genes are selectively activated by Cn. Transfection experiments demonstrated that Cn directly activates the mouse peroxisome proliferator-activated receptor α gene promoter. Co-transfection “add-back” experiments demonstrated that the transcription factors, myocyte enhancer factors 2C or 2D, were sufficient to confer Cn-mediated activation of the peroxisome proliferator-activated receptor α gene. Cn was also shown to directly activate a known peroxisome proliferator-activated receptor α target, muscle-type carnitine palmitoyltransferase I, providing a second mechanism by which Cn activates genes of cellular fatty acid utilization. Lastly, the gene expression of peroxisome proliferator-activated receptor γ coactivator 1a and peroxisome proliferator-activated receptor α was reduced in the hearts of mice with cardiac-specific ablation of the Cn regulatory subunit. These data support a role for calcium-triggered signaling pathways in the regulation of cardiac energetics and identify pathway-specific control of metabolic targets.  

Skeletal muscle fiber types are defined by contractile protein isoform composition and energy metabolic properties. Slow-twitch muscle fibers possess greater mitochondrial volume supporting higher oxidative metabolic capacity compared with fast-twitch fibers. Muscle metabolic phenotype is plastic, responding to numerous external stimuli. Calcium signaling serves an important role in the adaptive response of skeletal muscle to external stimuli, including fiber type determination. Evidence has emerged that calcium-triggered regulatory pathways acting through Cn (Cn), a serine/threonine protein phosphatase, and calcium/calmodulin-dependent protein kinases (CaMK), serve a major role in determining the functional and metabolic phenotype of skeletal muscle by transducing alterations in cytosolic calcium concentration. In support of this, Chin et al. (1) demonstrated that a constitutively active form of Cn (Cn*) is capable of activating transcription of slow fiber-specific gene promoters. Similarly, overexpression of Cn* in skeletal muscle results in an increase in slow muscle fiber types (2), and in Cnα and Aβ null mice there is a reduction in slow muscle fiber type composition (3). Administration of cyclosporine A (CsA), a specific inhibitor of Cn (4), to rats leads to reduced muscle mitochondrial respiration in vitro (5), and drives a slow-to-fast fiber type transition (1), although controversy remains because other groups have found no effect of Cn inhibition on fiber type differentiation (6). A constitutively active form of CaMK IV (CaMK*) was shown to activate transcription of slow fiber-specific gene promoters and overexpression in skeletal muscle activated the slow-twitch fiber program and mitochondrial biogenesis in transgenic mice (7). These data support a role for Cn and CaMK in the transduction of increased muscle activity to adaptive structural and metabolic gene regulatory responses.  

Recent studies have identified the transcriptional coactivator peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α) as a mediator of Cn/CaMK signaling in the regulation of skeletal muscle fiber type switches and mitochondrial energy metabolism. PGC-1α is an inducible coactivator of numerous nuclear receptor and non-nuclear receptor transcription factors known to control distinct components of mitochondrial energy metabolism.  

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* The abbreviations used are: Cn, Cn; FA, fatty acid; CaMK, calcium/calmodulin-dependent protein kinase IV; CsA, cyclosporine; MEF2, myocyte enhancer factor 2; PPARα, peroxisome proliferator-activated receptor α; PGC-1α, peroxisome proliferator-activated receptor γ coactivator 1α; NFAT, nuclear factor of activated T cells; NRF-1, nuclear respiratory factor 1; SRF, serum response factor; M-CPT I, muscle-type carnitine palmitoyltransferase I; MCAD, medium-chain acyl-CoA dehydrogenase; α-MHC, α myosin heavy chain; GFP, green fluorescent protein; DMEF, Dulbeco’s modified Eagle’s medium; RLU, relative light unit(s); RSV, Rous sarcoma virus.
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Adenoviral Expression Vectors—Adenovirus expressing both green fluorescent protein and constitutively active human CaMK IV was created by subcloning from the RSV-CaMKIV+ into pAdTrackCMV as described previously (23). The adenovirus expressing green fluorescent protein and constitutively active murine Cn was a gift from R. Sanders Williams and Rick Vega (University of Texas Southwestern).

Cell Culture and Transfection Studies

C6/C13 myoblasts were maintained at 37 °C and 5% CO2 in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal calf serum. Primary rat neonatal cardiac myocytes were harvested and maintained as previously described (19). CV-1 cells were maintained as previously described (24). For all cell types, cells were transfected by the calcium phosphate co-precipitation method as described (25). Briefly, reporter plasmids (4 μg/ml) were co-transfected with SV-40 β-galactosidase (500 ng/ml) to control for transfection efficiency and the appropriate mammalian expression vector (500 ng/ml) or its corresponding empty vector. After transfection, for C6/C13 cells, the medium was changed to DMEM containing 10% horse serum to promote differentiation. Cells were harvested 72 h later in cell lysis buffer (Promega), and luciferase activity was measured as previously described (22). All transfection data are presented as means (± S.E.) of at least three separate transfection experiments done in triplicate.

DNA Microarray

RNA isolated from cardiac myocytes was infected with adenovirus 24 h after initial plating at a multiplicity of infection sufficient to infect >95% of the cells based on green fluorescent protein fluorescence with minimal cell drop out. After infection, cells were maintained for 72 h in differentiation media as described (19).

RNA and Protein Blot Analyses

Total RNA was isolated from either adenovirus-infected cells or whole hearts using the RNAzol (Tel-Test, Inc., Friendswood, TX) method. Northern blot analysis was performed with QuikHyb (Stratagene, La Jolla, CA) using random-primed 32P-labeled cDNA probes. The probes were derived from cDNAs encoding mouse PGC-1α, mouse PPARα, rat M-CPT I, mouse MCAD, and human β-actin. Band intensities were quantified by phosphorimaging using a GS 525 Molecular Imager System (Bio-Rad) and normalized to the expression of β-actin.

For Western blotting studies to detect CnB, protein extracts were prepared from both ventricles as described (26). Blotting was performed using an antibody directed against the CnB subunit (Upstate Biotechnology Inc., Lake Placid, NY) or actin (Sigma). Band intensities were quantified by densitometric analysis, and CnB signal was normalized to actin levels.

Quantitative Real-time Reverse Transcription-PCR Analyses

First-strand cDNA was generated by reverse transcription using 500 ng of total RNA. Real-time reverse transcription-PCR was performed using the ABI Prism 7700 sequence detection system and the TaqMan kit following the manufacturer’s protocols (Applied Biosystems, Foster City, CA). FAM-labeled probes with corresponding upstream and downstream primer sets (shown below) were designed using the Primer Express software package and tailored to span exon splice borders. Arbitrary units of target mRNA were corrected by measuring the levels of glyceraldehyde-3-phosphate dehydrogenase mRNA in the same PCR reaction with the VIC-labeled probe set with the following primer/probe set was used to detect specific gene expression: PGC-1α forward, 5′-CAGAAATCATATCCACCACG-3′; PGC-1α reverse, 5′-TTAGGACCGTGACGTTTGG-3′; PGC-1α probe, 5′-TCATTCTCTCGCTTTCCTCAATATGTTGCGAGG-3′. Rodent glyceraldehyde-3-phosphate dehydrogenase (VIC) probe set was included in all reactions as internal control correction (Applied Biosystems).

EXPERIMENTAL PROCEDURES

Plasmids

Mammalian Expression Vectors—8Ro-Cn+ plasmid expressing constitutively active murine Cn was a gift from Stephen J. O’Keefe (Merck Research Laboratory); the RSV-CaMKIV+ plasmid expressing constitutively active human CaMK IV was a gift from Richard J. Williams (University of Texas Southwestern, Dallas, TX); and the pcDNA1-NFAT3 plasmid expressing constitutively active human CaMK IV was a gift from R. Sanders Williams and Rick Vega (University of Texas Southwestern). The pcDNA3.1-MEF2A, C, and D were a gift from Talal Chatila (Washington University School of Medicine, St. Louis, MO). pcDNA3.1-MEF2A, C, and D were a gift from Eric Olson (University of Texas Southwestern); and the pcDNA1-NFAT3 plasmid expressing constitutively active murine Cn was a gift from Stephen J. O’Keefe (Merck Research Laboratory); the RSV-CaMKIV+ plasmid expressing constitutively active human CaMK IV was a gift from Richard J. Williams (University of Texas Southwestern, Dallas, TX); and the pcDNA1-NFAT3 plasmid expressing constitutively active human CaMK IV was a gift from R. Sanders Williams and Rick Vega (University of Texas Southwestern).

Reporter Constructs—PGC-Luc.2112 was generated by PCR amplification followed by cloning into pGL3 Basic (Promega, Madison WI). PCR was performed using a BAC clone (Genome Systems, St. Louis, MO) as template. Primers were engineered to include MluI (5′) and BglII (3′) sites. The primers used to amplify the construct were: 1) 5′ primer: 5′-ATAAACATGCACGCGTTATAGCTAAGTG-3′ and 3′ primer: 5′-AATTGCCGCAGTAGATCTTTTCACCT-3′. The human PPARα promoter construct (PaH-H-plG3), a gift from Bart Stael (Institut Pasteur de Lille, Lille, France), has been described elsewhere (21) as has MCTP.Luc.781 (22).

drial oxidative metabolism. Through its activation of specific transcription factors, PGC-1α has been shown to play an important role in the regulation of mitochondrial capacity in oxidative tissues such as brown adipose, skeletal muscle, and heart (8). PGC-1α has been shown to coactivate nuclear respiratory factor 1 (NRF-1), a critical regulator of nuclear and mitochondrial genes involved in respiratory function and mitochondrial DNA replication (9). PGC-1α has also been shown to coactivate the nuclear receptor peroxisome proliferator-activated receptor α (PPARα), a transcriptional regulator of genes involved in cellular fatty acid utilization (10). The expression of PGC-1α is in turn regulated by upstream signaling events, including Ca2+-dependent signaling pathways (11). Specifically, Cn and CaMK have been shown to activate PGC-1α gene transcription through myocyte enhancer factor 2 (MEF2) and cAMP response element-binding protein (12). Furthermore, in transgenic mice skeletal muscle-specific overexpression of Cn* or CaMK* results in increased expression of PGC-1α (7, 13).

In contrast to skeletal muscle, the downstream metabolic effects of Cn and CaMK in cardiac muscle has not been characterized. It is not known if PGC-1α is downstream of Cn or CaMK in cardiac myocytes. Unlike skeletal muscle, forced expression of constitutively active forms of Cn or CaMK in cardiac muscle results in profound hypertrophy, heart failure, and death (14, 15). Isolated mitochondria from Cn*-expressing hearts demonstrated impaired oxidative function (16). These latter observations are inconsistent with the known metabolic responses in skeletal muscle given that, during pathological hypertrophic growth, the heart undergoes a reversion to a fetal energy metabolic program characterized by reduction of mitochondrial oxidative capacity and fatty acid utilization (17–19).

In contrast, with physiological forms of hypertrophy such as infection, cells were maintained for 72 h in differentiation media as previously described (22). Blotting was performed using an antibody directed against the CnB subunit (Upstate Biotechnology Inc., Lake Placid, NY) or actin (Sigma). Band intensities were quantified by densitometric analysis, and CnB signal was normalized to actin levels.

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DNA Microarray

RNA isolated from cardiac myocytes was infected with adenoviral infection was cleaned with the RNeasy kit (Qiagen, Valencia, CA). Double-stranded cDNA was synthesized from 12 μg of total RNA that was first reverse-trans-
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RESULTS

Cn and CaMK KinaseInduce PGC-1α Gene Expression in Cardiac Myocytes—To investigate the effects of Cn and CaMK kinase on PGC-1α expression in cardiac myocytes, primary neonatal rat ventricular myocytes were infected with green fluorescent protein (GFP)-expressing adenoviral vectors expressing a constitutively active form of either Cn A (Ad.Cn*) or CaMK IV (Ad.CaMK*). PGC-1α transcript levels in Ad.Cn*– and Ad.CaMK*-infected cells were compared with that in control cells expressing GFP alone (Ad.GFP). PGC-1α mRNA levels were significantly increased by Ad.Cn* (3-fold) or Ad.CaMK* (7-fold) (Fig. 1A). Additive effects were not observed by simultaneous infection with both constructs (data not shown).

To determine whether this regulatory effect occurred at the transcriptional level, transient co-transfection assays were performed in ventricular myocytes with a luciferase reporter vector driven by 2112 bp of the mouse PGC-1α gene promoter (PGC.Luc.2112) and expression plasmids for Cn* and/or CaMK*. Cn* induced a significant (3-fold) increase in reporter activity (Fig. 1B), which was reversed upon treatment with cyclosporine A (CsA), a specific inhibitor of Cn. CsA also reduced activity of the PGC-1α promoter by more than 50%, suggesting that its basal activity was influenced by Cn*. Surprisingly, overexpression of CaMK* did not increase reporter activity. This latter observation contrasts with C2C12 myotubes in which we found that addition of CaMK* activates the PGC-1α promoter similar to published reports (data not shown and Refs. 7 and 12). These latter results suggest that for activation of PGC-1α gene expression by CaMK* in cardiac myocytes, crucial factors are lacking in neonatal cells, elements are lacking in the reporter construct, or post-transcriptional mechanisms are involved. Co-transfection of CaMK* with Cn* did not activate PGC.Luc.2112 more than Cn* alone, indicating a lack of cooperativity (Fig. 1B).

Cn* and CaMK* Activate Distinct but Overlapping Gene Regulatory Programs in Cardiac Myocytes—To delineate the gene regulatory programs driven by Cn and CaMK in cardiac myocytes, transcriptional profiling experiments were performed using RNA isolated from neonatal cardiac myocytes infected with either Ad.Cn*, Ad.CaMK*, or Ad.GFP (control) in three independent trials. Given that both Cn* and CaMK*
Table I

Results of transcriptional profiling using DNA microarrays of cardiac myocyte genes following overexpression of Ca⁺⁺ or CaMK⁺⁺

“P” or “A” indicates a gene that was increased from being absent to present or decreased from being present to absent, respectively. Known PPARα target genes are noted with a @ symbol, NRF-1 target genes are noted with a § symbol.

A. Transcriptional Regulators and Signaling Molecules

| GenBank No. | Gene Name                                      | Cn⁺⁺ | CaMK⁺⁺ |
|------------|------------------------------------------------|------|--------|
| M88952     | Peroxisome proliferator activated receptor α   | ↑p   | -      |
| U15211     | Retinoic acid receptor α (RARα)                | ↑p   | -      |
| D86580     | Neuron-derived orphan receptor 1 (NOR1)        | -    | ↑p     |
| A1176710   | Small heterodimeric partner (SHP)              | -    | ↑p     |
| U17254     | Transcription factor NGFI-B                    | -    | ↑3.2   |
| AA956941   | Transcription factor 4                         | ↑p   | -      |
| AA891308   | P37 TRAP/SMCC/PC2 subunit                     | -    | ↑p     |
| AA900476   | Cbp/p300-interacting trans-activator           | -    | ↑p     |
| U73142     | p38 MAP kinase                                 | ↑p   | -      |
| M64301     | MAP kinase 6                                   | ↑2.6 | ↑2.8   |
| A1011376   | MAP kinase-activated protein kinase 2          | -    | ↑2.6   |
| M80633     | Adenyl cyclase 4                               | ↑p   | -      |
| U04835     | cAMP response element modulator                | -    | ↑p     |
| L22760     | GATA-binding protein 6                         | ↑p   | -      |
| U27767     | Regulator of G-protein signaling 4             | ↑p   | -      |
| M83676     | RAB12                                         | ↑p   | -      |
| A1177986   | Eukaryotic initiation factor 5 (eIF-5)         | -    | ↑2.2   |
| L24907     | CaM Kinase 1                                   | -    | ↑1.5   |
| U24282     | Deiodinase, iodothyronine, type 3              | -    | ↑p     |

|     | Early growth response 1                        | ↓₀.₂ | ↓₀.₄   |
|     | Early growth response 2                        | ↓₀.₄ | -      |
|     | growth arrest specific 7                       | ↓ₐ   | ↓₀.₄   |
|     | GATA-binding protein 4                         | -    | -      |

B. Cellular Fatty Acid Metabolism

| GenBank No. | Gene Name                                      | Cn⁺⁺ | CaMK⁺⁺ |
|------------|------------------------------------------------|------|--------|
| D43623     | Muscle carnitine palmitoyltransferase 1 (M-CPT I) | ↑₂.₈ | ↑₂.₅   |
| L07736     | Liver carnitine palmitoyltransferase 1 (L-CPT I) | ↑₂.₂ | -      |
| J05470     | Carnitine palmitoyltransferase II               | ↑₂.₃ | -      |
| J02791     | Medium chain acyl-CoA dehydrogenase (MCAD)      | ↑₂.₄ | ↑₁.₅   |
| J05029     | Long chain acyl-CoA dehydrogenase (LCAD)        | ↑₂.₈ | -      |
| D30647     | Very long chain acyl-CoA dehydrogenase (VLCAD)  | ↑₂.₃ | -      |
| AA799489   | Acyl-CoA oxidase                                | ↑p   | -      |
| AI237731   | Lipoprotein lipase                             | ↑₂.₄ | -      |
| AI071531   | Oxidized LDL receptor                          | ↑₃.₆ | -      |
| AA925752   | CD36                                           | ↑₂.₈ | ↑₁.₉   |
| J02773     | Fatty acid binding protein 3                   | ↑₃.₀ | ↑₄.₃   |
| X05341     | Acetyl-CoA acyltransferase 2                   | ↑₂.₆ | ↑₃.₃   |
| U26033     | Carnitine O-octanoyltransferase                | ↑₂.₃ | -      |
| AA800120   | Carnitine/acylcarnitine translocase            | ↑₂.₃ | -      |
| AA851223   | Enolase 3, beta                                | ↑p   | ↑p     |
| AA800851   | Carboxylesterase 3                             | ↑p   | -      |
| Y09333     | Mitochondrial acyl-CoA thioesterase 1          | ↑p   | -      |
| AA893242   | Fatty acid CoA ligase, long chain 2            | ↑₃.₀ | ↑p     |
| D85189     | Fatty acid CoA ligase, long chain 4            | -    | ↑₁.₇   |
| AA012933   | Fatty acid CoA ligase, long chain 5            | -    | ↑₂.₆   |
| AA892832   | Fatty acid elongase                            | -    | ↑₂.₈   |
| M29249     | 3-HMG CoA reductase                            | -    | ↑p     |
| A1177004   | 3-HMG CoA synthase                             | ↑₃.₅ | ↑₃.₃   |

|     | low density lipoprotein receptor-related protein 3 | ↓₀.₅ | -      |
activate expression of PGC-1α, we were particularly interested in the response of known PGC-1α target genes. Genes considered to be regulated exhibited either a ≥2-fold increase or ≥50% decrease in expression relative to GFP-infected controls. In addition, genes that were increased from being undetectable (absent) to detectable (present or "P") or decreased from being present to absent ("A") were considered to be regulated. Among the 8800 genes on the array, Cn* activated 213 (2.4%) genes and repressed 56 (0.6%) genes. CaMK* activated 290 (3.3%) genes and repressed 88 (1.0%) genes. A significant number of the regulated genes were involved in energy metabolic or regulatory pathways downstream of PGC-1α (Table I). Regulated genes sorted into several clusters, including transcriptional regulation and signaling, cellular fatty acid utilization, mitochondrial metabolism, and glucose metabolism (Table I). Notably, genes involved in nearly every stage of mitochondrial energy transduction were induced by both Cn* and CaMK*, many of which are known targets of PGC-1α. Strikingly, very few genes from this broad cellular energy metabolic program were down-regulated (Table I).

### C. Mitochondrial Metabolism

| GenBank No. | Gene Name                                      | Cn*  | CaMK* |
|-------------|------------------------------------------------|------|-------|
| AA799656    | Mitochondrial ribosomal protein S31            | ↑p   | ↑p    |
| A16939387   | Mitochondrial 28S ribosomal protein S6         | --   | ↑2.1  |
| AA892314    | Isocitrate dehydrogenase 1                     | ↑2.3 | ↑2.7  |
| AF093773    | Malate dehydrogenase                           | --   | ↑2.9  |
| K00750      | Cytochrome c, somatic                           | ↑2.0 | ↑2.0  |
| AA866477    | Cytochrome c oxidase subunit VIIb              | --   | ↑2.9  |
| U40836      | Cytochrome c oxidase subunit VIII              | --   | ↑3.6  |
| X64827      | Cytochrome c oxidase subunit VIII-H            | ↑4.0 | ↑5.3  |
| AI235358    | Cytochrome-c reductase                         | --   | ↑2.4  |
| L20427      | Coenzyme q                                     | ↑p   | --    |
| A176422     | Flavoprotein-ubiquinone oxidoreductase         | ↑3.1 | --    |
| X54510      | ATP synthase, mitochondrial F0 complex, subunit F6 | ↑2.2 | ↑2.2 |
| D13123      | ATP synthase, mitochondrial F0 complex, subunit c | --   | ↑4.4  |
| AA799276    | ATPase, Ca2+ transporting, cardiac muscle      | ↑3.1 | ↑3.2  |
| M10140      | Creatine kinase, muscle                        | --   | ↑3.5  |
| M57664      | Creatine kinase, brain                         | --   | ↑2.5  |

### D. Glucose Metabolism

| GenBank No. | Gene Name                                      | Cn*  | CaMK* |
|-------------|------------------------------------------------|------|-------|
| AA799760    | Glucose transporter 4 (GLUT4)                   | --   | ↑P    |
| U25651      | Phosphofructokinase, muscle (PFK)               | --   | ↑2.2  |
| AA892828    | Pyruvate dehydrogenase (PDH)                    | ↑2.5 | ↑2.8  |
| AF062740    | Pyruvate dehydrogenase phosphatase              | --   | ↑3.2  |
| U32314      | Pyruvate carboxylase                            | --   | ↓0.3  |

Specifically, Cn* activated the expression of known PPARα target genes involved in fatty acid uptake (e.g., CD36), mitochondrial β-oxidation (e.g., M-CPT I and II, VLCAD, LCAD, and MCAD), and peroxisomal β-oxidation (acyl-CoA oxidase) (Table IB). Similarly, Cn* increased expression of genes encoding lipoprotein lipase, mitochondrial acyl-CoA thioesterase 1, and several other genes involved in cellular fatty acid metabolism (Table IB). In contrast, CaMK* activated only a small subset of PPARα targets involved in fatty acid uptake (e.g., CD36) and oxidation (e.g., M-CPT I and MCAD). These results suggested that the activation of the PPARα gene by Cn* but not CaMK* mediates the selective regulation of genes involved in cellular FA uptake and oxidation.

The profile of metabolic genes activated by CaMK* exhibited an overlapping but distinct pattern compared with that of Cn*. CaMK* activated two major categories of metabolic genes. First, CaMK* activated a broad range of genes encoding mitochondrial enzymes, including enzymes involved in the tricarboxylic acid cycle (e.g., isocitrate dehydrogenase and malate dehydrogenase), electron transport (e.g., cytochrome c and cytochrome c oxidase subunits), and oxidative phosphorylation (ATP synthase subunits), many of which are known PGC-1α targets. In addition, CaMK* activated expression of the genes encoding muscle and brain isoforms of creatine kinase. Cn* activated only a subset of these mitochondrial energy metabolic genes (Table IC). Second, CaMK* activated genes involved in cellular glucose uptake and utilization, including glucose transport (GLUT4), glycolysis (phosphofructokinase, PFK), and glucose oxidation (pyruvate dehydrogenase). In contrast, only pyruvate dehydrogenase (PDH) was activated by Cn* (Table ID).
To validate the results of the microarray experiments, real-time PCR was used to determine mRNA expression levels of PPARα and one of its known targets, M-CPT I, following infection with Ad.Cn* or Ad.CaMK* in cardiac myocytes and C2C12 myotubes. Consistent with the transcriptional profiling results, Cn* activated endogenous PPARα expression in both cell types, whereas CaMK* infection had no effect (Fig. 2). Expression of M-CPT I was significantly induced by both Cn* and CaMK* in cardiac myocytes similar to the results of the gene expression array experiments. Interestingly, in contrast to the results in cardiac myocytes, CaMK* had no effect on M-CPT I expression in C2C12 myotubes, although it was robustly induced by Cn* (Fig. 2). In both cell types, there was no additive effect when both Cn* and CaMK* were co-expressed (data not shown).

Cn Activates the PPARα Promoter through MEF2 Transcription Factors—The transcriptional profiling results indicated that the effects of Cn on FA utilization pathways, in contrast with that of CaMK, were mediated at least in part by PPARα. Thus, we sought to characterize the mechanisms by which the Cn pathway was capable of activating PPARα gene expression. To determine whether Cn regulated transcription of the PPARα gene, we performed co-transfection experiments with a human PPARα promoter segment fused to a luciferase gene reporter (Po(H-H)-pGL3 (25)) in either primary rat neonatal cardiac myocytes or C2C12 myotubes with expression plasmids for Cn*, CaMK*, or both. In cardiac myocytes, Cn* induced a 2-fold increase in reporter activity, which was prevented by CsA. CsA also reduced basal activity by nearly 50%. In C2C12 myotubes, co-transfection with Cn* resulted in a nearly 7-fold increase in Po(H-H)-pGL3 activity (Fig. 3B). In striking contrast, co-transfection with CaMK* did not increase Po(H-H)-pGL3 activity. Rather, CaMK*, either alone or in combination with Cn*, reduced Po(H-H)-pGL3 activity in both cell types.

Inspection of 1664 bp of the mouse PPARα promoter region revealed the presence of several potential NFAT recognition and MEF2 response elements, both of which are known to mediate Cn activation (Fig. 3A (1, 29)). Co-transfection experiments utilizing the Po(H-H)-pGL3 reporter construct were repeated in CV1 cells, which are deficient in MEF2 and NFAT, to investigate the sufficiency of these transcription factors for Cn activation. “Add-back” co-transfection experiments were performed using the Po(H-H)-pGL3 construct, Cn*, and constructs expressing either NFAT3 or each of the three MEF2 isoforms (A, C, and D). Neither Cn*, NFAT3, nor MEF2 alone significantly stimulated activity of the Po(H-H)-pGL3 construct. Co-transfection of NFAT3 with either MEF isoform also had no stimulatory effect (data not shown). However, co-transfection of Cn* together with either the MEF2 isoform also had no stimulatory effect (data not shown). However, co-transfection of Cn* together with either MEF2C or MEF2D isoforms, but not NFAT3 or MEF2A, resulted in a greater than 10-fold induction of PPARα promoter activity (Fig. 3C). Collectively, these results demonstrate that MEF2C or MEF2D are sufficient to convey the Cn-mediated increase in transcriptional activity of PPARα.

**Fig. 2. Activation of PPARα and M-CPT I gene expression by Cn*.** Verification of the transcriptional profiling results using quantitative real-time PCR. The graphs depict the results of PPARα and M-CPT I gene expression in cardiac myocytes (A) and C2C12 myotubes (B) infected with control (Ad.GFP), Ad.Cn*, or Ad.CaMK* as indicated at the bottom. Bars represent mean ± S.E. levels of mRNA as arbitrary units normalized (= 1.0) to respective Ad.GFP controls. All data represent the means of at least three independent experiments; * indicates a significant difference from control (p < 0.05).
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Cn Directly Induces Transcription of the PPARα Target Gene, M-CPT I—Many of the fatty acid utilization genes induced by Cn* and CaMK* on the microarray are known targets for the PGC-1α/PPARα complex. The expression of one such gene, encoding muscle-type carnitine palmitoyltransferase I or M-CPT I, which catalyzes the rate-limiting step of fatty acid oxidation, was activated by both Cn and CaMK. We have shown previously that a promoter-reporter construct containing the human M-CPT I gene 5’-flanking region from −781 to −12 bp (MCPT.Luc.781) contains a well-characterized PPARα/PGC-1α-responsive element, FARE-1, located at −775 to −763 bp (Fig. 4A (10, 22)). MCPT.Luc.781 was used in cell co-transfection studies to determine whether the Cn- or CaMK-mediated induction of M-CPT I occurred via direct transcriptional activation. The M-CPT I promoter construct also allowed us to determine whether PPARα was involved. Co-transfection experiments were performed in either primary rat neonatal cardiac myocytes or C2C12 myotubes using MCPT.Luc.781 in the presence of CsA in rat primary neonatal cardiac myocytes or C2C12 myotubes. The bars represent mean RLU normalized (± 1.0) to the results with co-transfection of plasmid backbones alone. C, reconstitution experiments in CV1 cells using the PPARα promoter reporter and expression vectors for Cn*, NFAT3, MEF2A, MEF2C, or MEF2D as shown at the bottom. Bars represent mean (± S.E.) normalized to the control in which only vector backbone was co-transfected. All data represent the means of at least three independent experiments; * indicates a significant difference from control while † indicates a significant difference from Cn* stimulated (p < 0.05).

Fig. 3. Cn activates the PPARα promoter through MEF2 transcription factors. A, a schematic of the human PPARα promoter-reporter construct (Po(H-H)-pGL3). As shown, the human PPARα promoter contains a nuclear receptor-responsive element (HNF4-RE) as well as potential NFAT and MEF2 sites that may act as transducers of Cn signaling. B, the results of studies using co-transfection of Po(H-H)-pGL3 with expression plasmids for Cn* or CaMK* in the presence or absence of CsA in rat primary neonatal cardiac myocytes or C2C12 myotubes. The bars represent mean RLU normalized (= 1.0) to the results with co-transfection of plasmid backbones alone.
absence or presence of Cn*, CaMK*, or both. As anticipated, Cn* induced a significant increase in MCPT.Luc.781 activity in cardiac myocytes. CsA both prevented this activation and significantly reduced basal activity. In C2C12 myotubes, co-transfection with Cn* also resulted in a significant increase in reporter activity and CsA prevented this increase (Fig. 4B). In contrast to the results obtained with Cn*, CaMK* was unable to activate the reporter construct in either cell type and did not further increase Cn*-mediated activation (Fig. 4B). These results indicate that activation of the Cn pathway results in transcriptional induction of the M-CPT I gene. The inability of CaMK* to activate the M-CPT I promoter conflicts with the microarray results and suggests either that CaMK*-mediated increases in PGC-1α expression were insufficient to stimulate

![Diagram of human M-CPT I promoter-reporter construct (MCPT.Luc.781) with PPARα-responsive region (FARE-1) and consensus binding sites for NFAT and MEF2.](https://example.com/diagram.png)

**Fig. 4. Cn transactivates the M-CPT I promoter independent of PPARα.** A, a schematic of the human M-CPT I promoter-reporter construct (MCPT.Luc.781) depicting a well described PPARα-responsive region (FARE-1) as well as consensus binding sites for NFAT and MEF2. B, the results of co-transfection of MCPT.Luc.781 with expression vectors for Cn* or CaMK* in the presence or absence of CsA in rat primary neonatal cardiac myocytes and C2C12 myotubes. C, co-transfection studies in C2C12 myotubes using an expression vector for Cn* with either MCPT.Luc.781 (Wild-type promoter, left) or a mutant version of MCPT.Luc.781 in which a point mutation has rendered the construct unresponsive to PPARα (FARE-1 mutant, right). Bars represent mean RLU (±S.E.) normalized to the respective controls. All data represent the means of at least three independent experiments; * indicates a significant difference from control (p < 0.05).
the reporter construct alone or that elements critical for CaMK activation are not present in the 781-bp region of the M-CPT I promoter.

The gene expression array data demonstrated that Cn induced expression of the PPARα gene. Accordingly, a Cn-mediated increase in the levels of PPARα and its coactivator PGC-1α provides one mechanism whereby Cn activates PPARα targets such as M-CPT I. To investigate this further, co-transfection experiments were repeated in C6C132 myotubes using a mutant promoter-reporter construct, MCPT.Luc.781m1, which contains a point mutation in the upstream half site of the PPARα response element rendering it unable to bind PPARα (22). Surprisingly, Cn activated the MCPT.Luc.781m1 to a similar degree as MCPT.Luc.781 (Fig. 4C). These results indicate that the PPAR-RE is dispensable for Cn-mediated transcriptional activation of the M-CPT I gene. Potential elements mediating the direct Cn effect include consensus binding sites for NFAT and MEF2 that are located in close proximity to the FARE-1 (Fig. 4A). Additional potential NFAT REs are located in each of the first two introns, the first of which is also associated with two CCAC elements that have been previously shown to be involved in regulation of oxidative fiber-type genes (1).

Targeted Deletion of Cn Subunit B in Heart Muscle Reduces PPARα and PGC-1α Gene Expression in Vivo—To investigate the relevance of Cn-mediated control of the PGC-1α/PPARα regulatory axis in vivo, we examined mice in which Cn activity was reduced in heart muscle via targeted deletion of the regulatory subunit of Cn (CnB). To this end, mice in which the Cnb1 gene was flanked with loxP sites (28) were crossed with transgenic mice expressing Cre recombinase under the control of the α-MHC promoter. CnB protein expression in the hearts of Cre-positive mice was reduced by nearly 90% compared with non-transgenic littermate controls (Fig. 5A). The residual levels of CnB likely reflect expression of non-myocyte cells. The mice appeared grossly normal. We next assessed expression of PGC-1α, PPARα, and downstream target genes, M-CPT I and MCAD, using real-time PCR or Northern blot analysis. Both PGC-1α and PPARα gene expression were significantly reduced by ~30% in the hearts of mice lacking Cn activity (Fig. 5B). The PPARα target gene, medium-chain acyl-CoA dehydrogenase (MCAD) showed a similar reduction. Surprisingly, the expression of the M-CPT I gene was unaffected by disruption of Cn signaling. We speculate that this latter result reflects the convergence of multiple regulatory pathways, including CaMK, in the control of this gene (Fig. 5B). These results provide in vivo evidence that CnB is involved in the control of the PGC-1α/PPARα regulatory pathway in postnatal heart.

**DISCUSSION**

Despite emerging evidence for links between the Cn and CaMK signaling pathways, the transcriptional coactivator PGC-1α, and control of muscle energy metabolism, little is known about the biologic role of this regulatory network in high energy utilizing tissues such as heart. Moreover, knowledge of the specific downstream targets of these important signaling pathways is incomplete. In this study, we found that Cn and CaMK activate distinct but overlapping energy metabolic regulatory programs in cardiac myocytes. Consistent with previous studies, our results demonstrated that both Cn and CaMK induced expression of the PGC-1α gene, a known activator of multiple energy metabolic pathways, including mitochondrial biogenesis and respiratory capacity. However, despite similar activation of PGC-1α, Cn and CaMK exerted distinct effects upon downstream energy metabolic target genes, although some overlap was observed. Specifically, Cn activated many genes involved in cellular fatty acid uptake and oxidation, such as CD36, M-CPT I and II, several acyl-CoA dehydrogenases, and acyl-CoA oxidase. In contrast, CaMK stimulated the expression of genes involved in glucose metabolism (GLUT4,
PFN, and PDH) and mitochondrial respiratory function (tri-chloroacetic acid cycle enzymes and components of the electron transport chain).

Surprisingly, Cn but not CaMK was shown to activate a mouse PGC-1α promoter construct in cardiac myocytes despite the fact that CaMKα was shown to activate endogenous PGC-1α expression. The reason for this apparent discrepancy is unknown. It is possible that CaMK-mediated activation of PGC-1α gene expression does not occur via transcriptional mechanisms. However, others have shown that CaMK activates a similar PGC-1α promoter reporter in myogenic cell lines (7, 12). It is also possible that the regulatory elements necessary for CaMK-mediated activation of PGC-1α transcription are missing from the reporter construct used or that the duration or degree of CaMK activation achieved with the adeno-viral expression system is different than that with the transient expression plasmid. The C2C12 and cardiac myocyte culture systems employed in this study should prove useful for future studies aimed at characterizing the mechanisms responsible for the cell-specific behavior of CaMK on PGC-1α gene expression.

We identified several potential mechanisms involved in the selective activation of downstream targets by Cn. Cn, but not CaMK, activated expression of the PPARα gene in cardiac myocytes and C2C12 myotubes. PPARα is a known PGC-1α target and master regulator of genes involved in every step of cellular fatty acid utilization. Indeed, many of the genes shown to be induced by Cn in the transcriptional profiling experiments are known PPARα targets (Table I). The effect of Cn-mediated activation of PPARα gene expression was shown to occur through direct transcriptional activation and likely involves MEF2 transcription factors. MEF2 has been shown previously to mediate the transcriptional regulatory effects of Cn on the PGC-1α gene (12). In addition, the levels of mRNA encoding PGC-1α and PPARα were reduced in mice with cardiac-specific elimination of Cn activity confirming the relevance of this regulatory circuit in vivo.

We also found that Cn is capable of directly activating transcription of the PPARα target gene encoding M-CPT I, independent of PPARα. These results provide a second potential mechanism for the selective activation of cellular fatty acid utilization genes by Cn. M-CPT I catalyzes the rate-limiting step in mitochondrial fatty acid oxidation and is a nodal point of regulation at both the transcriptional and post-transcriptional levels. Multiple transcription factors have been shown to regulate M-CPT I gene expression, including GATA4, MEF2, SRF (30), and PPARα (22). Based on the presence of consensus binding sites in the human M-CPT I promoter, MEF2 and NFAT proteins are likely candidates for mediating the PPARα-independent activation of this gene by Cn. However, we cannot exclude the possibility that Cn-mediated activation of PGC-1α/PPARα regulates M-CPT I gene expression in certain contexts. It is possible that the direct regulation of M-CPT I gene expression by Cn is a mechanism for immediate activation of cardiac and skeletal muscle energy production in response to Ca2⁺-triggered signaling, whereas Cn-mediated induction of PGC-1α/PPARα could lead to a sustained adaptive response.

The results of recent studies have shown that calcium signaling events activate the metabolic and structural components of the skeletal muscle slow-twitch fiber program, including induction of mitochondrial biogenesis. However, the effects of calcium signaling through Cn and CaMK in cardiac muscle are less clear. Our results provide new information about the role of the Cn and CaMK signaling pathways in heart. Both Cn and CaMK signaling have been implicated in the cardiac hypertrophic growth response. Overexpression of activated Cn or CaMK in the hearts of transgenic mice results in profound cardiac hypertrophy leading to ventricular dysfunction (14, 15). Moreover, mitochondria isolated from Cn⁺-expressing, hypertrophied hearts exhibit impaired function (16). These results and additional studies showing that cardiac mitochondrial fatty acid oxidation is down-regulated in the hypertrophied and failing heart suggest that activation of Cn or CaMK leads to a reduction rather than augmentation in capacity for oxidative energy production. However, our gene expression profiling results indicate that both Cn and CaMK are capable of up-regulating a broad array of genes involved in mitochondrial

![Diagram](http://www.jbc.org/)

**Fig. 6. Scheme for the metabolic gene regulatory network downstream of Cn and CaMK.** Both Cn and CaMK signaling lead to enhanced expression of the transcriptional coactivator PGC-1α. Cn also directly activates expression of the PPARα gene and certain downstream target genes involved in cellular fatty acid utilization. CaMK up-regulates the expression of a broad program of mitochondrial oxidation genes as well as genes involved in glucose utilization through both NRF-1 and mechanisms that remain to be explored.
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energy production in cardiac myocytes. Indeed, our results were notable for a near complete absence of down-regulated metabolic genes. This conclusion was further supported by our observation that expression of the PGC-1α and PPARα genes was down-regulated in mice with cardiac-specific disruption of the CNB gene. These results, together with the known effects of these signaling pathways as activators of mitochondrial energy production capacity in skeletal muscle, are inconsistent with the predicted metabolic effects related to chronic activation of these pathways. We speculate, therefore, that the short term effects of Cn and CaMK signaling are distinct from the chronic activation that occurs in transgenic systems. Alternatively, distinct transcriptional targets could be activated in different physiological contexts in the heart. In support of this latter notion, a recent report utilizing NFAT reporter mice demonstrated that NFAT activity, an indirect measure of a select target of Cn signaling, is activated in pathological but not exercise-induced cardiac hypertrophy (31). Our results would predict that Cn acts through PPARα and MEF2 during developmental and physiological forms of cardiac hypertrophic growth in which mitochondrial energy production keeps pace with cardiac growth (32–34).

In summary, our results demonstrate a role for Cn and CaMK signaling in the gene regulatory circuitry controlling cardiac energy metabolism. We propose the following model for the gene regulatory effects of Cn and CaMK in the heart (Fig. 6). Both Cn and CaMK activate expression of PGC-1α, a key regulator of cellular energy metabolism through nuclear receptor (e.g. PPARα) and non-nuclear receptor (e.g. NRF-1) transcription factors. However, the metabolic programs activated by each factor exhibit distinct features: Cn directs the expression of genes encoding fatty acid utilization enzymes, whereas CaMK activates glucose utilization and mitochondrial respiratory program proteins. This selectivity may occur in part because PGC-1α alone does not stimulate gene transcription but must act in concert with nuclear receptors and transcription factors. One mechanism by which Cn could activate a distinct program is through the activation of PPARα, an important regulator of fatty acid utilization genes, which is not induced by CaMK. Second, Cn is able to directly activate transcription of at least a subset of fatty acid utilization enzyme genes, demonstrating multiple levels of regulation by Cn. Similar mechanisms are likely relevant to the selective activation of targets by CaMK.

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