Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 1305–1309, February 1992
Cell Biology

Transcriptional activation of the cardiac myosin light chain 2 and atrial natriuretic factor genes by protein kinase C in neonatal rat ventricular myocytes

HUDA E. SHUBEITA*, ELIZABETH A. MARTINSON†, MARC VAN BILSEN*, KENNETH R. CHIEN*‡§, AND JOAN HELLER BROWN†¶

Departments of †Pharmacology and *Medicine, ‡AHA-Bugher Foundation Center for Molecular Biology, and §Center for Molecular Genetics, University of California at San Diego, School of Medicine, La Jolla, CA 92093

Communicated by Daniel Steinberg, October 21, 1991

ABSTRACT **A cultured myocardial cell model was used to examine the role of protein kinase C-dependent pathways in the transcriptional activation of two cardiac muscle genes [myosin light chain 2 (MLC-2) and atrial natriuretic factor (ANF)] during α-adrenergic receptor-mediated hypertrophy. Phorbol ester (phorbol 12-myristate 13-acetate) and the α-adrenergic agonist phenylephrine both activate protein kinase C (PKC) and induce 4- to 5-fold increases in the expression of MLC-2 and ANF promoter/luciferase reporter genes with little effect on Rous sarcoma virus/luciferase or minimal prolactin promoter/luciferase genes. To further assess the role of PKC in cardiac gene regulation, PKC expression vectors encoding constitutively activated PKC-α or PKC-β, or a catalytically inactive PKC, were transiently cotransfected with the cardiac promoter/luciferase constructs. Cotransfection of either activated PKC-α or PKC-β cDNA induces the expression of MLC-2 and ANF promoter/luciferase genes and of a reporter gene responsive to the transcription factor AP-1. The Rous sarcoma virus/luciferase and minimal prolactin promoter/luciferase genes are not concomitantly induced by cotransfectin with the PKC genes, indicating specificity of the transcriptional effect. The finding that activated PKC increases cardiac gene transcription suggests that activation of this enzyme may be a proximal signal for coregulation of two cardiac genes, MLC-2 and ANF, during the course of myocardial cell hypertrophy.**

In response to a variety of trophic signals, including certain hormones and mechanical overload, ventricular cardiac muscle cells exhibit a hypertrophic response that is characterized by an up-regulation of constitutively expressed contractile protein genes and the reactivation of an embryonic gene program (1–5). The signaling mechanisms that mediate these alterations in cardiac gene expression are unknown. To define the molecular mechanisms that activate cardiac gene transcription during myocardial cell hypertrophy, recent studies have employed a neonatal rat ventricular cell model in which several features of hypertrophy can be induced by defined agonists (6–8). Stimulation of the α_1-adrenergic receptor leads to an increase in myocardial cell size (6, 9) and an increase in the accumulation and assembly of myosin light chain 2 (MLC-2) into organized contractile units (8). Accompanying these phenotypic alterations is the acceleration of protein synthesis (10, 11), the rapid and transient expression of immediate early genes (c-*fos*, c-*jun*, and *Egr-1*) (12), the induction of constitutively expressed contractile protein genes such as MLC-2 (8), and activation of the expression of embryonic genes such as atrial natriuretic factor (ANF) (13, 14), skeletal α-actin (15–17), and β-myosin heavy chain (18). As indicated by transient transfection assays with the MLC-2 or ANF promoter/luciferase reporter gene constructs, stimulation with the α-adrenergic agonist phenylephrine or with the cardioactive peptide endothelin 1 leads to transcriptional activation through defined promoter regions of these respective cardiac target genes (13, 19). The changes in MLC-2 and ANF expression do not reflect a generalized increase in transcription, but rather appear to be due to the presence of specific cis sequences within their respective promoters that mediate inducible expression (13, 20). Presumably, occupancy of the α_1-adrenergic receptor leads to the generation of signals that reach the nucleus and specifically activate the transcription of these cardiac genes. The biochemical nature of these signals is currently unclear.

Agonists that activate a hypertrophic response (phenylephrine, norepinephrine, endothelin 1, and angiotensin II) have all been demonstrated to stimulate inositol phospholipid hydrolysis in the cardiac system (19, 21–28). In neonatal rat cardiomyocytes, these agonists have been shown to cause the generation of the known second messengers inositol 1,4,5-trisphosphate and diacylglycerol (14, 19, 23, 28). The fact that these diverse hypertrophic stimuli share a common signaling pathway suggests the involvement of this pathway in the regulation of cardiac gene expression. Recent studies (29, 30) have shown that norepinephrine causes redistribution of protein kinase C (PKC) from a cytosolic to a particulate fraction in ventricular myocytes, consistent with the notion that PKC is also activated in this signaling cascade. In addition, phorbol esters, which directly activate PKC, induce many of the phenotypic features of hypertrophy (31), further implicating PKC in the hypertrophic response. However, there is no direct evidence that the activation of PKC is a necessary or sufficient signal for the induction of MLC-2 or ANF by hypertrophic agents.

The current study addresses this question by determining whether the activation of PKC by phorbol ester or by expression of genes encoding constitutively activated PKC leads to transcriptional activation of ANF and MLC-2 promoter/luciferase fusion genes. Our data demonstrate that both the MLC-2 and ANF promoter/luciferase genes as well as an AP-1 promoter/luciferase gene are induced in rat ventricular myocytes expressing constitutively activated PKC genes or stimulated with phorbol ester. This study identifies the activation of PKC as a common proximal signal for the coregulation of an embryonic and constitutive gene program in cardiac myocytes. These results have been reported in preliminary form (32).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: MLC-2, myosin light chain 2; ANF, atrial natriuretic factor; PKC, protein kinase C; RSV, Rous sarcoma virus; PRL, minimal prolactin promoter; CMV, cytomegalovirus; PMA, phorbol 12-myristate 13-acetate.

¶To whom reprint requests should be addressed at: Department of Pharmacology 0636, University of California at San Diego School of Medicine, La Jolla, CA 92093.

MATERIALS AND METHODS

Cell Culture. Neonatal ventricular cells were cultured and plated at a density of 3.4×10^4 cells per cm^2, in 4:1 Dulbecco's modified Eagle's medium/medium 199 (GIBCO) supplemented with 10% (vol/vol) horse serum, 5% (vol/vol) fetal calf serum, and antibiotics (ampicillin at 34 μg/ml and gentamicin at 3 μg/ml) as described (12).

PKC Assay. Twenty hours after plating in 35-mm plates, cells were serum-starved for 24 h. PKC was then assayed as described by Heasley and Johnson (33). Briefly, cells were exposed to agonists and then permeabilized with 0.01% saponin in an intracellular buffer in the presence of 50 μCi of [γ-^{32}P]ATP per ml and 150 μM PKC peptide substrate (Bachem Bioscience, Philadelphia) as described (34). The sequence of the peptide, Val-Arg-Lys-Arg-Thr-Leu-Arg-Arg-Leu, is based on the PKC-specific phosphorylation site of the epidermal growth factor receptor (35, 36). Reactions were terminated after various times by addition of trichloroacetic acid to a final concentration of 5%, and samples were applied to P-81 phosphocellulose filter paper strips (Whatman). After washing, the ^{32}P content of the strips was determined by liquid scintillation spectroscopy.

Isolation and Hybridization of RNA. Total RNA isolation and Northern blot analysis were performed as described (37, 38) using ANF (39) and MLC-2 (40) cDNA probes labeled with ^{32}P by random priming (41). The extent of hybridization was quantified by densitometry of the corresponding autoradiograms. Ribosomal RNA on the filters was stained with methylene blue to detect potential differences in loading and/or transfer efficiencies.

Plasmid Constructs. To assess the transcriptional activation of the MLC-2 and ANF promoters, the following plasmid constructs were used: pMLC(−2700 to +12)LΔ5′, an MLC-2/luciferase fusion gene consisting of the most proximal 2.7 kilobases of the MLC-2 5′-flanking region (42) inserted into the firefly luciferase reporter vector pSVOALΔ5′ (43); and pANF(−638)LΔ5′, an ANF/luciferase fusion gene (44) composed of a 638-base pair fragment of the ANF 5′-flanking region that was isolated from an ANF genomic clone (45) (generously provided by P. L. Davies, Department of Biochemistry, Queen's University, Kingston, Canada) and inserted into the luciferase reporter vector pSVOALΔ5′. As a negative control, we used an RSV/luciferase construct, pRSVLΔ5′, composed of the Rous sarcoma virus (RSV) promoter inserted into the luciferase reporter vector pSVOALΔ5′ (43). As a positive control, we used a 2×-AP-1/luciferase construct [TRE2PRL(−36), provided by M. G. Rosenfeld, University of California, San Diego], which contains the firefly luciferase cDNA under the control of a minimal rat prolactin promoter (46) that is preceded by two copies of the AP-1-responsive element that confers inducibility by phorbol ester. A PRL/luciferase construct [PRL(−36)] containing the firefly luciferase cDNA under the control of only a minimal prolactin promoter (PRL) (46) was used to control for the potential contributions from cryptic sequences within the plasmid vector in TRE2PRL(−36).

To examine the involvement of PKC in the inducible expression of MLC-2 and ANF, we used expression plasmids harboring different forms of constitutively activated PKC [PKAC and ΔPKCβ (47)] or a plasmid expressing a catalytically inactive form of PKC [PKCβΔOP (48)]. These PKC expression vectors were generous gifts of M. Muramatsu (DNAX Research Institute, Palo Alto, CA).

A β-galactosidase expression vector under the control of the human cytomegalovirus (CMV) promoter, pON249 (49), was cotransfected into cardiac myocytes and used as a control for transfection efficiency.

Transfection of Myocardial Cells and Assay of Reporter Gene Products. Eighteen to 20 h after plating, ventricular myocytes were switched to fresh medium containing serum. Two to 3 h later, the cells were transiently transfected for 18–20 h using a modified calcium phosphate method (50). The cells were then washed and cultured in serum-free medium in the presence or absence of agonist until time of harvest 48 h later. Transfected cells were washed twice with phosphate-buffered saline and then harvested in extraction buffer as described (19). Luciferase activity (43) in the cytosolic fraction was measured in triplicate using a Monolight 401 luminometer (Turner, Palo Alto, CA). β-Galactosidase activity in the same fraction was assayed as described (51).

RESULTS

Effects of Phenylephrine and Phorbol Ester on PKC Activity. α_1-Adrenergic receptor activation by phenylephrine stimulates inositol phospholipid hydrolysis and increases the diacylglycerol content of neonatal cardiomyocytes (14). To determine whether phenylephrine also increases PKC activity in this preparation, we exposed cells to phenylephrine for various time periods and subsequently assayed PKC activity by measuring phosphorylation of an exogenous PKC substrate peptide. As shown in Fig. 1*A*, phenylephrine treatment leads to a modest increase in the incorporation of ^{32}P into the peptide substrate, which is significantly above the control level at 15 min. Treatment with the phorbol ester phorbol 12-myristate 13-acetate (PMA) for 15 min leads to a 14-fold activation of PKC as assessed by phosphorylation of the peptide. When cells are treated with PMA for longer than 1 h, PMA-stimulated phosphorylation diminishes and is no longer significant after 6 h (Fig. 1*B*). These data are consistent with previous observations that PMA first stimulates but subsequently down-regulates PKC in a variety of cell types including cardiac myocytes (29, 52–54). In contrast, prolonged α-adrenergic receptor stimulation does not similarly

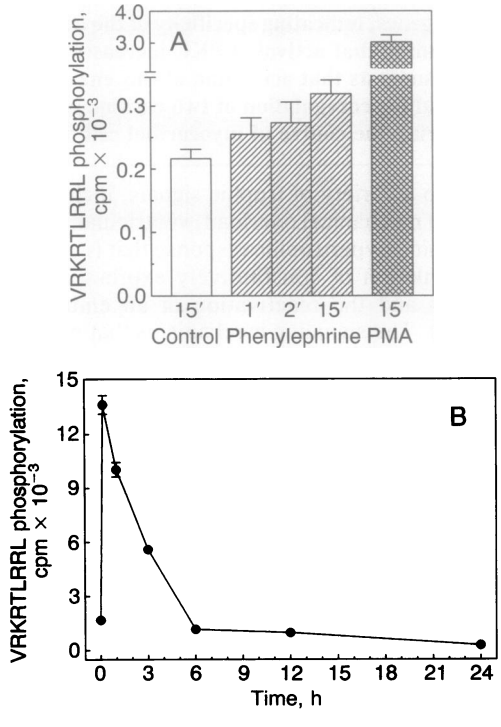

FIG. 1. Stimulation of cardiac myocyte PKC by phenylephrine and PMA. (*A*) Cells were exposed to 100 μM phenylephrine, 1 μM PMA, or control vehicle (0.1% dimethyl sulfoxide) in serum-free medium for the indicated times, permeabilized, and incubated with [γ-^{32}P]ATP and peptide substrate for 10 min. (*B*) Cells were exposed to PMA for the indicated times and assayed for PKC activity as in *A*. Similar results were obtained in three or four identical experiments.

down-regulate PKC (J. H. B., unpublished observations and ref. 29).

Transcriptional Activation of MLC-2 and ANF Promoter/Luciferase Constructs by Phenylephrine and Phorbol Ester. Treatment of rat ventricular myocytes with phenylephrine or the phorbol ester PMA for 48 h leads to a marked increase in cell size and a 2- to 3-fold increase in total cellular RNA content (data not shown). Phenylephrine treatment results in increased accumulation of ANF mRNA (17-fold) and MLC-2 mRNA (5-fold) [Fig. 2 and as described (8, 13)]. PMA also leads to a 12-fold increase in ANF mRNA levels. MLC-2 mRNA increases only 2- to 3-fold with PMA treatment, in parallel with the increase in total cellular RNA.

To determine whether phenylephrine and PMA lead to increased transcription of the respective cardiac genes, we evaluated the ability of phorbol esters and phenylephrine to activate the expression of luciferase reporter genes under the control of MLC-2 and ANF promoter sequences. The cardiac promoter constructs contain cis sequences that are sufficient to confer inducible expression by either phenylephrine or endothelin 1 in transient transfection assays of neonatal rat myocardial cells (13, 19). After transfection with the appropriate promoter/reporter gene constructs, cells were treated with PMA or phenylephrine and harvested 48 h later for the measurement of luciferase and β-galactosidase activities. Phenylephrine treatment results in a 5-fold increase in expression of both ANF and MLC-2/luciferase constructs. Both cardiac promoter/luciferase genes are also induced 4- to 5-fold by the PKC activator PMA. However, whereas PMA is more effective at inducing transcription through 2×AP-1 than through the cardiac promoters, phenylephrine is a more effective activator of ANF and MLC-2 promoters than of the PKC-regulated 2×AP-1 promoter (Fig. 3). Neither the PRL/luciferase gene nor the RSV/luciferase construct is induced

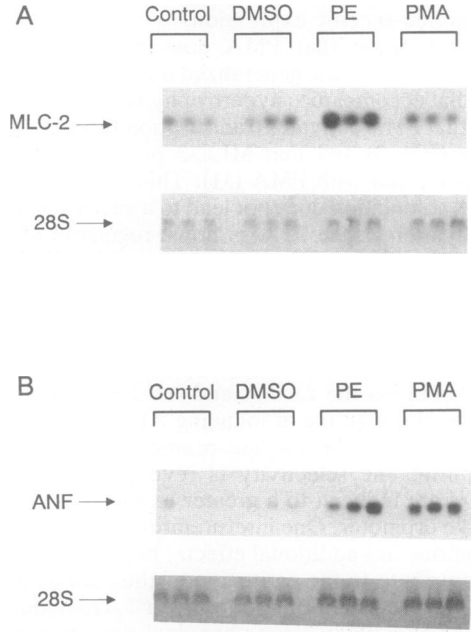

FIG. 2. MLC-2 and ANF mRNA levels of myocardial cells after stimulation with phenylephrine or PMA. Twenty-four hours after plating, triplicate 150-mm plates of cells were extensively washed and incubated in serum-free medium (Control) with 100 μM phenylephrine (PE), 100 nM PMA, or 0.1% dimethyl sulfoxide (DMSO) vehicle. Cells were harvested after 48 h, and total RNA was isolated. Effects of phenylephrine or PMA are shown in *A* for MLC-2 mRNA and in *B* for ANF mRNA. Methylene blue staining of the 28S ribosomal bands shows that loading and transfer of RNA samples were comparable between lanes. Results are representative of four additional experiments.

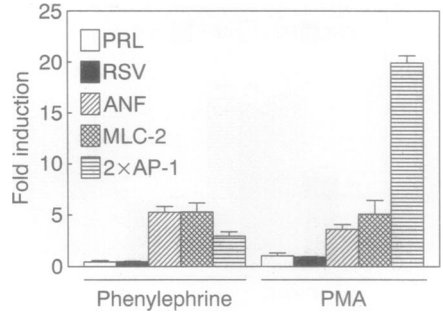

FIG. 3. Activation of ANF and MLC-2 promoters by phenylephrine and PMA in ventricular myocytes. Cells were cotransfected for 18–20 h with one of the indicated promoter/luciferase constructs (20 μg per dish) and the CMV/β-galactosidase construct (4 μg per dish) and then incubated in serum-free medium with vehicle (0.1% dimethyl sulfoxide), phenylephrine (100 μM), or PMA (100 nM) for 48 h. Cells were harvested and luciferase and β-galactosidase activities in the cell cytosolic fraction were then assayed. Luciferase activity was normalized to the corresponding β-galactosidase activity for each sample, and this value was used to calculate fold induction above activity in control cells. Basal expression of luciferase for vehicle-treated cells was 0.13 (PRL), 83 (RSV), 1.5 (ANF), 7.9 (MLC-2), and 1.5 (2×AP-1) in arbitrary light units. Data represent means ± SEM from three or four similar experiments.

by phenylephrine or PMA, indicating that the response to these agents occurs through cis regulatory elements specific to the ANF, MLC-2, and 2×AP-1 promoters.

Transcriptional Activation by the Expression of Constitutively Activated Forms of PKC. To determine directly whether PKC activation enhances transcription of the ANF and MLC-2 genes, cells were cotransfected with mutant PKC cDNA expression vectors in combination with the cardiac promoter/luciferase fusion genes. The mutant PKC vector PKAC encodes a PKC-α isoform in which the regulatory domain has been deleted. The unregulated gene product has been shown to be constitutively activated (47). Another constitutively activated PKC gene, ΔPKCβ, encodes a β isoform of PKC that is activated by truncation of the regulatory domain. A third construct contains the cDNA encoding PKC that is inactive due to deletion of residues in the catalytic domain (PKCβΔOP). Cotransfection of cardiac promoter/reporter genes with this latter construct results in low basal levels of luciferase activity. The levels of reporter gene activity obtained with PKCβΔOP were used as control values, and the activity in cells transfected with either PKAC or ΔPKCβ was expressed relative to that measured in PKCβΔOP-transfected cells. The data shown in Fig. 4 demonstrate that cotransfection with either of the two constitutively activated PKC vectors enhances the expression of the ANF and MLC-2 promoter/luciferase genes by 2- to 3-fold. There is no significant activation of RSV/luciferase in cells transfected with either PKAC or ΔPKCβ, thereby indicating that the effect is not due to a generalized increase in transcriptional activity. Expression of the constitutively activated PKC genes also induces luciferase activity in cells cotransfected with the 2×AP-1/luciferase construct (Fig. 4).

DISCUSSION

The observation that the ANF and MLC-2 genes are induced by ligands that activate the inositol phospholipid signaling pathway in neonatal ventricular myocytes suggested that the activation of PKC might be a proximal signal for the coregulation of these genes during myocardial cell hypertrophy. In the current study we provide several lines of evidence that activation of PKC regulates transcription of these two cardiac genes in the ventricular cardiac cell. First, we demonstrate that mRNA levels, at least for ANF, are significantly in-

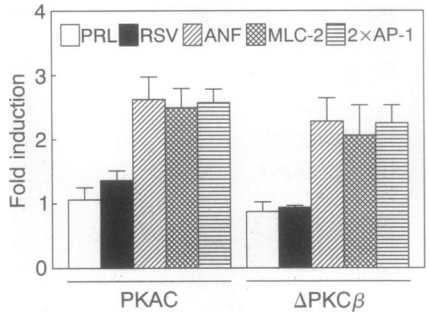

FIG. 4. Activation of ANF and MLC-2 promoters by expression of constitutively activated PKC isozymes in ventricular myocytes. Cells were plated and transfected with 4 μg of the indicated promoter/luciferase constructs, 4 μg of the CMV/β-galactosidase construct and 16 μg of the indicated PKC expression vectors. After transfection, cells were incubated for 48 h in serum-free medium, harvested, and assayed for reporter gene expression. Luciferase activity was normalized to the corresponding β-galactosidase activity for each sample as in Fig. 3. Data are expressed as fold induction above activity in cells cotransfected with PKCβΔOP and are means ± SEM from eight similar experiments. Luciferase activity in PKCβΔOP-transfected plates was 0.06 (PRL), 12 (RSV), 0.14 (ANF), 0.27 (MLC-2), and 0.083 (2 × AP-1) in arbitrary light units.

creased in cells treated with PMA to activate PKC. Secondly, using MLC-2 and ANF promoter/luciferase constructs, we demonstrate that PMA treatment leads to transcriptional activation of these cardiac genes. Finally, we present direct evidence that PKC activation leads to increased expression of these two genes in studies using expression vectors for constitutively activated PKC.

Rat heart has been shown to contain α, β, and γ forms of PKC (55, 56). In the present study, we find that either the activated α or the activated β isoform of PKC stimulate transcription through the ANF and MLC-2 promoters. This finding is consistent with the observation of Muramatsu *et al.* (47) that the two constitutively activated isoforms have comparable effects on c-*fos*/chloramphenicol acetyltransferase fusion gene expression and oocyte maturation. In a recent study by Simpson and colleagues (57), the two isoforms of PKC were also found to stimulate the expression of an AP-1/chloramphenicol acetyltransferase fusion gene in neonatal rat ventricular myocytes to a similar extent. These authors reported, however, that the β isoform of PKC is more effective at activating β-myosin heavy chain gene expression (57). It has been shown that PKC isoforms have different subcellular localizations in some tissues (30, 58–61), and it is hypothesized that the PKC isoforms might differ with respect to their endogenous substrates (62–64). If the isoforms of PKC differ in their capacity to regulate various target genes, this could account for differences between β-myosin heavy chain and MLC-2 or ANF gene regulation.

The mechanism by which PKC activation leads to increases in gene expression is a subject of considerable interest. Initially it was observed that treating cells with phorbol ester tumor promoters leads to the rapid induction of several protooncogenes (65, 66) as well as specific target genes (67, 68). A cis-acting element capable of conferring phorbol ester inducibility was identified in the metallothionein gene promoter and found to bear homology to sequences in other phorbol ester-inducible genes (69, 70). The transcriptional activator that binds to the phorbol ester-responsive elements was identified, and this factor, AP-1, has been shown to be largely composed of the protein product of the c-*jun* protooncogene (70, 71), which dimerizes with the product of the c-*fos* protooncogene (72–74). Another phorbol ester-sensitive site has been identified that binds a transcription factor termed AP-2 (75). Both AP-1 and AP-2-like sites are found within the promoter regions of the ANF and MLC-2 genes (13, 20), suggesting a potential role for PKC in the regulation of this gene during myocardial cell hypertrophy.

The mechanism by which phorbol esters regulate gene expression could involve PKC-mediated increases in c-*jun* transcription or posttranslational modification of the c-Jun protein. Phorbol esters have been shown to increase c-*jun* mRNA and protein in various cell types (76, 77), including cardiac myocytes (31). Changes in c-Jun protein phosphorylation may also be induced either directly through PKC or secondarily through PKC-dependent activation of other kinases or phosphatases (78). An additional mechanism by which PKC could regulate AP-1-responsive genes would be through modulation of c-*fos* protooncogene expression or posttranslational modification of c-Fos protein (65, 72, 79). In the cardiac myocyte, phorbol esters also induce c-*fos* mRNA accumulation (1, 31).

Alternatively, cardiac-specific factors that bind to other cis-regulatory elements within the 5′-flanking regions of the ANF and MLC-2 promoters may be required. These factors could work independently of, or synergistically with, putative AP-1 and AP-2 sites found in the ANF and MLC-2 promoters (13, 20). Recent studies have identified two conserved cis elements within a 250-base-pair fragment of the rat cardiac MLC-2 gene (HF-1 and HF-2) that mediate cardiac-specific and inducible expression during myocardial hypertrophy (20). The 28-base-pair element HF-1 is sufficient to confer both inducible and cardiac-specific responses to a neutral promoter, suggesting that the transcriptional factors that mediate muscle specificity might also be involved in inducible expression. It will be of interest to determine whether PKC-inducible expression is due to covalent modification of HF-1 or whether it reflects additional interactions between transcriptional factors. In addition, it will be of interest to determine if the factors that mediate inducible expression of an embryonic gene, such as ANF, will also confer cardiac-specific expression.

The observation that PMA does not increase MLC-2 mRNA levels above the generalized increase in cellular RNA content that accompanies hypertrophy is at variance with the findings that PMA activates transcription through the MLC-2 promoter (Fig. 3) and that MLC-2 protein is increased in myocytes treated with PMA (31). This may reflect the fact that PMA stimulation does not lead to a sustained increase in PKC activation, since PKC is down-regulated after 6 h of PMA treatment. Accordingly, an increase in MLC-2 mRNA may only be detectable for a brief period of time. The stability of the MLC-2 mRNA could also be concomitantly decreased by PMA treatment.

It is informative to compare the increases in gene expression mediated via the cardiac and AP-1 promoters (Fig. 3). PMA is highly effective at inducing AP-1 but is a relatively weak activator of the cardiac promoters. In contrast, for phenylephrine the selectivity is reversed and the cardiac promoters are induced to a greater extent than is the AP-1-responsive promoter. One interpretation of these data is that phenylephrine has additional effects, beyond those on PKC, which contribute to the activation of the cardiac genes. We recently reported that inhibition of Ca^{2+}/calmodulin kinase by W-7 can effectively block phenylephrine-stimulated ANF expression and that the calcium channel activator BAY K 8644 can stimulate ANF gene expression (14). These data suggest that a Ca^{2+}/calmodulin-dependent pathway may also be activated by phenylephrine and can potentially contribute to the effect of α_1-receptor stimulation on cardiac gene expression. On the other hand, inhibition of PKC by H-7 inhibits phenylephrine-induced increases in ANF mRNA and the associated production of this protein in neonatal ventricular myocytes (14), supporting a role for PKC in cardiac gene expression.

H.E.S. and E.A.M. made equal scientific contributions to this study. We would like to thank Dr. M. Muramatsu for providing the PKC expression vectors and Dr. M. G. Rosenfeld for providing the 2×AP-1/luciferase reporter construct. We also thank D. Goldstein and T. Wright for expert technical assistance. This work was supported by National Institutes of Health Grants HL28143 (J.H.B.) and HL36139, HL45609, and HL46345 (K.R.C.) and a grant from the Tobacco Related Disease Research Program (J.H.B.). K.R.C. is an Established Investigator of the American Heart Association. E.A.M. and M.V.B. are recipients of fellowships from the California Affiliate of the American Heart Association, and M.V.B. is a recipient of a NATO-Science Fellowship.

1. Komuro, I., Katoh, Y., Kaida, T., Shibazaki, Y., Kurabayashi, M., Hoh, E., Takaku, F. & Yazaki, Y. (1991) *J. Biol. Chem.* **266,** 1265–1268.
2. Izumo, S., Nadal-Ginard, B. & Mahdavi, V. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 339–343.
3. Schwartz, K., de la Bastie, D., Bouveret, P., Oliviero, P., Alonso, S. & Buckingham, M. (1986) *Circ. Res.* **59,** 551–555.
4. Schiaffino, S., Samuel, J. L., Sassoon, S. D., Lompre, A. M., Marotte, I. G. F., Buckingham, M., Rappaport, L. & Schwartz, K. (1989) *Circ. Res.* **64,** 937–948.
5. Chien, K. R., Knowlton, K. U., Zhu, H. & Chien, S. (1991) *FASEB J.* **5,** 3037–3046.
6. Simpson, P., McGrath, A. & Savion, S. (1982) *Circ. Res.* **51,** 787–801.
7. Simpson, P. C., Long, C. S., Waspe, L. E., Henrich, C. J. & Ordahl, C. P. (1989) *J. Mol. Cell. Cardiol.* **21,** Suppl. 5, 79–89.
8. Lee, H. R., Henderson, S. A., Reynolds, R., Dunnmon, P., Yuan, D. & Chien, K. R. (1988) *J. Biol. Chem.* **263,** 7352–7358.
9. Simpson, P. (1983) *J. Clin. Invest.* **72,** 732–738.
10. Simpson, P. (1985) *Circ. Res.* **56,** 884–894.
11. Meidell, R. S., Sen, A., Henderson, S. A., Slahetka, M. F. & Chien, K. R. (1986) *Am. J. Physiol.* **251,** H1076–H1084.
12. Iwaki, K., Sukhatme, V. P., Shubeita, H. E. & Chien, K. R. (1990) *J. Biol. Chem.* **265,** 13809–13817.
13. Knowlton, K. U., Baracchini, E., Ross, R. S., Harris, A. N., Henderson, S. A., Evans, S. M., Glembotski, C. C. & Chien, K. R. (1991) *J. Biol. Chem.* **266,** 7759–7768.
14. Sei, C. A., Irons, C. E., Sprenkle, A. B., McDonough, P. M., Brown, J. H. & Glembotski, C. G. (1991) *J. Biol. Chem.* **266,** 15910–15916.
15. Bishopric, N. H., Simpson, P. C. & Ordahl, C. P. (1987) *J. Clin. Invest.* **80,** 1194–1199.
16. Long, C. S., Ordahl, C. P. & Simpson, P. C. (1989) *J. Clin. Invest.* **83,** 1078–1082.
17. Bishopric, N. H. & Kedes, L. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 2132–2136.
18. Waspe, L. E., Ordahl, C. P. & Simpson, P. C. (1990) *J. Clin. Invest.* **85,** 1206–1214.
19. Shubeita, H. E., McDonough, P. M., Harris, A. N., Knowlton, K. U., Glembotski, C. C., Brown, J. H. & Chien, K. R. (1990) *J. Biol. Chem.* **265,** 20555–20562.
20. Zhu, H., Garcia, A. V., Ross, R. S., Evans, S. M. & Chien, K. R. (1991) *Mol. Cell. Biol.* **11,** 2273–2281.
21. Brown, J. H., Buxton, I. L. & Brunton, L. L. (1985) *Circ. Res.* **57,** 532–537.
22. Brown, J. H. & Jones, L. G. (1986) *Phosphoinositides and Receptor Mechanisms*, ed. Putney, J. W. (Liss, New York), pp. 245–270.
23. Baker, K. M., Singer, H. A. & Aceto, J. F. (1989) *J. Pharmacol. Exp. Ther.* **251,** 578–585.
24. Allen, I. S., Cohen, N. M., Dhallan, R. S., Gaa, S. T., Lederer, W. J. & Rogers, T. B. (1988) *Circ. Res.* **62,** 524–535.
25. Heathers, G. P., Evers, A. S. & Corr, P. B. (1989) *J. Clin. Invest.* **83,** 1409–1413.
26. Woodcock, E. A., White, L. B. S., Smith, A. I. & McLeod, J. K. (1987) *Circ. Res.* **61,** 625–631.
27. Poggioli, J., Sulpice, J. C. & Vassort, G. (1986) *FEBS Lett.* **206,** 292–298.
28. Steinberg, S. F., Kaplan, L. M., Inouye, T., Zhang, J. F. & Robinson, R. B. (1989) *J. Pharmacol. Exp. Ther.* **250,** 1141–1148.
29. Henrich, C. J. & Simpson, P. C. (1988) *J. Mol. Cell. Cardiol.* **20,** 1081–1085.
30. Mochly-Rosen, D., Henrich, C. J., Cheever, L., Khaner, H. & Simpson, P. C. (1990) *Cell Regul.* **1,** 693–706.
31. Dunnmon, P. M., Iwaki, K., Henderson, S. A., Sen, A. & Chien, K. R. (1990) *J. Mol. Cell. Cardiol.* **22,** 901–910.
32. Shubeita, H. E., Chien, K. R., Martinson, E. A. & Brown, J. H. (1990) *J. Cell. Biol.* **111,** 213 (abstr.).
33. Heasley, L. E. & Johnson, G. L. (1989) *J. Biol. Chem.* **264,** 8646–8652.
34. Trilivas, I., McDonough, P. M. & Brown, J. H. (1991) *J. Biol. Chem.* **266,** 8431–8438.
35. Davis, R. J. & Czech, M. P. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 1974–1978.
36. Cochet, C., Gill, G. N., Meisenhelder, J., Cooper, J. A. & Hunter, T. (1984) *J. Biol. Chem.* **259,** 2553–2558.
37. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. & Rutter, W. J. (1979) *Biochemistry* **18,** 5294–5299.
38. Sills, M. N., Xu, Y. C., Baracchini, E., Goodman, R. H., Cooperman, S. S., Mandel, G. & Chien, K. R. (1989) *J. Clin. Invest.* **84,** 331–336.
39. Zivin, R. A., Chondra, J. H., Dixon, R. A. F., Seidah, N. G., Chretien, M., Nemer, M., Chamberland, M. & Drouin, J. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 6325–6329.
40. Henderson, S. A., Xu, Y. & Chien, K. R. (1988) *Nucleic Acids Res.* **16,** 4722.
41. Feinberg, A. P. & Vogelstein, B. (1983) *Anal. Biochem.* **132,** 6–13.
42. Henderson, S. A., Spencer, M., Sen, A., Kumar, C., Siddiqui, M. A. Q. & Chien, K. R. (1989) *J. Biol. Chem.* **264,** 18142–18148.
43. deWet, J. R., Wood, K. V., DeLuca, M., Helinski, D. R. & Subramani, S. (1987) *Mol. Cell. Biol.* **7,** 725–737.
44. Knowlton, K. U., Ross, R. S. & Chien, K. R. (1990) *Clin. Res.* **38,** 776.
45. Argentin, S., Nemer, M., Drouin, J., Scott, G. K., Kennedy, B. P. & Davies, P. L. (1985) *J. Biol. Chem.* **260,** 4568–4571.
46. Nelson, C., Albert, V. R., Elsholz, H. P., Lu, L. I.-W. & Rosenfeld, M. G. (1988) *Science* **239,** 1400–1405.
47. Muramatsu, M., Kaibuchi, K. & Arai, K. (1989) *Mol. Cell. Biol.* **9,** 831–836.
48. Kaibuchi, K., Fukumoto, Y., Oku, N., Takai, Y., Arai, K. & Muramatsu, M. (1989) *J. Biol. Chem.* **264,** 13489–13496.
49. Cherrington, J. M. & Mocarski, E. S. (1989) *J. Virol.* **63,** 1435–1440.
50. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY), pp. 16.39–16.40.
51. Rosenthal, N. (1987) *Methods Enzymol.* **152,** 704–720.
52. Huang, F. L., Arora, P. K., Hanna, E. E. & Huang, K.-P. (1988) *Arch. Biochem. Biophys.* **267,** 503–514.
53. Rodriguez-Pena, A. & Rozengurt, E. (1984) *Biochem. Biophys. Res. Commun.* **120,** 1053–1059.
54. Fabbro, D., Mazurek, N., Borner, C., Conscience, J. F. & Erne, P. (1988) *J. Cardiovasc. Pharmacol.* **12,** Suppl. 5, S73–S79.
55. Kosaka, Y., Ogita, K., Ase, K., Nomura, H., Kikkawa, U. & Nishizuka, Y. (1988) *Biochem. Biophys. Res. Commun.* **151,** 973–981.
56. Qu, Y., Torchia, J., Phan, T. D. & Sen, A. K. (1991) *Mol. Cell. Biochem.* **103,** 171–180.
57. Kariya, K., Karns, L. R. & Simpson, P. C. (1991) *J. Biol. Chem.* **266,** 10023–10026.
58. Leach, K. L., Powers, E. A., Ruff, V. A., Jaken, S. & Kaufmann, S. (1989) *J. Cell Biol.* **109,** 685–695.
59. Rogue, P., Laburdette, G., Masmudi, A., Yoshida, Y., Huang, F. L., Huang, K.-P., Zwiller, J., Vincendon, G. & Malviya, A. N. (1990) *J. Biol. Chem.* **265,** 4161–4165.
60. Hocevar, B. A. & Fields, A. P. (1991) *J. Biol. Chem.* **266,** 28–33.
61. Fournier, A., Hardy, S. J., Clark, K. J. & Murray, A. W. (1989) *Biochem. Biophys. Res. Commun.* **161,** 556–561.
62. Huang, K.-P., Huang, F. L., Nakabayashi, H. & Yoshida, Y. (1988) *J. Biol. Chem.* **263,** 14839–14845.
63. Kikkawa, U., Kishimoto, A. & Nishizuka, Y. (1989) *Annu. Rev. Biochem.* **58,** 31–44.
64. Majumdar, S., Rossi, M. W., Fujiki, T., Phillips, W. A., Disa, S., Queen, C. F., Johnston, R. B., Rosen, O. M., Corkey, B. E. & Korchak, H. M. (1991) *J. Biol. Chem.* **266,** 9285–9294.
65. Greenberg, M. E. & Ziff, E. B. (1984) *Nature (London)* **311,** 433–438.
66. Kruijer, W., Cooper, J. A., Hunter, T. & Verma, I. M. (1984) *Nature (London)* **312,** 711–716.
67. Katz, A. & Kahana, C. (1987) *Mol. Cell. Biol.* **7,** 2641–2643.
68. Imbra, R. J. & Karin, M. (1987) *Mol. Cell. Biol.* **7,** 1358–1363.
69. Karin, M., Haslinger, A., Heguy, A., Deitlin, T. & Cooke, T. (1987) *Mol. Cell. Biol.* **7,** 606–613.
70. Karin, M. (1990) *The Cellular and Molecular Biology of Human Carcinogenesis* (Academic, San Diego), pp. 153–172.
71. Bohmann, D., Bos, T. J., Admon, A., Nishimura, T., Vogt, P. K. & Tjian, R. (1987) *Science* **238,** 1386–1392.
72. Chiu, R., Boyle, W. J., Meek, J., Smeal, T., Hunter, T. & Karin, M. (1988) *Cell* **54,** 541–552.
73. Rauscher, F. J., Voulalas, P. J., Franza, B. R. & Curran, T. (1988) *Genes Dev.* **2,** 1687–1699.
74. Smeal, T., Angel, P., Meek, J. & Karin, M. (1989) *Genes Dev.* **3,** 2091–2100.
75. Imagawa, M., Chiu, R. & Karin, M. (1987) *Cell* **51,** 251–260.
76. Trejo, J. & Brown, J. H. (1991) *J. Biol. Chem.* **266,** 7876–7882.
77. Angel, P., Hattori, K., Smeal, T. & Karin, M. (1988) *Cell* **55,** 875–885.
78. Boyle, W. J., Smeal, T., Defize, L. H. K., Angel, P., Woodgett, J. R., Karin, M. & Hunter, T. (1991) *Cell* **64,** 573–584.
79. Barber, J. R. & Verma, I. M. (1987) *Mol. Cell. Biol.* **7,** 2201–2211.