Differential Regulation of Na/K-ATPase α-subunit Isoform Gene Expressions in Cardiac Myocytes by Ouabain and Other Hypertrophic Stimuli

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L. Huang, P. Kometiani and Z. Xie. Differential Regulation of Na/K-ATPase α-subunit Isoform Gene Expressions in Cardiac Myocytes by Ouabain and Other Hypertrophic Stimuli. Journal of Molecular and Cellular Cardiology (1997) 29, 3157–3167. We showed before that partial inhibition of Na/K-ATPase by non-toxic concentrations of ouabain caused hypertrophic growth of neonatal rat cardiac myocytes, and induced several early- and late-response genes that are markers of cardiac hypertrophy. The aim of this study was to determine if the genes of the α-subunit isoforms of Na/K-ATPase were among those regulated by ouabain; and if so, to begin the characterization of the pathways regulating these genes. When neonatal myocytes, expressing α1- and α3-isoform messages, were exposed to 5–100 μM ouabain, α1 mRNA was not affected, but α3 mRNA was decreased in a dose- and time-dependent manner. Oubain-induced down-regulation of α3 mRNA was accompanied by a decrease in α3-protein content in these myocytes. There was a significant correlation between ouabain effects on α3-repression and skeletal α-actin induction; also, ouabain's transcriptional effects on both genes were antagonised by retinoic acid. These findings suggested the association of α3 repression with ouabain-induced hypertrophy. Phenylephrine and a phorbol ester, two hypertrophic stimuli that do not inhibit Na/K-ATPase, also down-regulated α3 mRNA without affecting α1 mRNA, suggesting that α3-repression is a common feature of the hypertrophic phenotype in these myocytes. Ouabain-induced repression of α3 required the influx of extracellular Ca2+, and was antagonized by inhibitors of protein kinase C, Ca2+-calmodulin kinase, and mitogen-activated protein kinase but not by inhibition of protein kinase A. These data, and prior findings on the mechanisms of hypertrophic effects of phenylephrine and phorbol esters, suggest that transcriptional repression of α3 by ouabain and other hypertrophic stimuli involves a common step regulated by a mitogen-activated protein kinase.

KEY WORDS: Cardiac glycosides; Na/K-ATPase; Hypertrophy; Ca2+ influx; Mitogen-activated protein kinases.

Introduction

Na/K-ATPase or sodium pump is an intrinsic plasma membrane enzyme that hydrolyses ATP to maintain the transmembrane gradients of Na+ and K+ found in most mammalian cells, and is inhibited specifically by cardiac glycosides such as ouabain (Mercer, 1993; Lingrel and Kuntzweiller, 1994). The enzyme consists of two non-covalently linked subunits. The α-subunit contains the catalytic and the ouabain binding sites, and the β-subunit is a glycoprotein that is essential for normal function and assembly of the enzyme (Mercer, 1993; Lingrel and Kuntzweiller, 1994). Three α-subunit isoforms have been identified and functionally characterized (Sweadner, 1989; Mercer, 1993; Lingrel and Kuntzweiller, 1994). The most striking differences between α1, α2, and α3 isoforms are in their sensitivities to cardiac glycosides (Sweadner, 1989; Mercer, 1993; Lingrel and Kuntzweiller, 1994) and oxygen free radicals (Xie et al., 1995), and in their tissue distribution patterns (Orlowski and Lingrel, 1993).
1990; Lucchesi and Sweadner, 1991; Sweadner et al., 1992; McDonough et al., 1994; Zahler et al., 1994). In rat, $\alpha_2$ isoform is relatively less sensitive than $\alpha_2$ and $\alpha_1$ isoforms to ouabain (Sweadner, 1989; Mercer, 1993; Lingrel and Kuntzweiler, 1994) and to oxidants (Xie et al., 1995). The expressions of these isoforms are subject to regulation by various hormones, and altered during development and under some pathological conditions (Herrera et al., 1988; Yamamoto et al., 1993; Zahler et al., 1993; Arystarkhova and Sweadner, 1994; Book et al., 1994; Charlemagne et al., 1994; Kim et al., 1994; Qin et al., 1994).

In the heart, Na/K-ATPase serves as the receptor for the positive inotropic effects of cardiac glycosides (Braunwald, 1985; Schwartz et al., 1988; Aker and Ng, 1991). Recently, using cultured neonatal rat cardiac myocytes, we have shown that partial inhibition of Na/K-ATPase by ouabain, at concentrations that increase myocyte contractility without causing overt toxicity, also transduces signals to the nucleus (Peng et al., 1991; Huang et al., 1997). Following the inductions of early response genes (Peng et al., 1996), ouabain produces hypertrophic growth and induces a number of late response genes that are also induced by other hypertrophic stimuli, and are considered to be markers of cardiac hypertrophic growth (Peng et al., 1996; Huang et al., 1997).

The discovery of the transcriptional regulation of growth-related cardiac genes by cardiac glycosides raises the important questions of whether the Na/K-ATPase genes are also among those regulated by cardiac glycosides, and if such feed-back regulation is involved in amplification or restriction of the drugs’ hypertrophic effects. Here, we present the results of our initial studies in these directions, showing that in neonatal rat cardiac myocytes, nontoxic concentrations of ouabain down-regulate $\alpha_2$, but not $\alpha_1$, isoform genes; and suggesting that down-regulation of $\alpha_2$ isoform may be a common feature of hypertrophic growth in these myocytes.

Materials and Methods

Materials

Chemicals of the highest purity available were from Sigma (St Louis, MO, USA) and Boehringer Mannheim (Indianapolis, IN, USA). TRI reagent for RNA isolation was from Molecular Research Center, Inc. (Cincinnati, OH, USA), and radio-nucleotides ($^{32}$P-labeled, about 3000 Ci/mmol) were from DuPont NEN (Boston, MA, USA). All protein kinase inhibitors were purchased from Calbiochem (San Diego, CA, USA).

Cell preparation and culture

Neonatal ventricular myocytes were prepared and cultured as described in our previous work (Peng et al., 1996; Huang et al., 1997). Briefly, myocytes were isolated from ventricles of 1-day-old Sprague-Dawley rats, and purified by centrifugation on Percoll gradients. Myocytes were then cultured at a density of $5 \times 10^4$ cells/cm$^2$ in a medium containing 4 parts of Dulbecco’s modified Eagle’s Medium (DMEM) and 1 part Medium 199 (Gibco), penicillin (100 units/ml), streptomycin (100 $\mu$g/ml), and 10% fetal bovine serum. After 24 h of incubation at 37°C in humidified air with 5% CO$_2$, medium was changed to one with the same composition as above, but without the serum. All experiments were done after 48 h of further incubation under serum-free conditions, and the great majority of these myocytes were quiescent or contracted infrequently (Peng et al., 1996). These cultures contain more than 95% myocytes as assessed by immunofluorescence staining with a myosin heavy chain antibody (Peng et al., 1996; Huang et al., 1997).

Northern blot and nuclear run-on assay

Northern blot was done as previously described (Peng et al., 1996; Huang et al., 1997). The same blots were analysed for several different mRNAs. After each measurement, the blots were stripped in 0.1 X SSC, 0.1% SDS solution at 95°C for 30 min, then rehybridized with other probes as previously described (Li et al., 1996). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and skeletal 2-actin (skACT) probes were made as before (Peng et al., 1996). The $\alpha$-subunit-specific probes (about 300 bp) were designed as previously described (Orlowski and Lingrel, 1990), and made by PCR amplification of the $\alpha$-subunit cDNAs using isoform-specific primers. In agreement with previous findings (Orlowski and Lingrel, 1990; Charlemagne, 1994), the specificities of the probes were established as follows. The $\alpha_1$ probe hybridized to a single species (about 3.7 kb) in both adult and neonatal rat heart RNA blots. The $\alpha_2$ probe detected two mRNA signals (about 5.3 and 3.4 kb) in adult, but not neonatal, rat heart RNA blots. On the other hand, the $\alpha_2$ probe hybridized with a single mRNA species in neonatal, but not adult rat heart RNA blots. Autoradiograms obtained at $-70°C$ were
scanned with a Bio-Rad densitometer. Multiple exposures were analysed to ensure that the signals were within the linear range of the film as we previously described (Peng et al., 1996). The relative amount of RNA in each sample was normalized to that of GAPDH mRNA to correct for differences in sample loading and transfer (Peng et al., 1996; Huang et al., 1997). For nuclear run-on assay, myocyte nuclei were isolated, and counted in 0.4% trypan blue using a hemocytometer as previously described (Peng et al., 1996; Huang et al., 1997). To label the nascent RNA transcripts, the nuclei (3 × 10^6) were incubated in 0.14 M KCl, 10 mM MgCl2, 1 mM MnCl2, 14 mM 2-mercapto-ethanol, 20% glycerol, 0.2 M Tris (pH 8.0), 0.1 mg/ml creatine kinase, 10 mM phosphocreatine, 1 mM each of ATP, GTP, CTP, 0.03 mM UTP, and 100 µCi of α-32P-UTP for 15 min at 30°C. The nuclei were collected, lysed, and digested with RNase-free DNase. Fifty µg of carrier yeast tRNA were added, and the 32P-labeled run-on RNA produced was then isolated using TRI reagent. Puriﬁed 32P-RNA was counted, then equal counts of 32P-labeled RNA from the different groups were used for hybridization (Peng et al., 1996; Huang et al., 1997). The probes were applied to Nytran membrane through slot blot apparatus, denatured, and immobilized by uv-cross linking.

Western blot and protein assay

Myocytes were washed twice with PBS (phosphate buffered saline), collected, and disrupted by sonication in 1 ml solution (0.3 M sucrose, 1 mM EDTA, and 20 µg/ml aprotinin) with a probe sonicator (Fishersonic-300) at a setting of 3 for 2 × 15 s. Cell lysate was centrifuged at 500 × g for 10 min, and the supernatant was removed and assayed for protein content by Lowry method using serum bovine albumin as standard (Lowry et al., 1951). Thirty µg of protein per lane was applied to 7.5% Laemmli gels for electrophoresis, and Western blot measurements were done as previously described (Xie et al., 1996). Rat x3 was detected using a polyclonal x3-speciﬁc antibody (Blanco et al., 1993) which was kindly provided by Dr Mercer (Washington University, St Louis, MO, USA).

Results

Ouabain differentially regulates Na/K-ATPase α-subunit mRNAs

When the steady-state levels of α1, α2, and α3 mRNAs were measured in myocytes after 48 h

Figure 1 Effects of ouabain on Na/K-ATPase subunit gene expression. (a) A representative autoradiogram of ouabain effects. The cells were treated with ouabain for 12 h as follows: lane 1, 0; lane 2, 50 µM; lane 3, 100 µM. Total RNA was isolated, and analysed for α1, α3, and GAPDH by Northern blot as described under Materials and Methods. Specific signals for α1, α3, and GAPDH were taken from three separate ﬁlms and combined as indicated. (b) Combined data from several experiments on α1. (c) Combined data from several experiments on α3. The mRNA values of α1 and α3 were normalized to those of corresponding GAPDH measured on the same blots and expressed relative to a control value of one. The values are mean ± s.e. of at least three independent experiments.
of culture in the absence of serum, those of $z_1$ and $z_3$ were readily detectable. In agreement with previous observations (Orlowski and Lingrel, 1990; Arystarkhova and Sweadner, 1994), however, the $z_2$ mRNA levels were very low and hardly detectable under these conditions. When these myocytes were exposed to different ouabain concentrations for 12 h, and assayed for isoform mRNAs, $z_3$ was down-regulated by ouabain in a dose-dependent manner, but $z_2$ mRNA was not significantly altered by ouabain [Figs 1(a)–(c)]. In these experiments, the levels of $z_2$ mRNA remained barely detectable (data not shown). When time-dependent changes in response to 100 $\mu$m ouabain were measured in these cells, a significant reduction of $z_1$ mRNA was observed after 6 h of exposure, reached a maximally reduced level at 12 h, and lasted for at least 48 h; but ouabain exhibited no significant effect on the levels of $z_3$ mRNA (Fig. 2). Taken together, these findings show that Na/K-ATPase $\alpha$-subunits are differentially regulated by ouabain in these myocytes.

**Ouabain-induced down-regulation of $z_3$ correlates with the up-regulation of skACT expression**

The skACT gene is one of the fetal genes that has been shown to be a marker of hypertrophic growth in rat cardiac myocytes, responding to various growth stimuli including ouabain (Calderone et al., 1995; Wollert et al., 1996; Huang et al., 1997). To compare the effects of ouabain on $z_3$ and skACT genes, the same blots obtained from the experiments of Figures 1 and 2 were also probed with skACT cDNA. Figure 3(a) is a representative autoradiograph showing ouabain-induced up-regulation of skACT. The same blot as in Figure 1(a) was used. (b) Combined data showing a correlation between $z_3$ down-regulation and skACT up-regulation. The same blots were assayed for $z_3$ and skACT mRNAs as in Figure 1. The relative units of $z_3$ mRNA were plotted on a logarithmic scale against the relative units of skACT mRNA.

**Figure 2** Time courses of the ouabain effects on the steady state levels of Na/K-ATPase subunit mRNAs. The cells were treated with 100 $\mu$m for various times, and assayed for $z_1$ (△) and $z_3$ (■) mRNAs as in Figure 1. The values are mean ± s.e. of three experiments.

**Figure 3** Correlation between $z_3$ down-regulation and skACT up-regulation. (a) A representative autoradiogram showing ouabain-induced up-regulation of skACT. The same blot as in Figure 1(a) was used. (b) Combined data showing a correlation between $z_3$ down-regulation and skACT up-regulation. The same blots were assayed for $z_3$ and skACT mRNAs as in Figure 1. The relative units of $z_3$ mRNA were plotted on a logarithmic scale against the relative units of skACT mRNA.
Regulation of Cardiac Na/K-ATPase Genes by Ouabain

GAPDH

\[ \alpha \]

1.5 kb

3.7 kb

Figure 5 Effects of PMA and phenylephrine on Na/K-ATPase genes. The cells were treated with PMA (100 nM) and phenylephrine (0.1 mM) for 12 h, and assayed for \( \alpha_1 \) and \( \alpha_3 \) mRNAs as in Figure 1(a). A representative autoradiogram from three independent experiments is shown. Lane 1, control; Lane 2, ouabain, 100 \( \mu \)M; Lane 3, PMA; Lane 4, phenylephrine.

Figure 6 Effects of ouabain and PMA on \( \alpha_3 \) protein levels. The cells were treated with 100 \( \mu \)M ouabain or 100 nM PMA for either 12 h (Lanes 2–4) or 24 h (Lanes 5–7), and assayed for \( \alpha_3 \) protein by Western blot as described under Materials and Methods. A representative blot from three independent experiments is shown. Lane 1, two protein markers (106 kDa and 80 kDa); Lanes 2 and 5, control; Lanes 3 and 6, ouabain; Lanes 4 and 7, PMA.

ouabain-induced increase in skACT mRNA, and antagonized ouabain-induced down-regulation of \( \alpha_1 \) mRNA.

Phorbol 12-myristate 13-acetate (PMA) and phenylephrine regulate Na/K-ATPase subunit gene expression in a pattern similar to that of ouabain

To test whether the down-regulation of \( \alpha_3 \) mRNA is a specific response to ouabain-induced inhibition of Na/K-ATPase, or a common feature of hypertrophic phenotype in these myocytes, cells were exposed to either PMA or phenylephrine, two well-known hypertrophic stimuli. PMA produces cardiac hypertrophy through activation of PKC in a pattern similar to that induced by volume-overload, whereas phenylephrine uses G-protein coupled receptors and causes cardiac hypertrophy similar to that of pressure-overload (Calderone et al., 1995; Wollert et al., 1996; Huang et al., 1997). As depicted in Figure 5, both PMA and phenylephrine down-regulated \( \alpha_3 \), but not \( \alpha_1 \), expression.

Ouabain and PMA decrease \( \alpha_3 \) protein in cardiac myocytes

To determine if down-regulation of \( \alpha_3 \) mRNA by ouabain and other stimuli is accompanied by a decrease in \( \alpha_3 \) protein in cardiac myocytes, cells were exposed to either ouabain or PMA. As shown in Figure 6, both ouabain and PMA causes a significant decrease in \( \alpha_3 \) protein content in cardiac myocytes.
Ouabain decreases α₃ transcription rate

To gain insight into how ouabain down-regulates α₃ mRNA, nuclear run-on experiments as depicted in Figure 7 were performed. The data from three independent experiments showed that ouabain decreased α₃ transcription rate significantly (P<0.01, Student’s t-test), but had no significant effect on α₁ transcription rate (P>0.05, Student’s t-test). These results indicate that ouabain-induced down-regulation of α₃ mRNA is, at least in part, due to a decrease in transcription rate of α₃ gene.

Net influx of extracellular Ca²⁺ and activations of calmodulin and PKC are required for ouabain-induced down-regulation of α₃

An increase in Ca²⁺ influx and activation of calmodulin and PKC are involved in ouabain-induced skACT expression in cultured neonatal cardiac myocytes (Huang et al., 1997). To determine whether the effects of ouabain on α₃ are also mediated by the pathways involved in skACT induction, the following studies were performed.

When cells were exposed to ouabain for 12 h in a nominally Ca²⁺-free medium, ouabain had no effect on α₁ mRNA (Fig. 8), clearly establishing the necessity of ouabain-induced net influx of extracellular Ca²⁺ for ouabain’s effect on α₃ gene. In agreement with the findings of others (LaPointe et al., 1990), there was no significant change in myocyte viability after 12 h of incubation in Ca²⁺-free medium. It is noteworthy that removal of extracellular Ca²⁺ also significantly decreased α₃ expression (data not shown) in these cultured cardiac myocytes, indicating the complexity of the role of Ca²⁺ in the pathway of α₃ expression.

To address the potential role of calmodulin in ouabain’s effect on α₃, W-7, a membrane-permeable inhibitor (Sei et al., 1991), was used. As shown in Figure 9, pretreatment of cells with W-7 completely blocked the effects of ouabain on α₃.

Two well-characterized membrane-permeable protein kinase inhibitors (H-7 and HA1004) were used to address the potential roles of these enzymes in ouabain-induced down-regulation of α₃ gene. When the cells were exposed to ouabain in the presence of H-7, which inhibits PKC and PKA with similar potencies (Sei et al., 1991), H-7 partially repressed ouabain-induced down-regulation of α₃ (Fig. 9). On the other hand, HA1004, which is much more selective for PKA and PKC (Sei et al., 1991), exhibited no significant effect on ouabain-induced α₃ down-regulation (Fig. 9). Taken together, the data suggest that activation of PKC, but...
not PKA, may be involved in ouabain’s signaling pathways leading to the down-regulation of α3 expression.

Inhibition of mitogen-activated protein kinase (MEK) by PD98059 blocks ouabain-induced effects on α3

Because α3 mRNA was down-regulated by several different hypertrophic stimuli (Figs 1 and 5), it seemed likely that the different initial signals generated by these stimuli must converge to a common point in order to suppress α3 expression. It has been shown that mitogen-activated protein kinase (MAPK) plays an important role in regulation of cell growth and gene expression, and that various hypertrophic stimuli including phenylephrine and PMA activate MAPK (Nishida and Gotoh, 1993; Alessi et al., 1995; Karin, 1995; Sadoshima et al., 1995; Eguchi et al., 1996; Post et al., 1996). To test for the involvement of MAPK in ouabain-induced down-regulation of α3 myocytes were pretreated with PD98059, a MEK inhibitor which blocks extracellular stimulus responsive kinase (ERK) type MAPK activation in various cells including cultured neonatal cardiac myocytes (Alessi et al., 1995; Post et al., 1996), before exposure to ouabain. As depicted in Figure 10, PD98059 completely blocked ouabain’s effect on α3 mRNA, supporting the notion that ouabain and other stimuli-initiated hypertrophic signals may converge to ERK1 and ERK2, resulting in α3 down-regulation. It was noteworthy, however, that PD98059 had no significant effect on ouabain-induced skACT expression (Fig. 10).

Discussion

The usefulness of the cultured neonatal rat cardiac myocytes as a model for the study of the mechanisms involved in the development of cardiac hypertrophy has been established by the extensive works of numerous laboratories (Simpson et al., 1982; Iwaki et al., 1990; Sadoshima et al., 1992; Sadoshima and Izumo, 1993; Thaik et al., 1995; Wollert et al., 1996). Using this model system, the aim of the present study was to determine if the recently discovered hypertrophic effect of ouabain...
(Peng et al., 1996; Huang et al., 1997) is accompanied by changes in the expression of the genes of the sarcolemmal ouabain receptors, i.e. the genes of the $\alpha$-subunits of Na/K-ATPase.

Differential regulation of $\alpha$-isoform genes by ouabain

The present findings show that of the two readily detectable $\alpha$-isoform messages of these myocytes, the $\alpha_3$ mRNA is down-regulated by ouabain, but that $\alpha_2$ mRNA level is not altered by ouabain under the conditions used (Figs 1 and 2). Down regulation of $\alpha_3$ mRNA is transcriptional (Fig. 7), and is accompanied by a decrease in $\alpha_3$ protein content (Fig. 6).

It is appropriate to address the apparent differences between the above findings and those of a previous study. Using neonatal rat cardiac myocytes similar to the cells used here, Yamamoto et al. (1993) reported that 1 mM ouabain caused a three- to four-fold increase in mRNAs of all three $\alpha$-isoforms. They also found, however, that these effects were independent of $\text{Ca}^{2+}$, but due to a large increase in intracellular concentration of $\text{Na}^+$ caused by this high concentration of ouabain. As we have discussed before (Peng et al., 1996), the lower non-toxic ouabain concentrations we have used here and in our previous work (Peng et al., 1996; Huang et al., 1997), cause significant increases in $[\text{Ca}^{2+}]$, are dependent on the presence of extracellular $\text{Ca}^{2+}$ for their transcriptional effects, and are known to produce only small changes in intracellular $\text{Na}^+$ concentration. As such, these ouabain concentrations are more comparable to those producing positive inotropic effects, but no toxicity, in the intact rat heart (Schwartz et al., 1988; Akera and Ng, 1991).

Freshly prepared and serum-starved neonatal rat cardiac myocytes contain only $\alpha_2$ and $\alpha_3$ isoform proteins (Arystarkhova and Sweadner, 1994). Based on this, the knowledge of the different ouabain sensitivities of the rat $\alpha_2$ and $\alpha_3$ isoforms (Sweadner, 1989; Lingrel and Kunzweiler, 1994), and the shape of the ouabain inhibition curve of the total Na/K-ATPase activity of these myocytes (Xie et al., 1989), it may easily be estimated that ouabain concentrations used in the present studies (5–100 $\mu$M) inhibit the $\alpha_3$ isoform completely, but cause less than 20–30% inhibition of the $\alpha_2$ isoform. Thus, the inhibition of the ouabain-sensitive $\alpha_3$ isoform may play an important role in initiating the pathways of ouabain-induced hypertrophy in these myocytes. It is reasonable, therefore, to consider the possibility that down-regulation of $\alpha_3$ may be an adaptive response to limit the extent of ouabain-induced hypertrophy. Clearly, this hypothesis needs to be tested by further experiments.

Similarities and differences between the signal transduction pathways of the ouabain-regulated genes

The clear correlation between the ouabain-initiated induction of skACT and repression of $\alpha_3$ (Fig. 3) suggests that the two events are not due to two unrelated effects of ouabain. Further comparison of the characteristics of the two events clearly indicates the common features of the signal pathways regulating the two genes. Both pathways require the ouabain-induced influx of extracellular $\text{Ca}^{2+}$, and are dependent on PKC, and most likely on a calmodulin kinase (Figs 8 and 9, and Huang et al., 1997). That the two pathways also diverge, however, is indicated by the fact that MEK inhibition blocks ouabain’s down-regulation of $\alpha_3$, but not its induction of skACT (Fig. 10). Evidently, the two pathways diverge at a point upstream of a MAPK-regulated step within the pathway of $\alpha_3$ regulation. These findings will be helpful to the future characterization of the details of the signal transduction pathways of the cardiac late-response genes regulated by ouabain.

An interesting aspect of the present study is the finding that an increase in extracellular $\text{Ca}^{2+}$, which is expected to raise the level of intracellular $\text{Ca}^{2+}$ (Ikenouchi et al., 1994), causes a significant increase in the level of $\alpha_3$ mRNA (Fig. 8). This may seem to be in conflict with the finding that ouabain-induced increase in intracellular $\text{Ca}^{2+}$ obtained in a $\text{Ca}^{2+}$-containing medium (Peng et al., 1996) is accompanied by decrease in $\alpha_3$ mRNA (Fig. 8). These apparently opposite effects of intracellular $\text{Ca}^{2+}$ are difficult to explain without the assumption of different compartments of intracellular $\text{Ca}^{2+}$. The existence of such compartments in myocytes has been suggested (Langer et al., 1990; Langer and Peskoff, 1996), and in cells other than myocytes it has been established that changes in intracellular $\text{Ca}^{2+}$ that are brought about through different mechanisms affect different steps of the signal pathways regulating the induction of c-fos (Ghosh and Greenberg, 1995).

Repression of $\alpha_3$ expression as a common feature of hypertrophic phenotype

A number of stimuli induce hypertrophic growth and alter gene expression in cardiac myocytes...
(Chien et al., 1993). Based on their effects on cardiac late response genes, they can be divided into two groups. Stimuli such as phenylephrine induce both atrial natriuretic factors (ANF) and skACT expression in a coordinate fashion, whereas other stimuli such as PMA and cardiotrophin increase ANF, but not skACT (Calderone et al., 1995; Wollert et al., 1996; Huang et al., 1997). Ouabain induces both skACT and ANF, thus exhibiting a phenotype similar to that of phenylephrine. However, because ouabain has significant effects on contractile protein genes skACT and myosin light chain-2, while it is less effective than phenylephrine in the induction of total protein synthesis and ANF expression (Calderone et al., 1995; Peng et al., 1996; Wollert et al., 1996; Huang et al., 1997), it is evident that ouabain and phenylephrine also act differently. Mechanistically, while ouabain initiates its hypertrophic effect on cardiac myocytes through inhibition of Na/K-ATPase, phenylephrine uses G protein-coupled receptors, and PMA activates PKC directly. The similar effects of ouabain, PMA, and phenylephrine on $z_1$ mRNA (Fig. 5), therefore, strongly suggest that transcriptional repression of $z_1$ is a common feature of the hypertrophic phenotype in these myocytes; and this is supported by the antagonism of the ouabain down-regulation of $z_1$ by retinoic acid (Fig. 4). The blockade of the ouabain-induced repression of $z_1$ by a specific inhibitor of MEK (Fig. 10), and the previously demonstrated MAPK activations induced by phenylephrine and PMA (Sadoshima et al., 1995; Post et al., 1996), suggest that a MAPK-activated step within the pathway of repression of $z_1$ is where the signals from ouabain, PMA, and phenylephrine converge.

Relation of the present findings to previous studies on Na/K-ATPase isoforms expressions in the intact rat heart

The relatively ouabain-insensitive $z_1$ isoform constitutes most of the sarcolemmal Na/K-ATPase both in the neonatal rat cardiac myocyte and in the adult rat heart (Sweedner, 1989; Arystarkhova and Swedner, 1994). However, while the predominant ouabain-sensitive isoform of the neonatal myocyte is $z_1$, that of the adult rat heart is the $z_2$ isoform; and the switch from $z_1$ to $z_2$ occurs during postnatal development (Sweedner, 1989; Arystarkhova and Swedner, 1994). A number of studies on the expressions of Na/K-ATPase isoforms of the adult myocardium in different rat models of pressure-overload hypertrophy have been conducted (Book et al., 1994; Charlemagne et al., 1994, and references therein). In spite of minor differences, the general pattern emerging from these studies is that at both mRNA and protein levels, there is no significant change in $z_1$ expression, but that $z_2$ expression is repressed. Thus, in the adult rat heart, as in the model of cultured neonatal myocyte used here, it seems that repression of the ouabain-sensitive isoform is the common feature of the hypertrophic phenotype. It is not known if ouabain, as a drug or as the suggested humoral agent (Blaustein, 1993), can cause hypertrophy and repression of the $z_2$ isoform in the intact adult rat heart.

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