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

Liuyu Huang, Peter Kometiani and Zijian Xie

Department of Pharmacology, Medical College of Ohio, Toledo, Ohio 43699-0008, USA

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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 α_1- and α_3-isoform messages, were exposed to 5–100 μM ouabain, α_1 mRNA was not affected, but α_3 mRNA was decreased in a dose- and time-dependent manner. Oubain-induced down-regulation of α_3 mRNA was accompanied by a decrease in α_3-protein content in these myocytes. There was a significant correlation between ouabain effects on α_3-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 α_3 repression with ouabain-induced hypertrophy. Phenylephrine and a phorbol ester, two hypertrophic stimuli that do not inhibit Na/K-ATPase, also down-regulated α_3 mRNA without affecting α_1 mRNA, suggesting that α_3-repression is a common feature of the hypertrophic phenotype in these myocytes. Ouabain-induced repression of α_3 required the influx of extracellular Ca^{2+}, and was antagonized by inhibitors of protein kinase C, Ca^{2+}-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 α_3 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; Ca^{2+} 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 Kuntzweiler, 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 Kuntzweiler, 1994). Three α-subunit isoforms have been identified and functionally characterized (Sweadner, 1989; Mercer, 1993; Lingrel and Kuntzweiler, 1994). The most striking differences between α_1, α_2, and α_3 isoforms are in their sensitivities to cardiac glycosides (Sweadner, 1989; Mercer, 1993; Lingrel and Kuntzweiler, 1994) and oxygen free radicals (Xie *et al.*, 1995), and in their tissue distribution patterns (Orlowski and Lingrel,

Please address all correspondence to: Zijian Xie, Department of Pharmacology, Medical College of Ohio, P.O. Box 10008, Toledo, OH 43699-0008, USA.

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1990; Lucchesi and Sweadner, 1991; Sweadner *et al.*, 1992; McDonough *et al.*, 1994; Zahler *et al.*, 1994). In rat, α_1 isoform is relatively less sensitive than α_2 and α_3 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; Akera 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 α_3, but not α_1, isoform genes; and suggesting that down-regulation of α_3 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 5×10^4 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 *M*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 α-subunit-specific probes (about 300 bp) were designed as previously described (Orlowski and Lingrel, 1990), and made by PCR amplification of the α-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 α_1 probe hybridized to a single species (about 3.7 kb) in both adult and neonatal rat heart RNA blots. The α_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 α_3 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 $MgCl_2$, 1 mM $MnCl_2$, 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 μM UTP, and 100 μCi of α-^{32}P-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 ^{32}P-labeled run-on RNA produced was then isolated using TRI reagent. Purified ^{32}P-RNA was counted, then equal counts of ^{32}P- 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 $500 \times \mathbf{g}$ 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 α_3 was detected using a polyclonal α_3-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 α_1, α_2, and α_3 mRNAs were measured in myocytes after 48 h

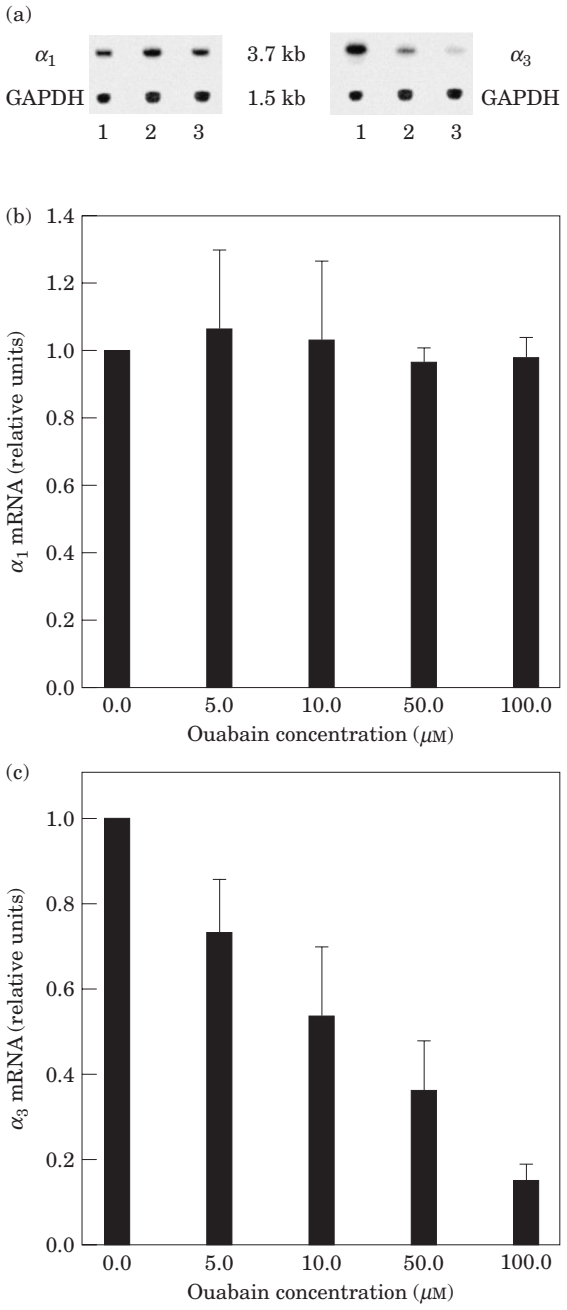

Figure 1 Effects of ouabain on Na/K-ATPase subunit gene expression. (a) A representative autoradiogram of ouabain effects. The cells were treated with ouabain for 12 h as follows: lane 1, 0; lane 2, 50 μM; lane 3, 100 μM. Total RNA was isolated, and analysed for α_1, α_3, and GAPDH by Northern blot as described under Materials and Methods. Specific signals for α_1, α_3, and GAPDH were taken from three separate films and combined as indicated. (b) Combined data from several experiments on α_1. (c) Combined data from several experiments on α_3. The mRNA values of α_1 and α_3 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 $\pm$ S.E. of at least three independent experiments.

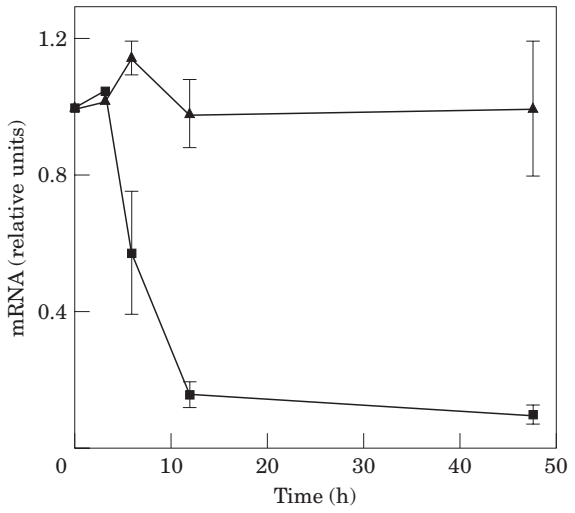

Figure 2 Time courses of the ouabain effects on the steady state levels of Na/K-ATPase subunit mRNAs. The cells were treated with 100 μM for various times, and assayed for α_1 (▲) and α_3 (■) mRNAs as in Figure 1. The values are mean ± S.E. of three experiments.

of culture in the absence of serum, those of α_1 and α_3 were readily detectable. In agreement with previous observations (Orlowski and Lingrel, 1990; Arystarkhova and Sweadner, 1994), however, the α_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, α_3 was down-regulated by ouabain in a dose-dependent manner, but α_1 mRNA was not significantly altered by ouabain [Figs 1(a)–(c)]. In these experiments, the levels of α_2 mRNA remained barely detectable (data not shown). When time-dependent changes in response to 100 μM ouabain were measured in these cells, a significant reduction of a_3 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 α_1 mRNA (Fig. 2). Taken together, these findings show that Na/K-ATPase α-subunits are differentially regulated by ouabain in these myocytes.

Ouabain-induced down-regulation of α_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

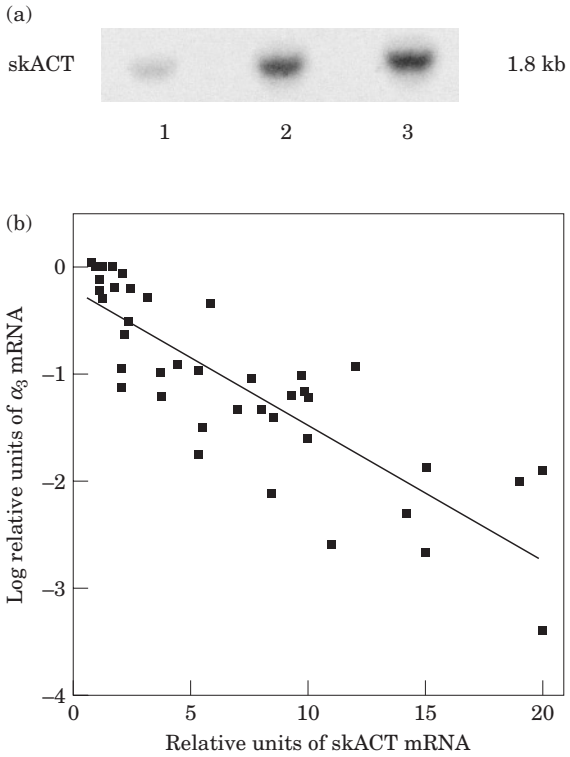

Figure 3 Correlation between α_3 down-regulation and skACT up-regulation. (a) A representative autoradiogram showing ouabain-induced up-regulation of skACT. The same blot as in Figure 1(a) was used. (b) Combined data showing a correlation between α_3 down-regulation and skACT up-regulation. The same blots were assayed for α_3 and skACT mRNAs as in Figure 1. The relative units of α_3 mRNA were plotted on a logarithmic scale against the relative units of skACT mRNA.

compare the effects of ouabain on α_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, as previously reported (Huang *et al.*, 1997). As shown in Figure 3(b), there was a significant inverse correlation between the mRNA levels of α_3 and skACT ($n=40$, $r=0.71$, $P<0.001$); suggesting that α_3 down-regulation may also be a marker of ouabain-induced hypertrophy.

To further test whether ouabain effects on α_3 are mediated by ouabain-induced growth signals, retinoic acid was employed, because retinoic acid has been shown to block hypertrophic phenotypes induced by various stimuli in rat cardiac myocytes (Zhou *et al.*, 1995). In experiments of Figure 4, the cells were treated with ouabain in the presence of different concentrations of retinoic acid, and assayed for mRNAs of skACT, α_3, and GAPDH. The results showed that retinoic acid suppressed

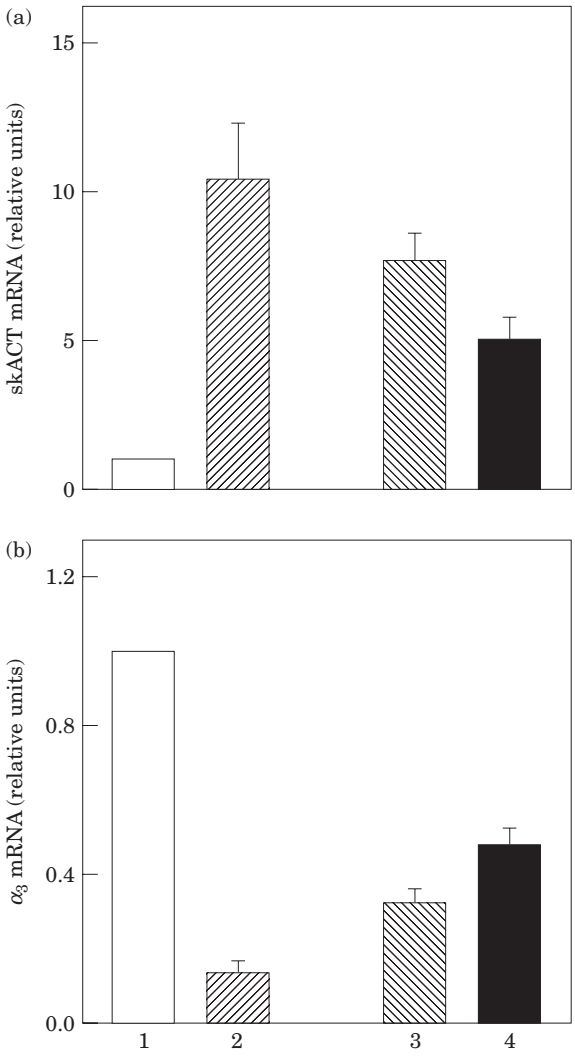

Figure 4 Retinoic acid represses ouabain effects on cardiac genes. The cells were treated with 100 μM ouabain in the presence and absence of retinoic acid for 12 h, and assayed for skACT and α_3 mRNAs as in Figure 1. The values are mean $\pm$ S.E. of three experiments. (□), control; (▨), ouabain (▧), ouabain and 0.1 μM retinoic acid; (■), ouabain and 1 μM retinoic acid.

ouabain-induced increase in skACT mRNA, and antagonized ouabain-induced down-regulation of α_3 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 α_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 α_3, but not α_1, expression.

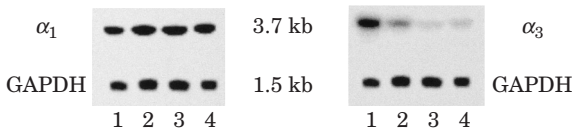

Figure 5 Effects of PMA and phenylephrine on Na/K-ATPase genes. The cells were treated with PMA (100 nM) and phenylephrine (0.1 mM) for 12 h, and assayed for α_1 and α_3 mRNAs as in Figure 1(a). A representative autoradiogram from three independent experiments is shown. Lane 1, control; Lane 2, ouabain, 100 μM; Lane 3, PMA; Lane 4, phenylephrine

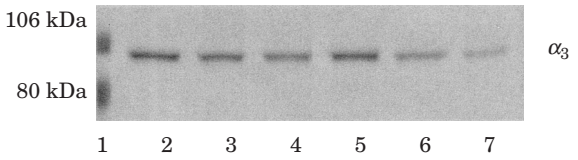

Figure 6 Effects of ouabain and PMA on α_3 protein levels. The cells were treated with 100 μM ouabain or 100 nM PMA for either 12 h (Lanes 2–4) or 24 h (Lanes 5–7), and assayed for α_3 protein by Western blot as described under Materials and Methods. A representative blot from three independent experiments 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 and PMA decrease α_3 protein in cardiac myocytes

To determine if down-regulation of α_3 mRNA by ouabain and other stimuli is accompanied by a decrease in α_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 α_3 protein content in cardiac myocytes.

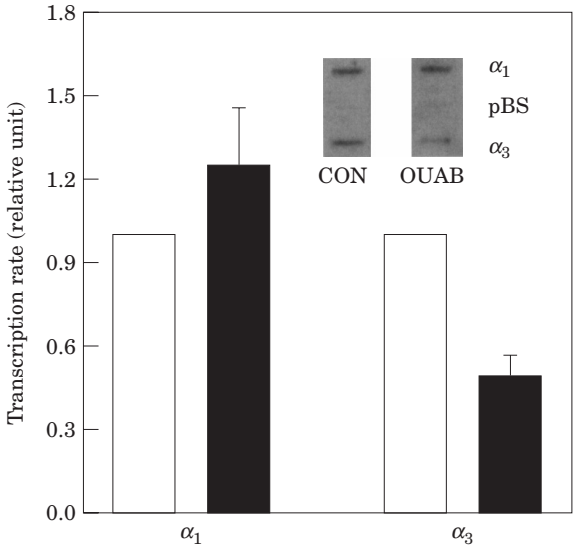

Figure 7 Nuclear run-on experiments showing the effects of ouabain on α_1 and α_3 transcription. The cells were treated with ouabain for 10 h, nuclei were isolated and labeled with α-^{32}P-UTP. Equal amount of radioactive run-on RNA was used in the hybridization. Insert, a representative autoradiogram. Combined data from three experiments are shown in the graph. The intensities of the signals of α_1 and α_3 were corrected by subtracting signals of a vector pBluescript, and the values are expressed as mean ± S.E. (□), control; (■) ouabain.

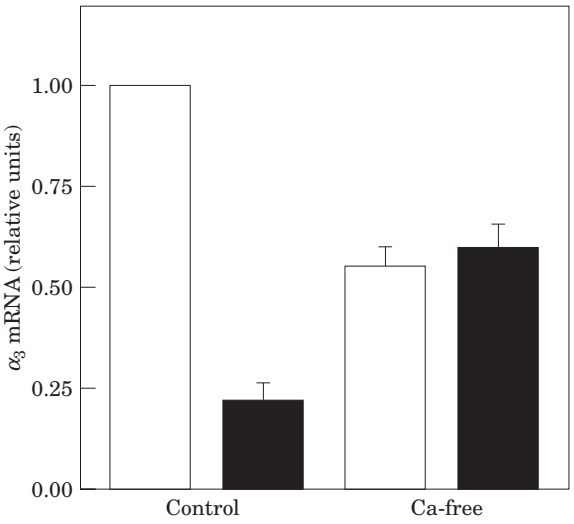

Figure 8 Effects of removal of extracellular Ca^{2+} on ouabain-induced down-regulation of α_3. The cells were treated with 100 μM of ouabain in normally calcium-free medium for 12 h, and assayed for α_3 and GAPDH mRNAs as in Figure 1. The values are expressed as mean ± S.E. of three experiments. (□), −ouabain; (■), +ouabain.

Ouabain decreases α_3 transcription rate

To gain insight into how ouabain down-regulates α_3 mRNA, nuclear run-on experiments as depicted in Figure 7 were performed. The data from three independent experiments showed that ouabain decreased α_3 transcription rate significantly ($P<0.01$, Student's *t*-test), but had no significant effect on α_1 transcription rate ($P>0.05$, Student's *t*-test). These results indicate that ouabain-induced down-regulation of α_3 mRNA is, at least in part, due to a decrease in transcription rate of α_3 gene.

Net influx of extracellular Ca^{2+} and activations of calmodulin and PKC are required for ouabain-induced down-regulation of α_3

An increase in Ca^{2+} 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 α_3 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 Ca^{2+}-free medium, ouabain had no effect on α_3 mRNA (Fig. 8), clearly establishing the necessity of ouabain-induced net influx of extracellular Ca^{2+} for ouabain's effect on α_3 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 Ca^{2+}-free medium. It is noteworthy that removal of extracellular Ca^{2+} also significantly decreased α_3 expression (Fig. 8), but not α_1 expression (data not shown) in these cultured cardiac myocytes, indicating the complexity of the role of Ca^{2+} in the pathway of α_3 expression.

To address the potential role of calmodulin in ouabain's effect on α_3, 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 α_3.

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 α_3 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 α_3 (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 α_3 down-regulation (Fig. 9). Taken together, the data suggest that activation of PKC, but

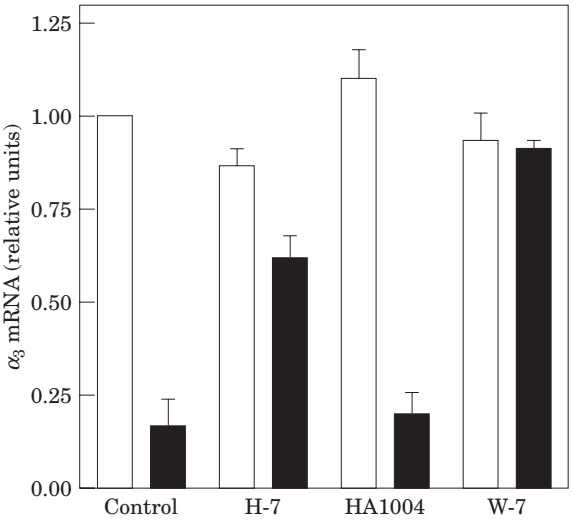

Figure 9 Effects of W-7, H-7 and HA1004 on ouabain-induced down-regulation of α_3. The cells were treated with 100 μM ouabain in the presence of 2 μM W-7 or 50 μM H-7 or 50 μM HA1004 for 12 h, and assayed for α_3 and GAPDH mRNAs as in Figure 1. The values are expressed as mean $\pm$ S.E. of three experiments. (□, −ouabain; (■), +ouabain.

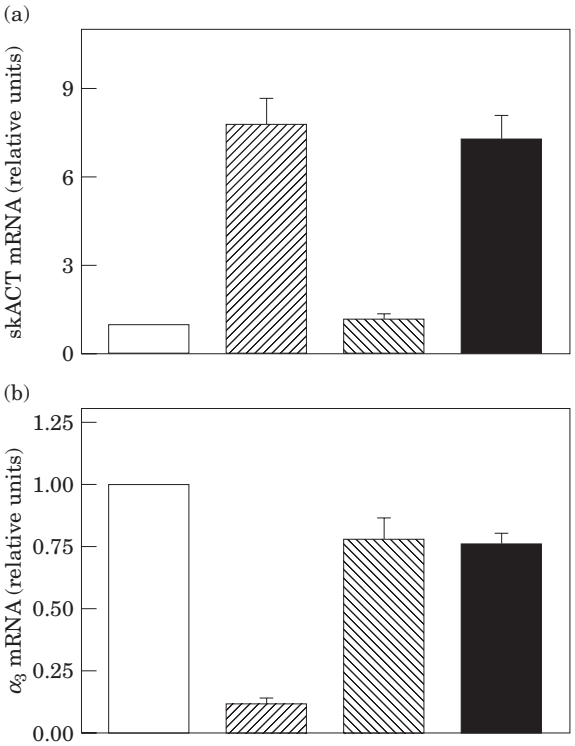

Figure 10 MEK inhibitor PD98059 blocks the effects of ouabain on α_3, but not skACT gene expression. The control cells and those pretreated with 10 μM PD98059 were exposed to 100 μM of ouabain for 12 h, and assayed for α_3, skACT, and GAPDH mRNAs as in Figure 1. The values are mean $\pm$ S.E. of four experiments. (□), control; (▨), ouabain 100 μM; (▧), PD 10 μM; (■), ouabain +PD 10 μM.

not PKA, may be involved in oubain's signaling pathways leading to the down-regulation of α_3 expression.

Inhibition of mitogen-activated protein kinase (MEK) by PD98059 blocks ouabain-induced effects on α_3

Because α_3 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 α_3 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 α_3 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 α_3 mRNA, supporting the notion that ouabain and other stimuli-initiated hypertrophic signals may converge to ERK1 and ERK2, resulting in α_3 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 α-subunits of Na/K-ATPase.

Differential regulation of α-isoform genes by ouabain

The present findings show that of the two readily detectable α-isoform messages of these myocytes, the α_3 mRNA is down-regulated by ouabain, but that α_1 mRNA level is not altered by ouabain under the conditions used (Figs 1 and 2). Down regulation of α_3 mRNA is transcriptional (Fig. 7), and is accompanied by a decrease in α_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 α-isoforms. They also found, however, that these effects were independent of Ca^{2+}, but due to a large increase in intracellular concentration of 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 $[Ca^{2+}]_i$, are dependent on the presence of extracellular Ca^{2+} for their transcriptional effects, and are known to produce only small changes in intracellular Na^{2+} 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 α_1 and α_3 isoform proteins (Arystarkhova and Sweadner, 1994). Based on this, the knowledge of the different ouabain sensitivities of the rat α_1 and α_3 isoforms (Sweadner, 1989; Lingrel and Kuntzweiler, 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 μM) inhibit the α_3 isoform completely, but cause less than 20–30% inhibition of the $\alpha_1$isoform. Thus, the inhibition of the ouabain-sensitive α_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 α_3 may be an adaptive response to limit the extent of oubain-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 α_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 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 α_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 α_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 Ca^{2+}, which is expected to raise the level of intracellular Ca^{2+} (Ikenouchi *et al.*, 1994), causes a significant increase in the level of α_3 mRNA (Fig. 8). This may seem to be in conflict with the finding that ouabain-induced increase in intracellular Ca^{2+} obtained in a Ca^{2+}-containing medium (Peng *et al.*, 1996) is accompanied by decrease in α_3 mRNA (Fig. 8). These apparently opposite effects of intracellular Ca^{2+} are difficult to explain without the assumption of different compartments of intracellular 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 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 α_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 α_3 mRNA (Fig. 5), therefore, strongly suggest that transcriptional repression of α_3 is a common feature of the hypertrophic phenotype in these myocytes; and this is supported by the antagonism of the ouabain down-regulation of α_3 by retinoic acid (Fig. 4). The blockade of the ouabain-induced repression of α_3 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 α_3 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 α_1 isoform constitutes most of the sarcolemmal Na/K-ATPase both in the neonatal rat cardiac myocyte and in the adult rat heart (Sweadner, 1989; Arystarkhova and Sweadner, 1994). However, while the predominant ouabain-sensitive isoform of the neonatal myocyte is α_3, that of the adult rat heart is the α_2 isoform; and the switch from α_3 to α_2 occurs during postnatal development (Sweadner, 1989; Arystarkhova and Sweadner, 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 α_1 expression, but that α_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 α_2 isoform in the intact adult rat heart.

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