



Selective Induction of Na,K-ATPase $\alpha 3$ Subunit mRNA Abundance in Cardiac Myocytes by Retinoic Acid

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(Received 17 March 1998, accepted in revised form 13 August 1998)

S. CHIN, H. HE AND G. GICK. Selective Induction of Na,K-ATPase $\alpha 3$ Subunit mRNA Abundance in Cardiac Myocytes by Retinoic Acid. *Journal of Molecular and Cellular Cardiology* (1998) 30, 2403–2410. Retinoic acid (RA) is a high affinity ligand for a nuclear receptor which regulates transcription in target cells. Specific effects of RA on cardiac development and myocardial cell hypertrophy have been demonstrated; however, little information exists concerning RA-mediated regulation of cardiac genes. This study was initiated to investigate whether RA regulates Na,K-ATPase subunit gene expression in primary cultures of neonatal rat cardiac myocytes. Northern blot analyses demonstrated that Na, K-ATPase $\alpha 3$ subunit mRNA content was stimulated three-fold by RA. The effect of RA on $\alpha 3$ subunit gene expression was selective as RA treatment had no effect on either Na,K-ATPase $\alpha 1$, $\alpha 2$ or $\beta 1$ subunit mRNAs. A stimulatory effect of RA on Na,K-ATPase $\alpha 3$ gene transcription was not evident in either transient transfection or nuclear run-on studies, suggesting that augmentation of $\alpha 3$ mRNA content by RA was due to a post-transcriptional mechanism. Finally, RA diminished the magnitude of the thyroid hormone (T3)-mediated increase in Na,K-ATPase $\beta 1$ subunit mRNA, while RA had no effect on the stimulation of $\alpha 3$ mRNA content by T3. © 1998 Academic Press

KEY WORDS: Post-transcriptional control; Thyroid hormone; $\beta 1$ subunit mRNA; Neonatal rat cardiac myocytes.

Introduction

Na,K-ATPase catalyzes the unequal transport of Na^+ and K^+ ions across the plasma membrane of all animal cells, thereby establishing an electrochemical gradient which is essential for a myriad of physiological processes including the regulation of myocardial contractility (Lingrel *et al.*, 1990). Na,K-ATPase consists of two subunits, α and β , and each subunit exists as several isoforms. The α subunit is responsible for catalytic activity and is the binding site for cardiac glycosides while the β subunit is essential in the assembly of the holoenzyme (Lingrel *et al.*, 1990). Na,K-ATPase α isoforms ($\alpha 1$, $\alpha 2$ and $\alpha 3$) display differential sensitivity to cardiac glycosides (Urayama and Sweadner,

1989) and affinity for Na^+ ions (Munzer *et al.*, 1994) suggesting that they are not functionally redundant but rather enable a cell to respond to a broad range of physiological stimuli. Indeed, the three distinct Na,K-ATPase α subunit genes exhibit a tissue- and development-specific pattern of expression (Lingrel *et al.*, 1990). For example, the $\alpha 1$ isoform is constitutively expressed throughout development in rat heart. In contrast, $\alpha 3$ mRNA is present at high levels only in the fetal and neonatal heart, whereas $\alpha 2$ expression occurs primarily in the juvenile and adult stages of cardiac development.

The molecular determinants underlying the developmentally-regulated expression of Na,K-ATPase α subunit genes in cardiac myocytes have not been

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delineated; however, we and others have demonstrated that thyroid hormone (T3)¹ regulates Na, K-ATPase subunit gene expression in a complex manner in primary cultures of neonatal rat cardiac myocytes (He *et al.*, 1996; Kamitani *et al.*, 1992; Orłowski and Lingrel, 1990). Since a subset of T3-responsive genes are also regulated by retinoic acid (RA) (Williams *et al.*, 1992) and RA has been implicated as an important regulatory factor in myocardial cell development (Kastner *et al.*, 1994; Sucov *et al.*, 1994), it was of interest to evaluate the effect of RA on Na,K-ATPase subunit gene expression in cultured neonatal rat cardiac myocytes. In this report, we demonstrate that RA selectively stimulates Na,K-ATPase $\alpha 3$ mRNA by a post-transcriptional mechanism.

Materials and Methods

Preparation of neonatal rat cardiac myocytes

Primary cultures of neonatal cardiac myocytes were prepared from 1–3-day-old Sprague-Dawley rats by dispersion of ventricles in 1.5 mg/ml trypsin and 20 μ g/ml DNase I as previously described (Huang *et al.*, 1994). Cardiac myocytes were maintained on tissue culture dishes coated with 1% gelatin for 1 day in Dulbecco's modified Eagles medium (DMEM) containing 5% charcoal-treated horse serum, 0.1 mM vitamin B₁₂ and antibiotics (100 U/ml penicillin G sodium, 100 μ g/ml streptomycin sulfate and 250 ng/ml amphotericin B as Fungizone). For Northern blot and nuclear run-on assays, 2.5×10^7 cells per 10-cm plate were incubated with 10 ml of serum-free DMEM containing 1.5 mM vitamin B₁₂, 10 μ g/ml insulin, 10 μ g/ml transferrin and antibiotics for 2 days prior to the addition of T3 and/or RA. For transient transfection studies, 1×10^7 cells per 6-cm plate were incubated with 4 ml of serum-containing DMEM for 3 h before transfection was performed. Cardiac myocytes were incubated at 37°C in a humidified environment (9% CO₂, 91% air).

Quantitation of Na,K-ATPase subunit mRNA levels

Cardiac myocytes were incubated in serum-free DMEM, treated with 1 μ M RA and/or 100 nM T3 and total cellular RNA was extracted using a previously described guanidinium thiocyanate method (Huang *et al.*, 1994). The abundance of Na,K-ATPase subunit mRNA was determined by Northern

blot analysis (Huang *et al.*, 1994). Briefly, 10–20 μ g of total RNA was subjected to electrophoresis in 1% agarose gels containing formaldehyde. RNA was transferred to nitrocellulose filters and equivalent loading and transfer of RNA was confirmed by staining of ribosomal RNA with ethidium bromide. Na,K-ATPase subunit cDNAs were labelled with [³²P] dCTP using a nick translation kit (GIBCO-BRL). Briefly, 0.1 μ g of either $\alpha 1$, $\alpha 2$, $\alpha 3$ or $\beta 1$ cDNA was incubated with 2 U DNA polymerase I/0.2 ng DNase I and 50 μ Ci [³²P] dCTP (3,000 Ci/mmol, ICN) at 16°C for 60 min. The labelled probes were purified by chromatography on Sephadex G-25 spin columns. Filters were hybridized in a solution containing $5 \times$ SSC, $1 \times$ Denhardt's 0.1% SDS, 1 mM EDTA, 10 mM NaH₂PO₄ (pH 6.9), 50% formamide and 100 μ g/ml of boiled sonicated salmon sperm DNA at 42°C for 1–3 days. Blots were washed twice with $0.1 \times$ SSC/0.1% SDS at 55°C for 15 min, dried and exposed to either X-ray film or a phosphorimager. Na,K-ATPase subunit mRNA abundance was quantitated by either densitometric analysis of the resultant autoradiograms or phosphorimaging analysis.

Transient transfection analysis

The effect of RA on $\alpha 3$ gene transcription was investigated in transient transfection studies using the $\alpha 3$ /luciferase chimeric construct pLUC34, containing the –1595/+80 region of the $\alpha 3$ gene (He *et al.*, 1996). Neonatal rat cardiac myocytes were transfected with 1 μ g of various constructs by calcium phosphate coprecipitation. DNA was mixed with 20 μ l of 2.5 M CaCl₂ and 200 μ l of $2 \times$ HBS buffer (280 mM NaCl, 50 mM HEPES and 1.5 mM Na₂HPO₄, pH 7.05) and was added to cultures in the presence of serum-containing DMEM. Cells were exposed to DNA precipitates overnight, washed twice in $1 \times$ PBS buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄ and 1.4 mM KH₂PO₄, pH 7.3) and incubated in serum-free DMEM for 1 day prior to exposure to 1 μ M RA for 2 days. In some experiments, cardiac myocytes were transfected with a luciferase construct containing a single copy of a palindromic DNA sequence element which confers both T3 and RA-responsiveness (Rohrer *et al.*, 1991). In studies examining the effect of overexpressed RA receptor, 1 μ g of a plasmid expressing human RA receptor α (Petrovich *et al.*, 1987) was co-transfected with luciferase fusion genes. In these experiments the total amount of transfected DNA was made equal by the addition of the RA receptor parental vector pSG5. Cells were

lysed by incubation for 15 min at room temperature with 200 μ l of reporter lysis buffer (PROMEGA) containing 1% Triton-X 100, 10% glycerol, 25 mM Tris-HCl, pH 7.8, 2 mM EDTA and 20 mM dithiothreitol. To measure luciferase activity, 25 μ l of cell lysate was combined with 100 μ l of substrate (470 μ M luciferin, 270 μ M coenzyme A, 530 μ M ATP, 20 mM tricine, pH 7.8 1.1 mM $(\text{MgCO}_3)_4\text{Mg}(\text{OH})_2 \cdot 5\text{H}_2\text{O}$, 2.7 mM MgSO_4 , 0.1 mM EDTA and 33.3 mM dithiothreitol) and photon emission was counted in a liquid scintillation counter within 20 sec. Luciferase activity was expressed per total cellular protein as previously detailed (Huang *et al.*, 1994).

Nuclear run-in analysis

The rate of Na,K-ATPase $\alpha 3$ gene transcription was measured as previously described (Huang *et al.*, 1994). Briefly, neonatal rat cardiac myocytes were cultured in serum-free DMEM medium, treated with 1 μ M RA for 1 day and nuclei were isolated following cell lysis with Nonidet P-40. Nuclei ($1-2 \times 10^7$) were incubated with 100 μ l of a NTP mixture (1.25 mM CTP, 1.25 mM GRP, 2.5 mM ATP and 200 μ Ci [^{32}P]-UTP (3,000 Ci/mmol)) and 100 μ l of a 4 \times salt solution (160 mM Tris-HCl, pH 8.3, 600 mM NH_4Cl and 30 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$) at 27°C for 10 min. Reaction products were treated with DNase 1 (1 mg/ml, RNase free), proteinase K (4 mg/ml) and extracted with phenol/chloroform. RNA was precipitated and transferred onto nitrocellulose filters and extensively washed with 10 ml of an ice-cold TCA/NaPPi solution (3% TCA in 10 g/L NaPPi). RNA was eluted with a buffer containing 150 mM NaCl, 5 mM EDTA and 50 mM Tris-HCl, pH 8.0 at 60–65°C and precipitated with ethanol at -20°C . RNA pellets were resuspended and incubated with 10 μ g of $\alpha 3$ and $\alpha 2$ cDNAs and pBR322 which had been immobilized on separate nitrocellulose filters. After hybridization at 45°C for 4 days, filters were washed in $0.1 \times \text{SSC}$, 0.1% SDS once at room temperature for 20 min and twice at 50°C for 15 min. Hybridization of labelled RNA to DNA-containing filters was quantitated by phosphorimaging analysis. Binding to pBR322 was considered non-specific and subtracted from $\alpha 3$ and $\alpha 2$ values. Corrected $\alpha 3$ and $\alpha 2$ values were then divided by the total input ^{32}P -labelled RNA determined by liquid scintillation counting.

Statistical analysis

To determine the statistical significance of results, unpaired Student's *t*-test (2-tailed) were utilized.

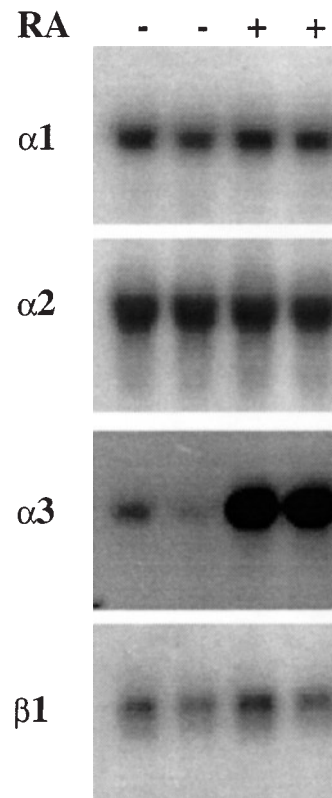


Figure 1 Northern blot analysis of the effect of RA on Na,K-ATPase subunit mRNA levels. Neonatal rat cardiac myocytes were isolated and incubated in serum-free DMEM as detailed in Materials and Methods. Cardiac myocytes were exposed to 1 μ M RA for 1 d. Total RNA was extracted from separate plates and resolved on a 1% agarose gel. RNA was transferred to nitrocellulose membranes and blots were hybridized with ^{32}P -labelled Na,K-ATPase $\alpha 1$, $\alpha 2$, $\alpha 3$ and $\beta 1$ subunit cDNAs.

Results

RA selectively increases Na,K-ATPase $\alpha 3$ mRNA abundance

To evaluate whether RA regulates Na,K-ATPase subunit gene expression, Northern blot analysis was conducted with total RNA isolated from primary cultures of neonatal rat cardiac myocytes incubated for 1 day in the absence and presence of RA. A representative Northern blot depicting the effect of RA on Na,K-ATPase $\alpha 1$, $\alpha 2$, $\alpha 3$ and $\beta 1$ subunit mRNA is shown in Figure 1. In cardiac myocytes incubated in serum-free medium, $\alpha 1$, $\alpha 2$, $\alpha 3$ and $\beta 1$ mRNA expression was evident. Exposure to 1 μ M RA for 1 day, however, yielded a selective increase in the content of $\alpha 3$ mRNA. An assessment of the time-course of the effect of RA on $\alpha 3$ mRNA revealed that a maximum three-fold induction

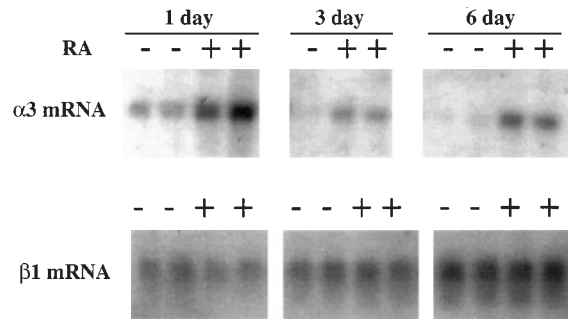


Figure 2 Time-course of the effect of RA on Na,K-ATPase $\alpha 3$ and $\beta 1$ subunit mRNA. Neonatal rat cardiac myocytes were cultured as described in Figure 1 and exposed to $1 \mu\text{M}$ RA for either 1, 3 or 6 d. Total RNA was extracted from individual plates of cells, resolved on a 1% agarose gel and transferred to nitrocellulose membranes. Blots were hybridized with ^{32}P -labelled rat Na,K-ATPase $\alpha 3$ or $\beta 1$ subunit cDNA.

occurred within 1 day, and that prolonged exposure to RA still had no effect on $\beta 1$ mRNA expression (Fig. 2, Table 1).

RA increases Na,K-ATPase $\alpha 3$ mRNA content by a post-transcriptional mechanism

To investigate whether RA-mediated stimulation of Na,K-ATPase $\alpha 3$ subunit mRNA levels in cardiac myocytes was via a transcriptional mechanism, transient transfection analysis was conducted with an $\alpha 3$ gene construct which contained 1595 base pairs of 5'-flanking DNA (pLUC34) in the absence and presence of a co-transfected RA receptor expression plasmid. Cardiac myocytes were cultured in serum-free DMEM for 1 day and then incubated in the presence of $1 \mu\text{M}$ RA for 2 days. Cells were lysed, luciferase activity was measured and the RA

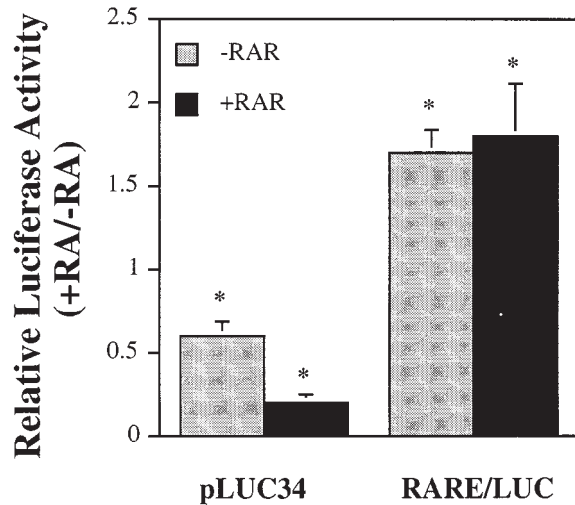


Figure 3 Effect of RA on Na,K-ATPase $\alpha 3$ /luciferase chimeric gene expression. Neonatal rat cardiac myocytes were cultured as described in Materials and Methods and transfected with $1 \mu\text{g}$ of either the $\alpha 3$ gene construct pLUC34 or a luciferase plasmid containing a RA response element (RARE/LUC) with or without cotransfection of $1 \mu\text{g}$ of a RA receptor α expression vector. Cells were treated with $1 \mu\text{M}$ RA for 2 d and luciferase activity was determined as detailed in Materials and Methods. Data ($n=8-10$) are presented as the ratio of the normalized luciferase activity in the presence of RA relative to that in the absence of RA, which is set to 1.0. * $P<0.05$.

values were expressed relative to the minus RA controls. As depicted in Figure 3, pLUC34 activity was reduced 40% by RA, whereas expression of a plasmid containing a positive RA response element was augmented 1.7-fold. Since we and others have shown that both T3 and RA receptors can be limiting in transient transfection studies in neonatal rat cardiac myocytes and thereby either blunt or abolish a hormone-dependent activation of reporter gene expression (Huang *et al.*, 1994; Rohrer *et al.*,

Table 1 The effect of RA on the abundance of Na,K-ATPase subunit mRNAs

Duration of Treatment	Relative mRNA content				
	RA	$\alpha 1$	$\alpha 2$	$\alpha 3$	$\beta 1$
1 d	-	1.00 ± 0.16	1.00 ± 0.07	1.00 ± 0.05	1.00 ± 0.21
	+	1.01 ± 0.14	1.03 ± 0.05	$3.29 \pm 0.77^*$	1.34 ± 0.58
3 d	-	ND**	ND	1.00 ± 0.03	1.00 ± 0.16
	+	ND	ND	$2.63 \pm 0.63^*$	1.10 ± 0.26
6 d	-	ND	ND	1.00 ± 0.04	1.00 ± 0.18
	+	ND	ND	$4.56 \pm 1.35^*$	1.38 ± 0.26

Neonatal rat cardiac myocytes were prepared as described in Figure 1 and incubated in serum free medium in the absence and presence of $1 \mu\text{M}$ RA for 1, 3 and 6 d. Total RNA was extracted and analyzed by Northern blot hybridization. The values shown represent the relative Na,K-ATPase subunit mRNA induction as a ratio of densitometric intensity in the presence of RA divided by the intensity in the absence of RA. Values in the absence of RA have been set to equal 1.00. Data ($n=4-10$) are presented as mean and standard error of the mean. * $P<0.05$; **ND=not determined.

1991), we investigated the effect of RA on chimeric gene activity in the presence of cotransfected RA receptor (Fig. 3). Over-expression of RA receptor did not, however, result in a RA-mediated increase in pLUC34 expression. In contrast, a 1.8-fold stimulation of luciferase activity driven by the RA response element was evident in cells cotransfected with a RA receptor expression plasmid. These results suggest that a region within 1595 base pairs of the 5'-flanking region of the $\alpha 3$ gene does not mediate an increase in transcription in response to RA.

To determine if RA affects the overall transcription rate of the Na,K-ATPase $\alpha 3$ subunit gene, nuclear run-on assays were performed. Neonatal rat cardiac myocytes were incubated in serum-free medium for 2 days and then exposed to 1 μ M RA for 1 day. This time-point represents a new steady-state since RA treatment for 6 days yielded a stimulation of $\alpha 3$ mRNA which was statistically the same as the increase observed at 1 day (Table 1). Nuclei were isolated from control and RA-treated cells and incubated with [³²P] UTP. [³²P] UTP-labelled newly synthesized mRNA was extracted, hybridized to either Na,K-ATPase $\alpha 3$ or $\alpha 2$ subunit cDNAs and detected by phosphorimaging analysis (Fig. 4). Although filters containing subunit cDNA hybridized significantly more ³²P-labelled RNA than those with pBR322 (Fig. 4A), incubation of cardiac myocytes with 1 μ M RA did not influence the rate of either $\alpha 3$ or $\alpha 2$ gene transcription (Fig. 4B). Thus, up-regulation of Na,K-ATPase $\alpha 3$ mRNA content by RA most likely occurs at a post-transcriptional level in neonatal rat cardiac myocytes.

T3-mediated augmentation of Na,K-ATPase $\beta 1$ mRNA content is diminished by RA

To examine whether RA and T3 have a combinatorial action on regulating Na,K-ATPase subunit gene expression, we assessed the effect of both RA and T3 on $\alpha 3$ and $\beta 1$ mRNA levels. Primary cultures of neonatal rat cardiac myocytes were preincubated in serum-free medium and exposed to 1 μ M RA, 100 nM T3, or both for 1 day. Replicate Northern blots were prepared with total RNA isolated from separate plates of cells. The quantitation of these results are shown in Figure 5. Consistent with previous reports (Kamitani *et al.*, 1992; Liu *et al.*, 1993; Orłowski and Lingrel, 1990), we found that incubation with T3 yielded an increase in the abundance of both Na,K-ATPase $\alpha 3$ and $\beta 1$ subunit mRNAs. The effect of T3 and RA on the content of $\alpha 3$ mRNA, however, was indistinguishable from

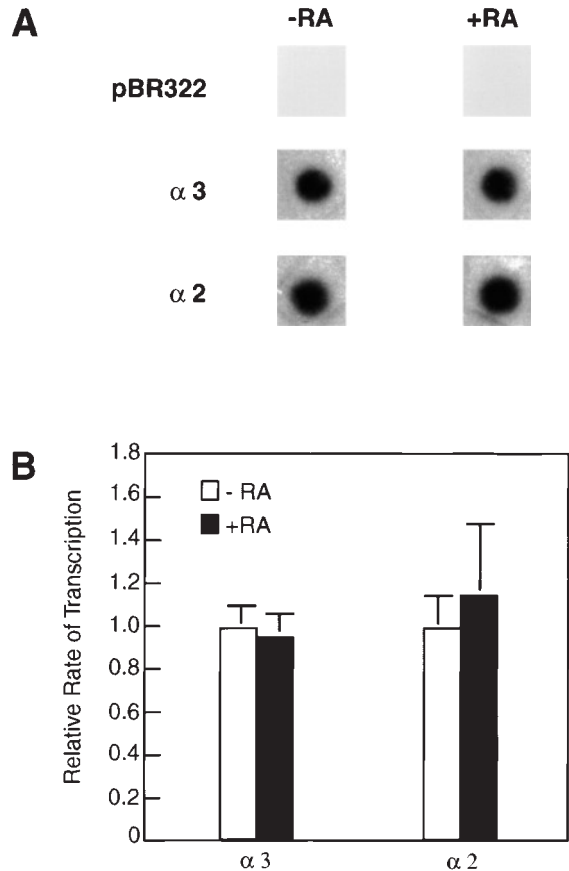


Figure 4 Nuclear run-on analysis of the effect of RA on Na,K-ATPase $\alpha 3$ and $\alpha 2$ subunit gene transcription. Cultures of neonatal cardiac myocytes were pre-incubated in serum free medium as in Figure 1 and exposed to 1 μ M RA for 1 d. The nuclei were isolated and used in nuclear run-on assays as described in Materials and Methods. A: Data shown are from a single experiment in which cells were incubated in the absence or presence of 1 μ M RA for 1 d. *In vitro* ³²P-labelled RNA was hybridized in a single reaction to filters containing pBR322, $\alpha 3$ and $\alpha 2$ cDNAs. B: Pooled data from two independent experiments ($n=6$). Within each experiment -RA values were averaged and normalized to 1.00 and +RA values were expressed as the ratio to the -RA group. Data are presented as mean and standard error of the mean.

the induction seen with either T3 or RA alone. Interestingly, although RA by itself had no effect on $\beta 1$ mRNA content, it inhibited the upregulation of $\beta 1$ mRNA by T3.

Discussion

In the present study we have utilized primary cultures of neonatal rat cardiac myocytes to investigate the effect of RA on Na,K-ATPase subunit gene expression. To our knowledge, the demonstration

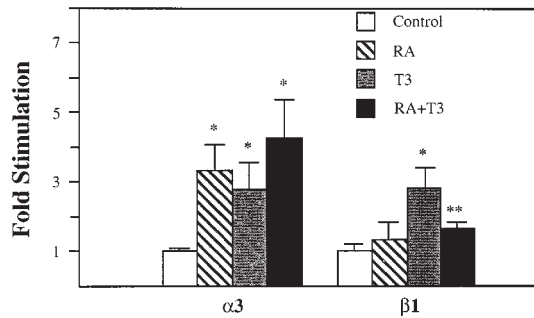


Figure 5 Effect of RA and T3 on Na,K-ATPase $\alpha 3$ and $\beta 1$ subunit mRNAs. Neonatal rat cardiac myocytes were incubated as described in Figure 1 and exposed to either vehicle, 1 μ M RA, 100 nM T3, or both RA and T3 for 1 d. Total RNA was prepared and subjected to Northern blot analysis with labelled Na,K-ATPase $\alpha 3$ or $\beta 1$ cDNAs. Results from multiple experiments were pooled and represented as the relative effect of each treatment to control. Data ($n=4-8$) are presented as mean and standard error of the mean. * $P<0.05$ (vs control), ** $P<0.05$. (The magnitude of stimulation of $\beta 1$ mRNA by T3 alone is significantly different from that by T3 plus RA.) The data which represents induction of $\alpha 3$ and $\beta 1$ mRNA contents by RA for 1 d are the same as shown in Table 1.

that Na,K-ATPase $\alpha 3$ subunit mRNA levels are upregulated by RA in these cells is a novel finding. The stimulatory effect of RA on Na,K-ATPase $\alpha 3$ mRNA was selective since $\alpha 1$, $\alpha 2$ and $\beta 1$ mRNA levels were not increased (Fig. 1). In contrast, upregulation of Na,K-ATPase activity by either serum, T3 or hyperoxia have been found to elicit a co-ordinate induction of subunit mRNA levels (Bhutada *et al.*, 1990; Gick *et al.*, 1988; Nici *et al.*, 1991). It has been frequently observed, however, that there is either a quantitative difference in the extent of upregulation of subunit mRNAs (Bhutada *et al.*, 1991; Gick *et al.*, 1990; Pressley *et al.*, 1988) or similar to our observation with RA, a Na,K-ATPase subunit gene(s) which is totally non-responsive to a given inducer (Farman *et al.*, 1994; Gick, *et al.*, 1988). The physiological role of the selective induction of Na,K-ATPase $\alpha 3$ subunit mRNA by RA is unclear, however, since in rat heart, Na,K-ATPase $\alpha 3$ subunit mRNA is upregulated in the fetus and neonate (Lingrel *et al.*, 1990), we propose that retinoid action contributes, in part, to the development-related expression pattern of the $\alpha 3$ gene.

We have shown that RA had no stimulatory effect on Na,K-ATPase $\alpha 3$ gene transcription in either transient transfection experiments (Fig. 3) or nuclear run-on assays (Fig. 4). These findings suggest that the stimulation of $\alpha 3$ mRNA content by RA is a result of a post-transcriptional mechanism in neonatal rat cardiac myocytes. The

post-transcriptional effect of RA on Na,K-ATPase $\alpha 3$ gene expression is consistent with other studies of RA action. For example, RA exerted post-transcriptional control of gene expression by altering the stability of cytoplasmic mRNAs encoding tyrosine aminotransferase (Pan *et al.*, 1992) and proteolipid (Lopez-Barahona, *et al.*, 1993). These observations support the contention that post-transcriptional control may represent a significant mechanism whereby RA modulates gene expression in mammalian cells.

In transient transfection experiments utilizing an $\alpha 3$ gene construct containing 1595 base pairs of 5'-flanking DNA, RA treatment repressed $\alpha 3$ chimeric gene expression both in the absence and presence of co-transfected RA receptor. These results are consistent with our previous demonstration that the -116 to -6 base pair region of the Na,K-ATPase $\alpha 3$ gene contains a negative RA response element (He *et al.*, 1996). The physiological significance of this negative RA response element in the $\alpha 3$ gene is unclear. It may modulate RA's stimulatory effect on $\alpha 3$ mRNA abundance at specific stages during cardiac cell differentiation. We also speculate that the negative RA response element might, in part, be responsible for the tissue-restricted suppression of $\alpha 3$ gene expression (Lingrel *et al.*, 1990). It is not uncommon to identify a single gene which is regulated by both positive and negative mechanisms. We have demonstrated that both stimulatory and inhibitory mechanisms contribute to T3-mediated expression of rat Na,K-ATPase $\alpha 3$ and $\alpha 2$ genes in primary cultures of neonatal rat cardiac myocytes (He *et al.*, 1996; Huang *et al.*, 1994). Similarly, positive and negative T3 response elements have been localized in the promoter regions of the rat growth hormone (Crone *et al.*, 1990) and apolipoprotein A1 genes (Taylor *et al.*, 1996).

In the present study, we found that the incubation of primary cultures of neonatal rat cardiac myocytes in the presence of either RA or T3, or both RA and T3, yields a three-fold stimulation of Na,K-ATPase $\alpha 3$ subunit mRNA content (Fig. 5). The lack of either an additive or synergistic effect of exposure to both RA and T3 on $\alpha 3$ mRNA content implies that the RA and T3 receptors are not functionally co-operative in the regulation of $\alpha 3$ gene expression. Similarly, no co-operativity was evident in an investigation in cultured cardiac myocyte of the response of slow sarcoplasmic reticulum Ca^{2+} -ATPase and myosin heavy chain α mRNA content to RA and T3 (Rohrer *et al.*, 1991). Interplay between RA and T3-mediated pathways of gene expression, however, was evident in the inhibitory effect of RA on the T3-mediated increase in Na,K-ATPase $\beta 1$

subunit mRNA abundance (Fig. 5). Since a post-transcriptional mechanism has been proposed to regulate the stimulatory effect of T3 on $\beta 1$ mRNA in neonatal rat cardiac myocytes (Liu *et al.*, 1993), we hypothesize that RA represses the transcription of a factor which is responsible for increasing $\beta 1$ mRNA content at a post-transcriptional level.

RA plays a fundamental role in cell growth, differentiation and vertebrate development via binding to nuclear receptors and subsequent modulation of gene transcription (Giguere, 1994). RA receptors are capable of forming heterodimers with retinoid X receptors resulting in enhanced binding to RA response elements. Targeted disruption of RA receptors and retinoid X receptor genes in mice demonstrated that RA receptor/retinoid X receptor heterodimers are essential for normal heart morphogenesis (Kastner *et al.*, 1994; Sucov *et al.*, 1994). Moreover, cardiac cell hypertrophy induced by either phenylephrine or endothelin treatment of primary cultures of neonatal rat cardiac myocytes was suppressed by RA (Wu *et al.*, 1996; Zhou *et al.*, 1995). It is important to note, however, that little information is available regarding specific genes which are regulated by RA during either heart development or cardiac hypertrophy. Our data implicate Na,K-ATPase $\alpha 3$ subunit gene expression as an early target for retinoid action in cardiac cell development. Interestingly, a recent study reported that Na,K-ATPase $\alpha 3$ mRNA was selectively repressed during ouabain-mediated hypertrophy of neonatal rat cardiac myocytes, and RA was found to antagonize the suppression of $\alpha 3$ mRNA by ouabain (Huang *et al.*, 1997). Taken together, these findings suggest that the regulation of Na,K-ATPase $\alpha 3$ subunit gene expression by retinoids plays an important functional role in cardiac myocytes.

Acknowledgements

We thank Dr. J. Lingrel for the gift of rat Na,K-ATPase subunit cDNAs. We also thank Dr. G. Gill for providing a RA response element/luciferase construct. We are also grateful to Dr. P. Chambon for providing a human RA receptor α expression vector. This work was supported by grants to GG, from the National Science Foundation and an Investigatorship and Grant-in-Aid from the American Heart Association, New York City Affiliate.

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