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Regulation of Na/K-ATPase β_1-subunit gene expression by ouabain and other hypertrophic stimuli in neonatal rat cardiac myocytes

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Abstract

Partial inhibition of Na/K-ATPase by ouabain causes hypertrophic growth and regulates several early and late response genes, including that of Na/K-ATPase α_3 subunit, in cultured neonatal rat cardiac myocytes. The aim of this work was to determine whether ouabain and other hypertrophic stimuli affect Na/K-ATPase β_1 subunit gene expression. When myocytes were exposed to non-toxic concentrations of ouabain, ouabain increased β_1 subunit mRNA in a dose- and time-dependent manner. Like the α_3 gene, β_1 mRNA was also regulated by several other well-known hypertrophic stimuli including phenylephrine, a phorbol ester, endothelin-1, and insulin-like growth factor, suggesting involvement of growth signals in regulation of β_1 expression. Ouabain failed to increase β_1 subunit mRNA in the presence of actinomycin D. Using a luciferase reporter gene that is directed by the 5′-flanking region of the β_1 subunit gene, transient transfection assay showed that ouabain augmented the expression of luciferase. These data support the proposition that ouabain regulates the β_1 subunit through a transcriptional mechanism. The effect of ouabain on β_1 subunit induction, like that on α_3 repression, was dependent on extracellular Ca^{2+} and on calmodulin. Inhibitions of PKC, Ras, and MEK, however, had different quantitive effects on ouabain-induced regulations of β_1 and α_3 subunits. The findings show that partial inhibition of Na/K-ATPase activates multiple signaling pathways that regulate growth-related genes, including those of two subunit isoforms of Na/K-ATPase, in a gene-specific manner. (Mol Cell Biochem **215**: 65–72, 2000)

Key words: Na/K-ATPase, calcium, signal transduction, ouabain, Ras, mitogen-activated protein kinase

Introduction

Na/K-ATPase catalyzes the coupled active transport of Na^+ and K^+ across the plasma membranes of most mammalian cells [1, 2]. The enzyme consists of two noncovalently linked subunits. The α subunit (about 112 kD) contains the ATP, digitalis, and other ligand binding sites, and is considered as the 'catalytic subunit'. It spans the membrane 10 times, with both N- and C-terminals on the cytoplasmic side. The β subunit, spanning the membrane once, is a glycoprotein (about 55 kD) that is essential for normal function and assembly of the pump. Several isoforms of both α and β subunits have been identified [3–5]. While rat α isoforms exhibit different sensitivities to ouabain and oxygen free radicals, all β isoforms appear to be able to assemble with an α subunit to form functional enzyme. Expression of both α and β subunits are regulated by hormones and changes in intracellular ion composition [6–8].

In the heart, Na/K-ATPase serves as the receptor for the positive inotropic effects of cardiac glycosides [9–12]. Mechanistically, the partial inhibition of the myocardial enzyme by ouabain and related drugs causes a small increase in in-

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tracellular Na^+ which in turn affects the sarcolemmal Na^+/Ca^{2+} exchanger, leading to a significant increase in intracellular Ca^{2+} and in the force of contraction. This effect on cardiac contractility is the basis of the major therapeutic use of these drugs in the treatment of congestive heart failure. Recently, using cultured neonatal rat cardiac myocytes, we showed that partial inhibition of Na/K-ATPase by ouabain also activates multiple signaling pathways, thus stimulating the hypertrophic growth of these myocytes [13–16]. Clearly, the altered activity of Na/K-ATPase by digitalis drugs must now be considered as a potential signal for hypertrophic growth along with other hormonal, mechanical, and pathological stimuli of cardiac hypertrophy. Cardiac hypertrophy is not only a beneficial adaptive response to increased workload, but also an independent risk factor for the development of heart failure [17]. Hence, it is important to study the potential hypertrophic effects of drugs that are widely used in the treatment of heart failure. Since the enzyme serves as a receptor for ouabain, our recent new findings prompted us to determine whether ouabain and other hypertrophic stimuli regulate genes of Na/K-ATPase in cardiac myocytes. Neonatal cardiac myocytes, when cultured under serum-free conditions, express α_1, α_3, and β_1. When these cells were exposed to ouabain and other hypertrophic stimuli, α_1 mRNA was not affected but α_3 mRNA was decreased in a dose- and time-dependent manner [15]. Since inhibition of Na/K-ATPase in cardiac myocytes by lowering extracellular K^+ up-regulates β_1 expression [18], the aim of this work was to determine if ouabain and other hypertrophic stimuli affect β_1 expression and to define the signal pathways of ouabain-mediated regulation of the β_1 gene.

Materials and methods

Materials

Chemicals of the highest purity available were purchased 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). Radio-nucleotides (^{32}P-labeled, about 3000 Ci/mmol) were from Dupont NEN (Boston, MA, USA). Rabbit polyclonal Anti-ACTIVE MAPK (mitogen-activated protein kinases) pAb and anti-p44/42 MAPK antibodies were obtained from Promega (Madison, WI, USA) and New England Biolabs (Beverly, MA, USA), respectively. 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 [15]. Briefly, myocytes were isolated from ventricles of 1-day-old Sprague-Dawley rats, and purified by centrifugation on Percoll gradients. Myocytes were then cultured in a medium containing 4 parts of DMEM (Dulbecco's modified Eagle's medium) and 1 part Medium 199 (Gibco), penicillin (100 units/ml), streptomycin (100 μg/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 serum. All experiments were done after 48 h of further incubation under serum-free conditions. These cultures contain more than 95% myocytes as assessed by immunofluorescence staining with a myosin heavy chain antibody (data not shown).

Northern blot

Northern blot was done as we previously described [15]. The same blots were analyzed for several different mRNAs. After each measurement, the blots were stripped, then rehybridized with other probes [14, 15]. The glyceraldehyde-3-phosphate dehydrogenase (GADPH) and the α_3 probes were made as before [15]. To probe β_1 mRNA, a full length rat β_1 cDNA was used. Routinely about 20 μg of total RNA was subjected to gel electrophoresis, transferred to a Nytran membrane, UV-immobilized, and hybridized to ^{32}P-labeled probes. Autoradiograms obtained at –70°C were scanned with a Bio-Rad densitometer. Multiple exposures were analyzed to assure that the signals are within the linear range of the film. The relative amount of RNA in each sample was normalized to that of GAPDH mRNA to correct for differences in sample loading and transfer.

Transient transfection assay

A firefly luciferase chimeric gene (EP-pXP1) directed by the Na/K-ATPase β-1 promoter was constructed as we previously described [18]. After 12 h culture in serum containing medium, myocytes were transfected with 5 μg EP-pXP1 plasmid DNA using a modified calcium phosphate method [19]. After 48 h incubation, the transfected cells were subjected to ouabain and other treatments. Cell lysate was prepared and assayed for luciferase activity as described [18]. All transfections were conducted in triplicate in 6 cm tissue culture dishes and control experiments with a Rous sarcoma virus (RSV)-β-galactosidase reporter plasmid indicated that transfection efficiency varied less than 15% within a given experiment under our experimental conditions.

Fluorescence microscopic measurements of intracellular Ca^{2+} concentration

Myocytes were cultured on glass coverslips. Intracellular Ca^{2+} was measured by fura-2 as we previously described [20].

Fura-2 fluorescence was recorded using an Attofluor imaging system (Atto Instruments) at excitation wavelength of 340/380 nm and at emission wavelength of 505 nm. Under each experimental condition time-averaged signals were obtained from about 40 single cells. Relative Ca^{2+} concentration was calculated based on the fluorescence ratio and Ca^{2+} calibration curve.

Preparation of replication-defective adenovirus Asn^{17} Ras and adenovirus infection of cardiac myocytes

A replication-defective adenovirus expressing dominant negative Asn^{17} Ras was generated as previously described [16]. Virus was amplified in human kidney 293 cells, and the viral particles were purified from 293 cell lysates by cesium chloride gradient ultracentrifugation then desalted by dialysis to HBS. The concentration of recombinant adenovirus was determined based on the absorbance at 260 nm where 1 optical density unit corresponds to 10^{12} particles/ml. An identical adenovirus containing the β-galactosidase gene instead of the Asn^{17} Ras was used as a virus control. Myocytes were infected with the viruses at a dose of 1,000 viral particles/cell for 12 h, and used for experiments as we previously described [16].

Sodium pump activity assay

Sodium pump activity was measured by ouabain-sensitive $^{86}Rb^+$ uptake as previously described [13]. Briefly, myocytes cultured in 12-well plates were washed and treated with different stimuli. Both control and treated cells were then incubated with the same medium in the presence or absence of 1 mM ouabain at 37°C for 5 min. Monensin (25 μM) and $^{86}Rb^+$ as tracer for K^+ (1 μCi/well) were added to start the uptake experiment. After 15 min, cells were washed 3 times with ice-cold 100 mM $MgCl_2$ solution, dissolved in SDS, assayed for protein and, counted by conventional procedures.

Statistics

Data are given as mean ± S.E. Statistical analysis was performed using Student's *t*-test, and significance was accepted at $p < 0.05$.

Results

Stimulation of β_1 expression by ouabain and other hypertrophic stimuli in cardiac myocytes

When the steady-state levels of Na/K-ATPase subunit mRNAs were measured in myocytes after 48 h of culture in the serum-free medium, those of α_1 and α_3 were readily detectable [15]. Several β_1 mRNA species (e.g. 2.7, 2.4, and 1.8 kb) were detected in cultured neonatal cardiac myocytes when blots were hybridized with a full-length β_1 cDNA probe (Fig. 1). Under our experimental conditions the 2.7 and 2.4 kb β_1 mRNAs were not well separated. Over-exposure revealed additional low molecular weight β_1 mRNAs. When cardiac myocytes were exposed to different non-toxic concentrations of ouabain, in contrast to down-regulation of α_3 mRNA [15], all three species of β_1 mRNAs were increased by ouabain in a dose-dependent manner (Fig. 1). When time-dependent changes were measured after the cells were exposed to 100 μM ouabain, a significant increase was noted after 4 h and maximal stimulation was reached after 12 h (Fig. 2). These findings are in agreement with the prior studies indicating that

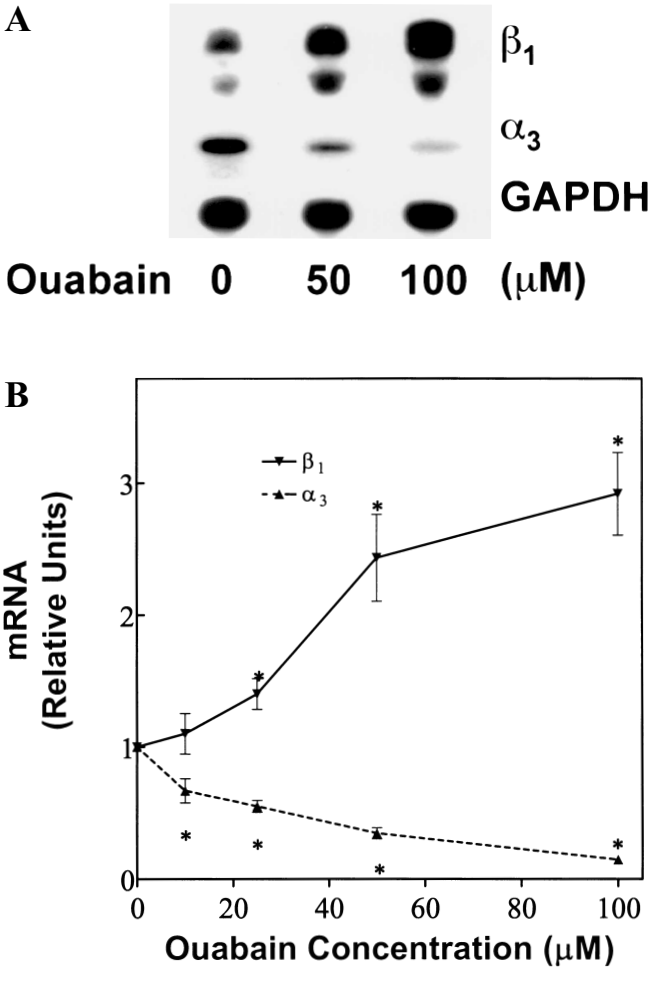

Fig. 1. Effects of ouabain on β_1 and α_3 mRNAs. (A) A representative autoradiogram of ouabain effects. The cells were treated with ouabain for 12 h. Total RNA was isolated, and analyzed for β_1, α_3, and GAPDH by Northern blot as described under 'Materials and methods'. (B) The mRNA values of β_1 and 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 ± S.E. of 4 experiments. *$p < 0.05$ vs. control cells.

inhibition of Na/K-ATPase by lowering extracellular K^+ can stimulate β_1 expression in cardiac myocytes [18].

To determine whether up-regulation of β_1 mRNA is specific to ouabain-induced sodium pump inhibition or ouabain-activated growth-related pathways, we determined the effects of several well-known hypertrophic stimuli on β_1 mRNA in cardiac myocytes. In experiments of Fig. 3, myocytes were exposed to PMA (phorbol 12-myristate 13-acetate, 100 nM), PE (phenylephrine, 0.1 mM), ET-1 (endothelin-1, 100 nM) and IGF (insulin like growth factor, 10 nM) for 12 h and measured for β_1 mRNA. Concentrations of these stimuli were chosen based on prior studies on other hypertrophic marker genes [15, 21–23]. Like ouabain, all of these hypertrophic stimuli increased β_1 mRNA abundance in cardiac myocytes (Fig. 3). Of these stimuli PMA caused the highest stimulation of β_1 expression. Unlike ouabain, however, none of the stimuli inhibited sodium pump activity measured as ouabain-sensitive $^{86}Rb^+$ uptake in these cardiac myocytes after 30 min incubation (data not shown).

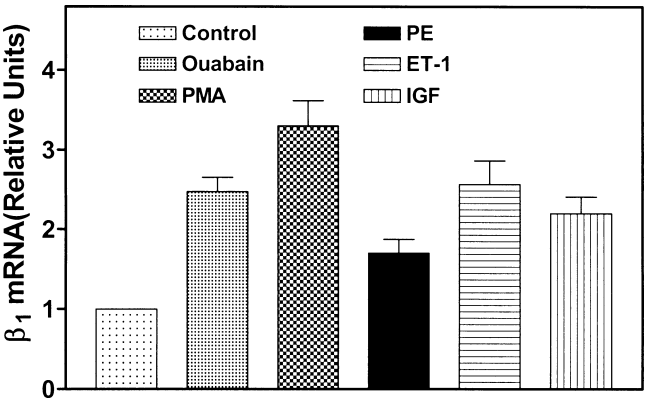

Fig. 3. Effects of hypertrophic stimuli on β_1 mRNA. Myocytes were treated with different stimuli as indicated for 12 h. Total RNA was isolated and measured for β_1 mRNA as in Fig. 1. The values are mean ± S.E. of 3 experiments.

Net influx of extracellular Ca^{2+} and activation of calmodulin are required for ouabain-induced up-regulation of β_1 mRNA

We have demonstrated that ouabain-activated hypertrophic pathways are initiated by increases in Ca^{2+} influx and activation of CaM (calmodulin) [14]. To determine whether these factors are required for ouabain-induced up-regulation of β_1 mRNA abundance, the experiments of Fig. 4 were done. When cells were exposed to ouabain in a nominally Ca^{2+}-free medium, ouabain showed no effect on β_1 mRNA (Fig. 4). As expected, after 30 min incubation ouabain raised steady state Ca^{2+} from 119 ± 17 nM to 229 ± 28 nM ($p < 0.01$) in the presence of extracellular Ca^{2+}. Ouabain-induced increases in intracellular Ca^{2+} lasted for at least 12 h (data not shown). However, removal of extracellular Ca^{2+} blocked ouabain-induced increases in intracellular Ca^{2+} (control 86 ± 8 nM vs. ouabain 90 ± 12 nM, $p > 0.05$). These data indicate that net influx of extracellular Ca^{2+} is required for ouabain-induced up-regulation of β_1 mRNA in cardiac myocytes (Fig. 4). Incubation of cardiac myocytes in nominally Ca^{2+}-free medium significantly decreased intracellular Ca^{2+}. After 30 min incubation, intracellular Ca^{2+} decreased from 119 ± 16 nM to 86 ± 8 nM ($p < 0.05$). Interestingly, removal of extracellular Ca^{2+} also reduced basal β_1 mRNA abundance (Fig. 4), further sup-

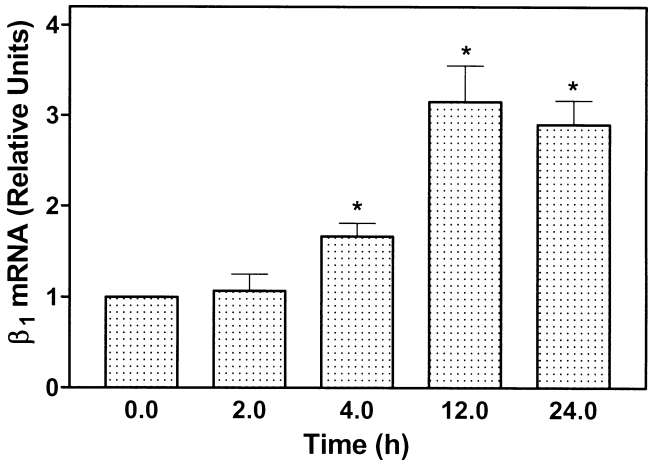

Fig. 2. Time course of the ouabain effects on the steady state levels of β_1 mRNA. The cells were treated with 100 μM ouabain for various times, and assayed for β_1 and α_3 mRNAs as in Fig. 1. The values are mean ± S.E. of 3 experiments. *$p < 0.05$ vs. control cells.

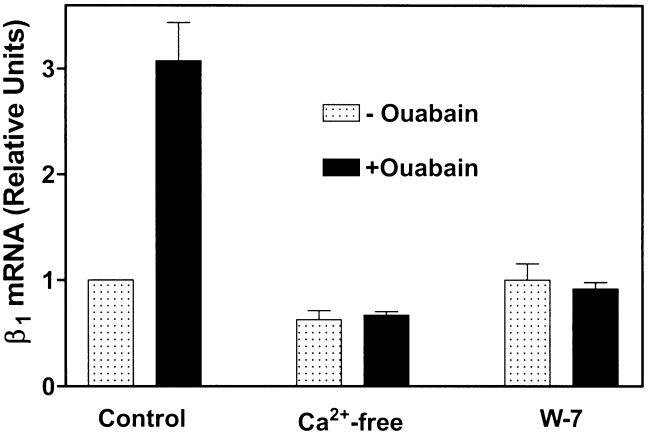

Fig. 4. Effects of H-7 and W-7 on ouabain-induced luciferase activity. Myocytes were transfected with a β_1 promoter-luciferase chimeric gene for 12 h, washed and incubated for an additional 36 h. Transfected cells were then exposed to 100 μM of ouabain in the presence or absence of either 2 μM W-7 or 25 μM H-7 for 17 h. Cell lysates were prepared and assayed for luciferase activity as described under 'Materials and methods'. The values are mean ± S.E. of 6 measurements.

porting an involvement of Ca^{2+}-dependent pathways in regulation of β_1. To determine the role of CaM in ouabain-induced up-regulation of β_1, myocytes were exposed to ouabain in the presence of W-7 (N-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide hydrochloride) for 12 h. As depicted in Fig. 4, W-7 completely blocked the effect of ouabain on β_1 mRNA.

The role of protein kinases in ouabain-induced up-regulation of β_1

We have demonstrated that ouabain-induced down-regulation of α_3 involves the participation of PKC (protein kinase C). To determine the role of PKC in ouabain-induced up-regulation of β_1 mRNA, myocytes were treated with ouabain in the presence of H-7 (1-(5-isoquinolinesulfonyl)-2-methyl-piperazine dihydrochloride), a PKC and PKA inhibitor [15]. As shown in Fig. 5, while the effect of ouabain on α_3 was partially blocked by H-7, this kinase inhibitor completely blocked ouabain-induced up-regulation of β_1 mRNA. On the other hand, HA1004 (HA-1004, N-(2-guanidinoethyl)-5-isoquinoline-sulfonamide hydrochloride), a PKA-specific inhibitor, showed no effect on ouabain-induced up-regulation of β_1. Interestingly, H-7 also significantly decreased basal levels of β_1 mRNA in myocytes. These data and those on PMA (Fig. 3) clearly indicated that PKC plays a key role in regulation of both basal and stimulated expression of β_1.

The roles of Ras and MAPK in ouabain-induced up-regulation of β_1

Exposure of cardiac myocytes to ouabain activates Ras and p42/44 MAPK [16]. Activation of Ras and p42/44 MAPK is required for ouabain-induced down-regulation of α_3 in cardiac myocytes [16]. To determine if Ras and p42/44 MAPK are also involved in pathways of β_1 regulation, the experiments of Fig. 6 were done. Myocytes were first transduced with a dominant negative Ras Asn^{17} adenoviral vector, then exposed to 100 μM ouabain. Unlike its effect on α_3, expression of dominant negative Ras only caused a partial suppression of ouabain-induced β_1 expression. Concordantly, when activation of p42/44 MAPK was blocked by a MEK (MAPK kinase) inhibitor PD 98059, the effects of ouabain on β_1 were partially blocked.

Ouabain transactivates β_1-promoter-directed luciferase expression in a W-7- and H-7-dependent manner

To determine whether ouabain regulates β_1 expression through a transcriptional mechanism, myocytes were transfected with a luciferase reporter gene that is directed by the 5′-flanking region of the β_1 subunit gene. As shown in Fig. 7, ouabain significantly increased luciferase expression, supporting a proposition that ouabain regulates β_1 through a transcriptional mechanism. To corroborate this, myocytes were exposed to ouabain for 12 h in the presence of actinomycin D, a RNA synthesis inhibitor. While 100 μM ouabain caused a significant increase in β_1 mRNA (2.9 ± 0.4-fold relative to control value of one, $p < 0.01$) in the absence of actinomycin D, it failed to increase β_1 mRNA (1.4 ± 0.3-fold relative to control value of one, $p > 0.05$) in the presence of 5 μg/ml of actinomycin D.

To further test the role of CaM and PKC in ouabain-stimulated transcriptional regulation of the β_1 gene, the effects of W-7 and H-7 on ouabain-induced luciferase activity was

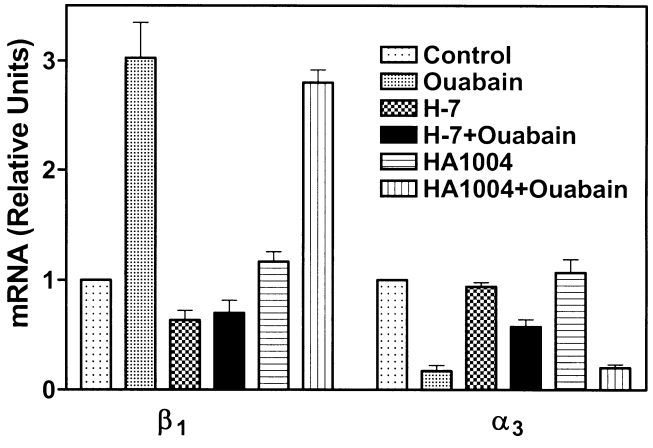

Fig. 5. Effects of extracellular Ca^{2+} and W-7 on ouabain-induced β_1 mRNA. Myocytes were either preincubated in a nominally Ca^{2+}-free medium or pretreated with 2 μM W-7 for 15 min, then exposed to ouabain for 12 h. β_1 mRNA was measured as in Fig. 1 and the values are mean ± S.E. of 3 experiments.

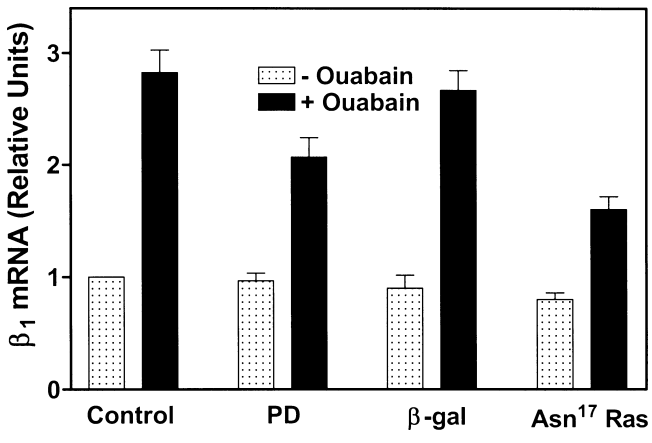

Fig. 6. Inhibition of PKC shows different effects on changes of β_1 and α_3 mRNA in response to ouabain. Myocytes were pretreated with either 25 μM H-7 or 25 μM HA 1004 for 15 min, then exposed to ouabain for 12 h. mRNAs of β_1 and α_3 were measured as in Fig. 1. The values are mean ± S.E. of 3 experiments.

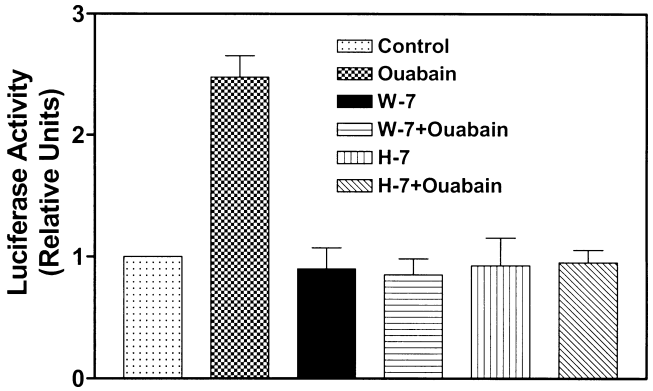

Fig. 7. Inhibition of Ras and MEK partially blocks ouabain-induced up-regulation of β_1 mRNA. Myocytes were infected with a dominant negative Asn^{17} Ras adenoviral vector or a β-gal virus at concentration of 1000 particles/cell for 12 h, washed, then exposed to 100 μM ouabain for 12 h. In separate experiments myocytes were pretreated with 20 μM PD98059 for 15 min, then exposed to ouabain. β_1 mRNA was measured as in Fig. 1. The values are mean ± S.E. of 3 experiments.

determined. As depicted in Fig. 7, both W-7 and H-7 completely blocked ouabain-induced increases in luciferase activity.

Discussion

The β subunit of Na/K-ATPase is essential for the maturation and assembly of the functional enzyme [2]. Inhibition of Na/K-ATPase by lowering extracellular K^+ increases β_1 expression in several different cells including neonatal rat cardiac myocytes [18, 24]. We have demonstrated that ouabain activates hypertrophic pathways and regulates several growth-related genes including the α_3 subunit of Na/K-ATPase in cardiac myocytes [15, 16]. The present work was prompted by these earlier observations to determine if ouabain and other hypertrophic stimuli regulate β_1 expression. We demonstrated here that partial inhibition of sodium pump by ouabain stimulated β_1 expression through a transcriptional mechanism in cardiac myocytes and that transcriptional stimulation of β_1 expression was mediated through ouabain-activated pathways of hypertrophic growth.

Stimulation of β_1 expression by ouabain and other hypertrophic stimuli

Ouabain increased β_1 mRNA in a dose-dependent manner in cardiac myocytes (Fig. 1). Significant increases were observed when the cells were exposed to 25 μM ouabain. Based on the dose-response curve of ouabain inhibition of Na/K-ATPase in cardiac myocytes, it is estimated that ouabain concentrations used in the experiments of Fig. 1 inhibit about 30–60% of the total Na/K-ATPase [25]. Because exposure of cardiac myocytes to such ouabain concentrations activates hypertrophic growth, it was of interest to determine whether ouabain-stimulated β_1 expression is specific to Na/K-ATPase inhibition or related to hypertrophic growth of these myocytes [14, 15]. Three groups of well-known hypertrophic stimuli were used to address this issue. PMA was chosen because it directly activates PKC. PE and ET-1 use G protein-coupled receptors whereas IGF activates receptor tyrosine kinases [26–28]. These stimuli also activate different signaling pathways and cause distinct phenotypic changes in cardiac myocytes. Although in cells other than cardiac myocytes activation of PKC by PMA was shown to phosphorylate and inhibit Na/K-ATPase [29], we found that PMA and other stimuli exhibited no inhibitory effect on ouabain-sensitive ^{86}Rb uptake in these neonatal myocytes under our experimental conditions. These seemingly different results on PMA may be due to its well-established cell-specific effects on the enzyme [30]. Like ouabain, however, all of these stimuli increased β_1 mRNA abundance (Fig. 3). These data support that β_1 is regulated by growth signals, and raises an intriguing question as to whether up-regulation of β_1 represents a common feature of cell growth. Interestingly, prior studies in a liver cell line showed that β_1 could be stimulated by different growth factors including serum and PMA [31]. An *in vivo* study also showed a significant increase in β_1 mRNA in the aorta of hypertensive rats [32]. However, *in vivo* studies on hypertrophied hearts produced mixed results [33]. An increase in β_1 mRNA was noted by Northern blot in hypertrophied hearts that were subjected to pressure-overload; but dot blot analysis failed to demonstrate statistical significance between control and hypertrophied hearts due to sample variations [32, 34].

Since inhibition of Na/K-ATPase by lowering extracellular K^+ also stimulated β_1 expression in cardiac myocytes, our current findings suggest that low K^+ may act as ouabain, stimulating hypertrophic growth in these cultured myocytes. Indeed, when myocytes were cultured in a low K^+ medium, expression of skeletal α-actin and atrial natriuretic factor, two of the hypertrophic marker genes, were induced [35]. Like ouabain, low K^+ also decreased expression of Na/K-ATPase α_3 subunit, and stimulated protein tyrosine phosphorylation and mitogen-activated protein kinases in cardiac myocytes [35, 36]. However, unlike ouabain, low K^+ failed to stimulate protein synthesis in cardiac myocytes [35]. Clearly, while ouabain and low K^+ share some of the pathways, they also activate different, stimulus-specific pathways in cardiac myocytes.

The functional significance of the increased expression of β_1 mRNA remains to be determined. Our previous work showed that ouabain and other hypertrophic stimuli decreased α_3, but not α_1 in cardiac myocytes [15]. It is well established

that the β_1 subunit is required for functional assembly of Na/K-ATPase. If ouabain also increases β_1 protein levels, this may serve as a feedback mechanism to compensate for ouabain-induced down-regulation of α_3 by increasing the assembly of the enzyme in cardiac myocytes. Consistent with this notion, exposure of cardiac myocytes to 100 μM ouabain for 24 h showed no effect on the total Na/K-ATPase activity in these cultured myocytes although the α_3 protein was significantly reduced [15].

Similarities and differences in regulation of α_3 and β_1 by ouabain

A number of physiological and pharmacological stimuli are known to cause cardiac hypertrophy [26, 37]. To determine how these stimuli work in concert to regulate cardiac growth, remodeling and failure, it is necessary to define not only stimulus-specific, but also gene-specific signal pathways. To this end, we showed that exposure of cardiac myocytes to non-toxic concentrations of ouabain partially inhibits Na/K-ATPase, and raises intracellular Ca^{2+}, resulting in activations of multiple signaling pathways including Ras and p42/44 MAPK [15, 16]. Down-regulation of α_3 and up-regulation of β_1 are all initiated by increases in Ca^{2+}-influx, thus sharing the same early segment of these pathways. Several studies have demonstrated that Ras and p42/44 MAPKs of cardiac myocytes are key mediators of cardiac hypertrophy and are involved in regulation of some but not all growth-related genes in cardiac myocytes [16, 38, 39]. The effects of ouabain on α_3 are completely reversed by blockade of Ras or by inhibition of MEK [15, 16]. On the other hand, expression of dominant negative Ras and addition of PD 98059 only caused a partial blockade of ouabain-induced up-regulation of β_1. These data indicate an involvement of both Ras/p42/44 MAPK-dependent and Ras/p42/44 MAPK-independent mechanisms in regulation of β_1 expression and support that α_3 and β_1 are regulated by gene-specific signals. In comparison to other ouabain-regulated late response genes, the role of Ras in regulation of β_1 appears to be similar to that of ANF (atrial natriuretic factor), but different from that of skACT (skeletal α actin) [16].

Based on the present data and our previous findings [15, 16] we have demonstrated that both CaM and PKC are involved in ouabain-mediated regulation of several late response genes such as α_3, β_1, and skACT in cardiac myocytes. Inhibition of CaM completely suppressed the effects of ouabain on mRNAs of every late response gene we have determined. Furthermore, we demonstrated that inhibition of CaM also blocked ouabain-induced luciferase activity in cells transfected with a β_1 promoter-luciferase reporter gene. These data clearly show that CaM plays an essential role in ouabain-specific regulation of cardiac genes. When the role of PKC was determined, it was found that inhibition of PKC also caused a complete blockade of ouabain-induced up-regulation of β_1 mRNA as well as luciferase activity. Interestingly, under the same experimental conditions inhibition of PKC only partially blocked ouabain-induced repression of α_3. These data suggest that activation of PKC may be a downstream event from ouabain-induced increases in Ca^{2+} and activation of CaM.

In summary, we demonstrated that partial inhibition of Na/K-ATPase by ouabain activated multiple signaling pathways and stimulated β_1 expression in a CaM- and PKC-dependent manner in cardiac myocytes. Questions remain as to how activation of CaM and PKC leads to a transcriptional regulation of β_1 in cardiac myocytes.

Acknowledgements

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