Summer 7-2-1999

Intracellular Reactive Oxygen Species Mediate the Linkage of Na+/K+-ATPase to Hypertrophy and its Marker Genes in Cardiac Myocytes

Joseph I. Shapiro

Amir Askari

Zijian Xie

Peter Kometiani

Jie Li

See next page for additional authors

Recommended Citation

Xie, Z., Kometiani, P., Liu, J., Li, J., Shapiro, J. I., & Askari, A. (1999). Intracellular reactive oxygen species mediate the linkage of Na+/K+-ATPase to hypertrophy and its marker genes in cardiac myocytes. Journal of Biological Chemistry, 274(27), 19323-19328.

Authors
Joseph I. Shapiro, Amir Askari, Zijian Xie, Peter Kometiani, Jie Li, and Jiang Liu

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 274, No. 27, Issue of July 2, pp. 19323–19328, 1999

Printed in U.S.A.

Intracellular Reactive Oxygen Species Mediate the Linkage of Na^+/K^+-ATPase to Hypertrophy and Its Marker Genes in Cardiac Myocytes*

(Received for publication, January 20, 1999, and in revised form, April 7, 1999)

Zijian Xie‡, Peter Kometiani, Jiang Liu, Jie Li, Joseph I. Shapiro, and Amir Askari

From the Departments of Pharmacology and Medicine, Medical College of Ohio, Toledo, Ohio 43614

We showed before that in cardiac myocytes partial inhibition of Na^+/K^+-ATPase by nontoxic concentrations of ouabain causes hypertrophy and transcriptional regulations of growth-related marker genes through multiple Ca^{2+}-dependent signal pathways many of which involve Ras and p42/44 mitogen-activated protein kinases. The aim of this work was to explore the roles of intracellular reactive oxygen species (ROS) in these ouabain-initiated pathways. Ouabain caused a rapid generation of ROS within the myocytes that was prevented by preexposure of cells to *N*-acetylcysteine (NAC) or vitamin E. These antioxidants also blocked or attenuated the following actions of ouabain: inductions of the genes of skeletal α-actin and atrial natriuretic factor, repression of the gene of the α_3-subunit of Na^+/K^+-ATPase, activation of mitogen-activated protein kinases, activation of Ras-dependent protein synthesis, and activation of transcription factor NF-κB. Induction of c-*fos* and activation of AP-1 by ouabain were not sensitive to NAC. Ouabain-induced inhibition of active Rb^+ uptake through Na^+/K^+-ATPase and the resulting rise in intracellular Ca^{2+} were also not prevented by NAC. A phorbol ester that also causes myocyte hypertrophy did not increase ROS generation, and its effects on marker genes and protein synthesis were not affected by NAC. We conclude the following: (*a*) ROS are essential second messengers within some but not all signal pathways that are activated by the effect of ouabain on Na^+/K^+-ATPase; (*b*) the ROS-dependent pathways are involved in ouabain-induced hypertrophy; (*c*) increased ROS generation is not a common response of the myocyte to all hypertrophic stimuli; and (*d*) it may be possible to dissociate the positive inotropic effect of ouabain from its growth-related effects by alteration of the redox state of the cardiac myocyte.

Na^+/K^+-ATPase (sodium pump) catalyzes the active transport of Na^+ and K^+ across the plasma membranes of most mammalian cells (1, 2). In the heart, partial inhibition of Na^+/K^+-ATPase by ouabain and related cardiac glycosides causes a modest increase in intracellular Na^+, which in turn leads to significant increases in intracellular Ca^{2+} through the Na^+/Ca^{2+} exchanger and in myocardial contractility through the effects of Ca^{2+} on contractile proteins (3). This effect on cardiac contractility is the basis of the continued therapeutic use of these drugs in the management of congestive heart failure (4, 5). Recently, we demonstrated that in cultured cardiac myocytes the same nontoxic concentrations of ouabain that cause partial inhibition of the sodium pump and increase intracellular Ca^{2+} also stimulate hypertrophic growth and transcriptionally regulate early- and late-response genes that are markers of cardiac hypertrophy (6–8). This clearly indicated that the altered activity of cardiac Na^+/K^+-ATPase by cardiac glycoside drugs or by endogenous ouabain-like putative hormones (9) must be considered as a potential signal for cardiac hypertrophy and its transition to failure, along with other hormonal, mechanical, and pathological stimuli of hypertrophy (10). While exploring the mechanism of linkage between the plasma membrane sodium pump and four marker genes (c-*fos* and the genes of skACT,[1] ANF, and the α_3 subunit of Na^+/K^+-ATPase), we also found that ouabain interaction with the pump initiates multiple signal transduction cascades and that many of these gene regulatory pathways involve activations of the GTP-binding protein Ras and p42/44 MAPKs (11).

ROS, such as the superoxide radical, the hydroxyl radical, and H_2O_2, are continuously produced in most cells, and their levels are regulated by a number of enzymes and physiological antioxidants (12). It has been known for some time that excessive generation of ROS is associated with cell injury in a variety of pathological conditions including those of the cardiovascular system (12). More recently, there has been rapidly growing evidence indicating that ROS also have normal roles as second messengers within several signal pathways involved in the control of gene transcription (13–15). Because Ras has been shown to be implicated in a number of such ROS-regulated pathways (15–18) and in view of our findings on the central role of Ras in ouabain-induced regulation of the growth-related genes of cardiac myocytes (11), we were prompted to determine the possible involvement of ROS in these ouabain effects. Here we present the results of our initial studies showing that intracellular ROS are indeed essential mediators of the ouabain-induced hypertrophic phenotype and that antioxidants are capable of preventing the growth-related effects of ouabain in cultured cardiac myocytes.

EXPERIMENTAL PROCEDURES

Materials—Chemicals of the highest purity available were obtained from Sigma. TRI reagent for RNA isolation was from Molecular Re-

* This work was supported by NHLBI National Institutes of Health Grant HL-36573, by a grant-in-aid from the American Heart Association, and by funds contributed in part by the American Heart Association, Ohio-West Virginia Affiliate. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Pharmacology, Medical College of Ohio, 3035 Arlington Ave., Toledo, OH 43614-5804. Tel.: 419-383-4182; Fax: 419-383-2871; E-mail: ZXie@mco.edu.

[1] The abbreviations used are: skACT, skeletal α-actin; ANF, atrial natriuretic factor; CAT, chloramphenicol acetyltransferase; CM-DCF, 5-(and 6) chloromethyl-2′,7′-dichlorofluorescein; MAPK, mitogen-activated protein kinase; NAC, *N*-acetylcysteine; PMA, phorbol 12-myristate 13-acetate; ROS, reactive oxygen species; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; CM-DCFH, reduced CM-DCF.

search Center, Inc. (Cincinnati, OH). Radionucleotides (^{32}P-labeled, about 3000 Ci/mmol) and $^{86}Rb^+$ were obtained from NEN Life Science Products. [^{3}H]Phenylalanine and the ECL kit were from Amersham Pharmacia Biotech. Rabbit polyclonal Anti-ACTIVE MAPK pAb and anti-p42/44 antibodies were obtained from Promega (Madison, WI) and New England Biolabs (Beverly, MA), respectively. Fura-2 AM and CM-DCFH diacetate were obtained from Molecular Probes (Eugene, OR). Optitran and Nytran membranes were obtained from Schleicher and Schuell.

Cell Preparation and Culture—Neonatal ventricular myocytes were prepared and cultured as described in our previous work (6). 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 Dulbecco's modified Eagle's medium and 1 part of Medium 199 (Life Technologies, Inc.), 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 the 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.

Fluorescence Microscopic Measurements of Intracellular Ca^{2+} Concentration and ROS Production—Myocytes were cultured on glass coverslips. Intracellular Ca^{2+} was measured by fura-2 as we previously described (6). Fura-2 fluorescence was recorded using an Attofluor imaging system (Atto Instruments) at an excitation wavelength of 340/380 nm and at an 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 (6). To measure intracellular ROS production, cells were loaded with 10 μM CM-DCFH diacetate for 15 min at room temperature in the dark. The coverslip was affixed to a culture chamber and perfused with the same culture medium without phenol red. During loading the acetate groups on CM-DCFH diacetate are removed by intracellular esterase, trapping the probe inside the myocytes. Several dihydrofluorescein derivatives have been used for measuring intracellular ROS generation (19–21). CM-DCFH was chosen because it exhibited better retention in cells than other derivatives. Production of ROS was measured by changes in fluorescence because oxidation of CM-DCFH produced fluorescent product CM-DCF in cardiac myocytes. Under each experimental condition about 15 single myocytes were imaged with an Attofluor imaging system (Atto Instruments), and CM-DCF fluorescence was measured at an excitation wavelength of 480 nm and an emission wavelength of 520 nm.

Northern Blot—Northern blot was done as described previously (6–8). 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 glyceraldehyde-3-phosphate dehydrogenase mRNA to correct for differences in sample loading and transfer.

Measurement of Phosphorylation of p42/44 MAPKs—Activation of p42/44 MAPKs in cultured myocytes was determined by Western blot using a rabbit polyclonal antibody raised against dually phosphorylated p42/44 MAPKs (11). In brief, after cells were exposed to ouabain, reaction was terminated by the replacement of medium with 200 μl of ice-cold lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM NaF, 1 mM Na_3VO_4, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 50 mM tetrasodium pyrophosphate, 10 nM okadaic acid, 1% Triton X-100, 0.25% sodium deoxycholate, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). For Western blot analysis, cell lysates (60 μg/lane) were electrophoresed on 10% SDS-polyacrylamide gels and transferred to an Optitran membrane. The membranes were probed with Anti-ACTIVE MAPK pAb that detects p42/44 MAPKs only when they are activated by phosphorylation at Thr^{202} and Tyr^{204}. To ensure equal loading and protein transfer, the same blots were stripped and probed with a polyclonal antibody recognizing both phosphorylated and nonphosphorylated p42/44 MAPKs. These membranes were developed with a secondary anti-rabbit antibody using the ECL method as instructed by the manufacturer (Amersham Pharmacia Biotech). Autoradiograms were scanned with a Bio-Rad densitometer to quantitate MAPK signals.

Assay of AP-1 Activation—A CAT reporter gene under the control of three copies of the AP-1 *cis*-element (22) was kindly provided to us by Dr. P. A. Baeuerle. Myocytes were transfected with 5 μg of plasmid DNA using a modified calcium phosphate method (23). After 48 h of incubation, the transfected cells were subjected to ouabain and other treatments. Cell lysate was prepared and assayed for CAT as described previously (24). All transfections were conducted in triplicate in 6.0-cm tissue culture dishes. Control experiments with Rous sarcoma virus·β-galactosidase reporter plasmid indicated that transfection efficiency varied less than 15% within a given experiment under our experimental conditions.

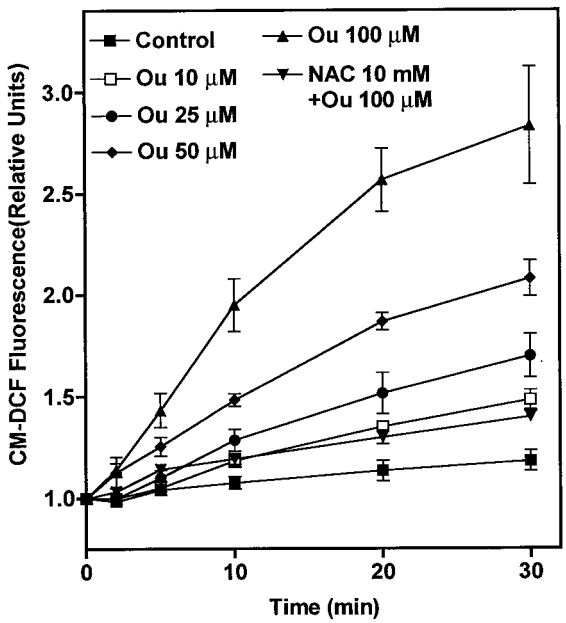

FIG. 1. **Time courses of the effects of varying ouabain concentrations on intracellular ROS production and the prevention of these effects by NAC.** Cardiac myocytes were loaded with the fluorogenic probe, exposed to ouabain (*Ou*), and assayed for the fluorescence of the oxidized probe as indicated under "Experimental Procedures." When NAC was used, cells were incubated with it for 30 min prior to the addition of ouabain. Each value is the mean ± S.E. of determinations on 40–60 cells in four independent experiments.

Detection of NF-κB by Immunofluorescence—This was done as described before (35) using an antibody against the p65 subunit of NF-κB (Santa Cruz Biotechnology).

Preparation of Replication-defective Adenovirus Asn^{17} Ras and Infection of Myocytes—The adenovirus expressing the dominant-negative Ras was generated, amplified, and purified as described before (11). An identical virus containing the β-galactosidase gene was used as virus control. Myocytes were transduced by the same experimental procedures used before (11). Under these conditions viral infection had no effect on cell viability (11).

Protein Synthesis—Protein synthesis in these cultured myocytes was assayed by [^{3}H]phenylalanine incorporation as we previously described (7). Cells were cultured in a 12-well plate, treated with ouabain and other agents as indicated for 44 h, and continued for an additional 4 h in the presence of 0.5 μCi of [^{3}H]phenylalanine/well before total protein and phenylalanine incorporation were assayed as indicated (7).

Assay of $^{86}Rb^+$ Uptake by Myocytes—The initial rate of ouabain-sensitive uptake of Rb^+ through the Na^+/K^+-ATPase of the intact myocytes was measured with modification of our previously described procedures (6). Monensin (20 μM) was added to the medium prior to the initiation of the Rb^+ uptake assay to assure that the maximal capacity of active uptake was measured (25).

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

RESULTS

Ouabain Increases Intracellular ROS in Cardiac Myocytes—To determine if ouabain increases ROS production in cardiac myocytes, cells were incubated with 10 μM CM-DCFH diacetate for 15 min at room temperature in the dark, washed with fresh medium, exposed to ouabain, and monitored for the production of the fluorescent CM-DCF. As shown in Fig. 1,

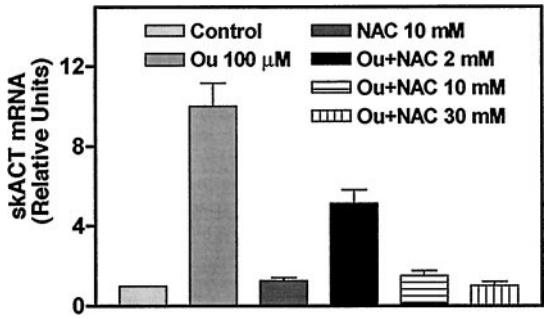

FIG. 2. **Effects of NAC on ouabain-induced expression of the skACT gene.** Cells were pretreated with NAC for 15 min, exposed to ouabain (*Ou*) for 12 h, and assayed for mRNAs of skACT and glyceraldehyde-3-phosphate dehydrogenase as described under "Experimental Procedures." Values, normalized to those of corresponding glyceraldehyde-3-phosphate dehydrogenase, are mean ± S.E. of five experiments.

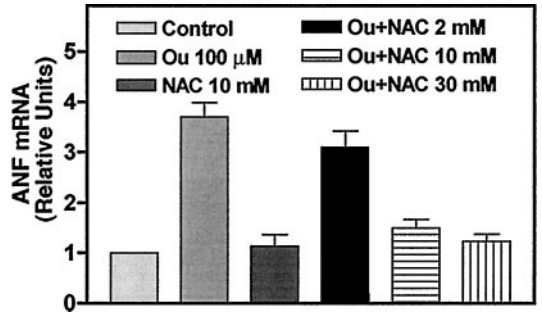

FIG. 3. **Effects of NAC on ouabain-induced expression of the ANF gene.** Experiments were done as in Fig. 2. *Ou*, ouabain.

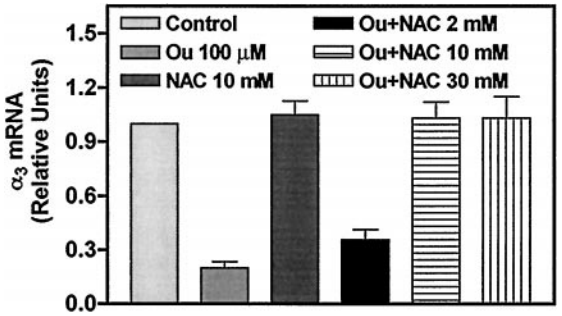

FIG. 4. **Effects of NAC on ouabain-induced down-regulation of the gene of the α_3-subunit of Na^+/K^+-ATPase.** Experiments were done as in Fig. 2. *Ou*, ouabain.

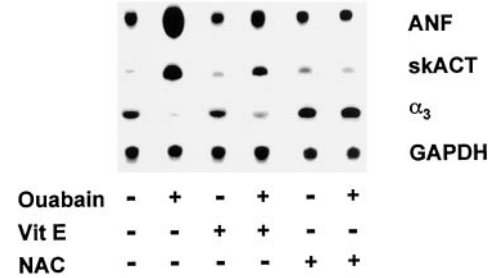

FIG. 5. **Comparison of NAC and vitamin E effects on ouabain-induced regulations of the genes of ANF, skACT, and α_3-subunit of Na^+/K^+-ATPase.** Cells were pretreated with 10 mM NAC or 0.5 mM vitamin E for 15 min, exposed to 100 μM ouabain for 12 h, and assayed for mRNAs as indicated under "Experimental Procedures." A representative Northern blot of four different experiments is shown. *Vit E*, vitamin E; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

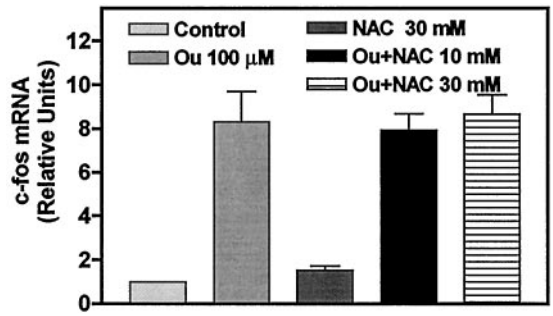

FIG. 6. **Lack of effect of NAC on ouabain-induced expression of c-*fos*.** Cells were pretreated with NAC for 30 min, exposed to ouabain (*Ou*) for 45 min, and assayed for mRNAs as in Fig. 2. Values are mean ± S.E. of three experiments.

ouabain caused dose- and time-dependent increases in fluorescence, indicating stimulated ROS production in these cultured myocytes. The highest ouabain concentration used was 100 μM to avoid loss of viable cells (6). As expected, myocytes loaded with the fluorogenic probe also exhibited increased fluorescence when exposed to H_2O_2 (data not shown). The maximal fluorescence increase obtained with ouabain in experiments of Fig. 1 was about the same as that produced by the addition of 2 μM H_2O_2. Preincubation of myocytes with the antioxidant NAC (10 mM) suppressed the fluorescence increases induced by ouabain (Fig. 1) and by H_2O_2 (not shown). Preincubation with 0.5 mM α-tocopherol (vitamin E) had about the same effect as that of NAC shown in Fig. 1.

Effects of Antioxidants on the Gene Regulatory Effects of Ouabain—The experiments shown in Figs. 2–6 were done to assess the relation of the ouabain-induced generation of ROS to the regulations of the four genes that we had previously shown to be transcriptionally regulated by ouabain (6–8, 11). The results showed that NAC blocked, in a dose-dependent manner, the ouabain-induced increases in skACT and ANF mRNAs (Figs. 2 and 3) and the ouabain-induced decrease in mRNA of the α_3-subunit of Na^+/K^+-ATPase (Fig. 4). Experiments depicted in Fig. 5 showed that vitamin E and NAC similarly antagonized the effects of ouabain on skACT, ANF, and α_3-subunit genes. In contrast to the antioxidant effects on these late-response genes, induction of the early-response gene c-*fos* by ouabain was not affected by either NAC (Fig. 6) or vitamin E (not shown). The combined data of Figs. 2–6 clearly indicate that ouabain initiates ROS-dependent and ROS-independent gene regulatory pathways in these myocytes.

We showed before that induction of c-*fos* by ouabain is accompanied by an increase in the transcription factor AP-1, which has been implicated in the regulation of cardiac growth-related genes (6). In view of the lack of effect of NAC on the induction of c-*fos* by ouabain (Fig. 6) but the known activation of cardiac myocyte AP-1 by exogenous H_2O_2 (35), in the experiments shown in Fig. 7 we examined the effects of ouabain and NAC on myocytes that were transfected with a CAT gene construct containing three AP-1 elements. Ouabain-induced increase in CAT expression was also unaffected by NAC (Fig. 7), providing further support for the conclusion that some gene regulatory effects of ouabain are through ROS-independent pathways.

NF-κB is another transcription factor that, along with AP-1, is regulated by the redox state in a variety of cell types (13–15, 34) and is known to be activated by exogenous H_2O_2 in cardiac myocytes (35). Exposure of myocytes to ouabain caused the nuclear translocation of the p65 subunit of NF-κB (Fig. 8), indicative of its activation. Preincubation of myocytes with NAC blocked this ouabain-induced translocation (Fig. 8), suggesting a role of intracellular ROS in ouabain-induced activation of NF-κB.

In control experiments, the results of which are not shown, it was established that antioxidants, at the concentrations and

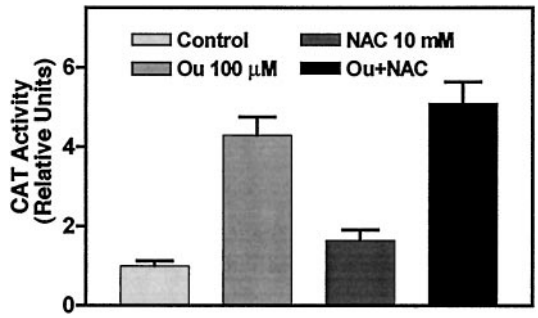

FIG. 7. **Lack of effect of NAC on ouabain-induced activation of AP-1.** Myocytes were transfected with a report CAT gene directed by AP-1 elements, exposed to ouabain (*Ou*) for 16 h in the presence or absence of NAC, and assayed for CAT as described under "Experimental Procedures." Values are mean ± S.E. of three experiments.

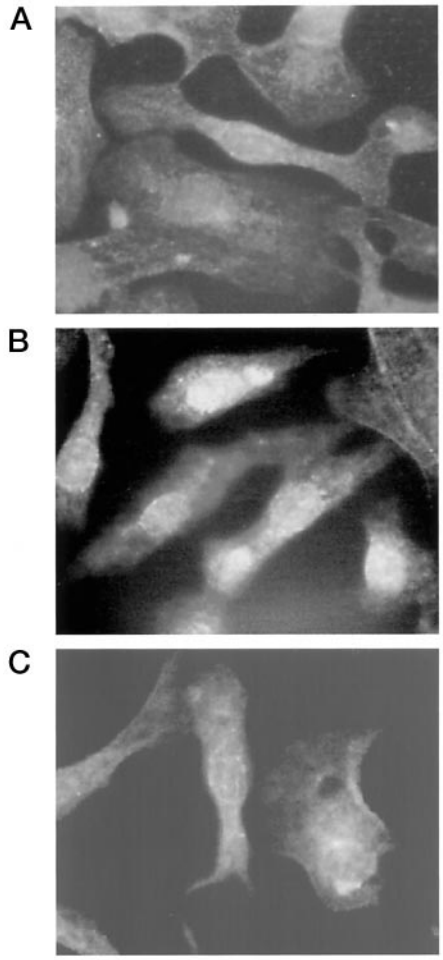

FIG. 8. **Effect of NAC on ouabain-induced nuclear translocation of NF-κB.** Immunostaining of myocytes with anti-p65 antibody was done as indicated under "Experimental Procedures." *A*, control myocytes; *B*, myocytes treated with 100 μM ouabain for 1 h; *C*, myocytes pretreated with 10 mM NAC for 30 min and then exposed to ouabain as in *B*.

for the durations used in the experiments shown in Figs. 2–8, had no significant effects on myocyte viability as measured by lactate dehydrogenase release (6).

The Roles of Ras and ROS in Ouabain Stimulation of Protein Synthesis—We showed before that nontoxic concentrations of ouabain cause moderate hypertrophy of the myocytes as evidenced by ouabain-induced increases in total cell protein (6) and in the rate of [^{3}H]phenylalanine incorporation into cell proteins (7). Because we had shown the involvement of Ras in

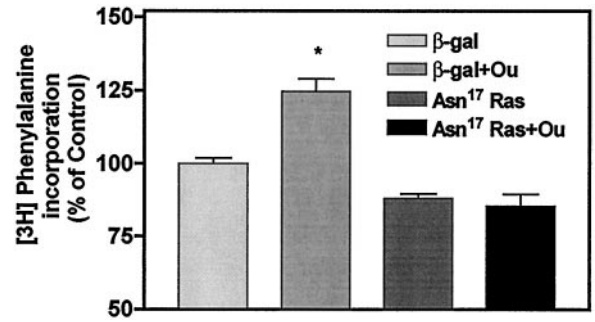

FIG. 9. **Prevention of ouabain effect on protein synthesis by dominant-negative Ras.** Myocytes were transduced either with the adenoviruses expressing an Asn^{17} dominant-negative mutant of Ras or with the control virus containing the β-galactosidase (*β-gal*) gene for 12 h and then assayed for [^{3}H]phenylalanine incorporation in the absence or presence of 100 μM ouabain (*Ou*) as indicated under "Experimental Procedures." Values are mean ± S.E. of three experiments. *, $p < 0.05$ against control.

several signal pathways activated by ouabain (11), it was of interest to know if Ras was also involved in the effect of ouabain on protein synthesis. Experiments depicted in Fig. 9 showed that the adenoviral-mediated expression of a dominant-negative Ras blocked ouabain stimulation of [^{3}H]phenylalanine incorporation into myocyte proteins. Experiments of Fig. 10 showed that ouabain stimulation of the protein synthesis was also blocked by NAC. The combined data of Figs. 9 and 10 indicate that ouabain-induced hypertrophy, like ouabain-induced regulations of the late-response genes (skACT, ANF, and α_3-subunit), involves both Ras and ROS. Interestingly, NAC also significantly decreased the [^{3}H]phenylalanine incorporation in the absence of ouabain (Fig. 10), suggesting that basal levels of ROS may be important signals for the regulation of protein synthesis.

Different Roles of ROS in Ouabain-induced and PMA-induced Effects on Growth-related Genes and on Protein Synthesis—PMA is a well established hypertrophic stimulus for the cultured neonatal myocytes (6, 7, 26). Under the same conditions where ouabain-induced effects on ANF and α_3-subunit genes were blocked by NAC, the antioxidant had no effect on PMA-induced effects on the same genes (Fig. 11). Stimulation of protein synthesis by PMA was also not blocked by NAC, in contrast to the antioxidant effect on ouabain-stimulated protein synthesis (Fig. 10). In experiments similar to those shown in Fig. 1, the same concentrations of PMA used the experiments shown in Figs. 10 and 11 did not cause increased generation of ROS (data not shown). These results clearly show that increased production of ROS is not a common response of the cardiac myocytes to all hypertrophic stimuli.

Effect of NAC on Ouabain-induced Activation of p42/44 MAPKs—The antagonistic effects of antioxidants on ouabain-induced actions, as noted in Figs. 2–10, occurred in experiments with durations of several hours. On the other hand, the rapid onset of the ouabain effect on the intracellular production of CM-DCF (Fig. 1) suggested that increased generation of ROS may represent an early event in ouabain-initiated signal transduction pathways. Because the maximal activation of the Ras-Raf-MEK-p42/44 MAPK cascade occurs within 5–15 min after exposure to ouabain (11), it was of interest to examine the effect of NAC on this rapid action of ouabain. Experiments depicted in Fig. 12 showed that ouabain activation of p42 MAPK was indeed attenuated by NAC.

NAC and Ouabain Effects on $^{86}Rb^+$ Uptake and on Intracellular Ca^{2+}—Because all of the hypertrophic and gene regulatory effects of ouabain are dependent on ouabain-induced inhibition of the sodium pump and the resulting rapid rise in

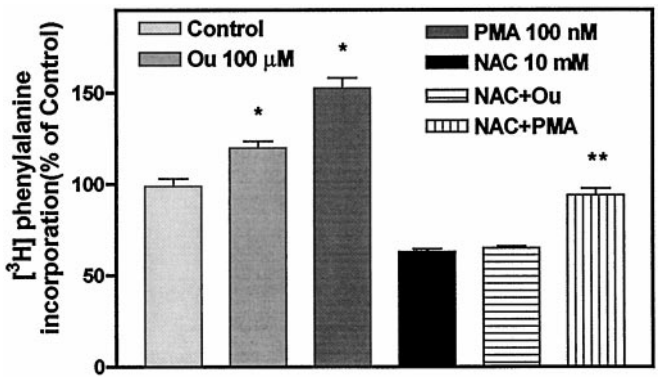

FIG. 10. **Effects of NAC on ouabain-induced and PMA-induced activations of protein synthesis.** Myocytes exposed to the indicated concentrations of ouabain (*Ou*), PMA, and NAC were assayed for [^{3}H]phenylalanine incorporation as described under "Experimental Procedures." Values are mean ± S.E. of three experiments. *, $p < 0.05$ against control; **, $p < 0.05$ against 10 mM NAC.

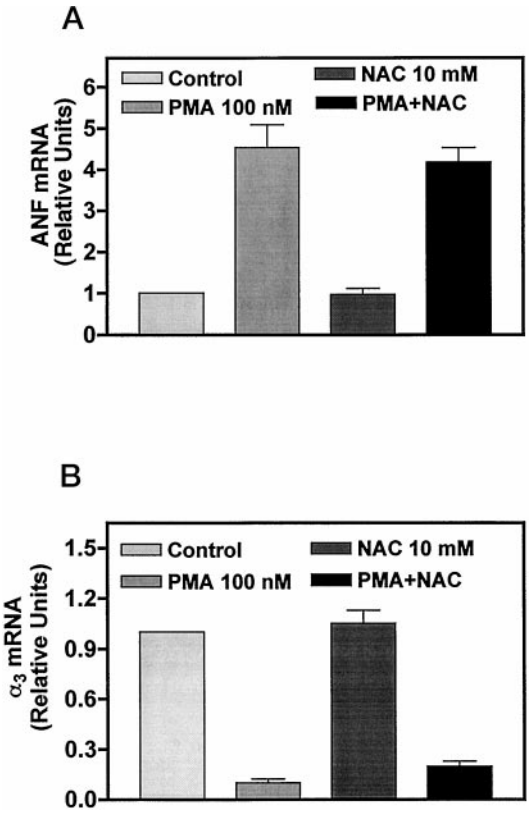

FIG. 11. **Lack of effect of NAC on PMA-induced regulations of ANF (*A*) and α^3-subunit (*B*) of Na^+/K^+-ATPase genes.** Control and NAC-treated cells were exposed to PMA and assayed for mRNAs as in Fig. 2.

intracellular Ca^{2+} (6–8, 11), we examined the combined effects of NAC and ouabain on the transport function of the intact myocyte Na^+/K^+-ATPase as measured by $^{86}Rb^+$ uptake, and on intracellular Ca^{2+} concentrations of the cultured myocytes. In experiments similar to those we have reported before (6), neither the maximal inhibitory effect of 1 mM ouabain nor the partial inhibitory effect of 100 μM ouabain on the initial rate of $^{86}Rb^+$ uptake was prevented or reduced by preincubation of myocytes with 10 mM NAC for 15 min (data not shown). In agreement with our previous results (6), 100 μM ouabain increased intracellular Ca^{2+} concentration from 89 ± 18 nM to 167 ± 24 nM. In myocytes that were pretreated with 10 mM NAC, 100 μM ouabain raised Ca^{2+} concentration from 104 ± 25 nM to 191 ± 34 nM.

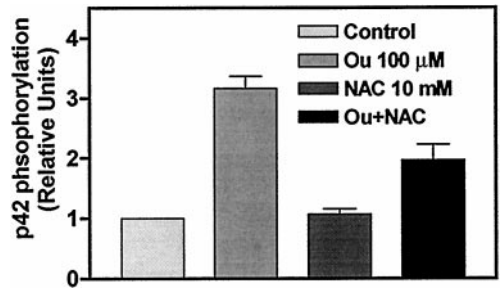

FIG. 12. **Effect of NAC on ouabain-induced activation of p42 MAPK.** Myocytes were pretreated with NAC for 30 min, exposed to ouabain (*Ou*) for 15 min, and assayed for MAPK activation as described under "Experimental Procedures." Values are mean ± S.E. of four experiments.

DISCUSSION

The fact that ROS are second messengers within a variety of intracellular signal transduction pathways has been indicated by a large number of studies in cells other than cardiac myocytes (13–18). The signaling roles of intracellularly generated ROS in cardiac myocytes have been suggested by previous studies when myocytes were subjected to hypoxia or hypoxia reoxygenation (21, 27, 28) and when myocytes were exposed to tumor necrosis factor-α or angiotensin II, two stimuli that induce cardiac myocyte hypertrophy (29). Our findings presented here clearly establish the essential roles of ROS-regulated signaling pathways in ouabain-induced hypertrophy, and in transcriptional regulations of several growth-related genes of these cardiac myocytes by ouabain. However, our findings showing that PMA-induced hypertrophy does not involve ROS also establish that increased intracellular generation of ROS is not a requirement for the induction of myocyte hypertrophy *per se*. Clearly, the potential signaling roles of ROS need to be explored for each physiological and pharmacological stimulus of cardiac hypertrophy to allow the determination of how the various stimuli interact to cause pathological cardiac hypertrophy and to permit the assessment of the roles of ROS and antioxidants in the development of cardiac hypertrophy and failure.

Of particular interest are our findings on the different roles of ROS in the ouabain-induced regulations of the four growth-related genes. Although the regulations of three genes (those of skACT, ANF, and the α_3 subunit of Na^+/K^+-ATPase) are dependent on ROS generated in response to ouabain, these ROS do not seem to be involved in the induction of c-*fos* by ouabain. A plausible explanation for this apparent pathway specificity is that within the complex network of multiple pathways activated by ouabain (11), the target of the generated ROS is not a proximal component that is shared by all the pathways of the network. Alternatively, because there are two branches within the ouabain-activated pathways leading to the induction of c-*fos* (11), it may be that repression of a ROS-regulated branch is accompanied by a compensatory change in the activity of a ROS-independent branch.

The nature and the source of the ouabain-induced ROS remain to be determined. Although available data suggest that the fluorogenic compound used here for the detection of ROS is more sensitive to H_2O_2 and the hydroxyl radical than to the superoxide radical (21), the insufficient selectivity of the probe and the interconversions of the generated ROS do not permit firm conclusions on the nature of the species that act as second messengers. In cells other than cardiac myocytes, mitochondria and NADH·NADPH oxidase complexes, which include Rac as a component, have been implicated as sources of signaling ROS (15–17). In cardiac myocytes, mitochondria seem to be the source of ROS involved in hypoxia-induced signaling (30). The

existence of a cytosolic NADH oxidase system responsible for the production of superoxide in cardiac myocytes has also been suggested (31), and the presence of Rac in cardiac myocytes has been shown (32, 33). Because we have shown Ras activation to be involved in all ouabain-initiated pathways (11) and because others have implicated a signal cascade involving Ras-Rac in the development of cardiac myocyte hypertrophy (33), it is reasonable to suspect that a source of ouabain-induced ROS generation may indeed be a Ras-Rac-NAD(P)H oxidase cascade. Our studies on the relative roles of NAD(P)H complexes and mitochondria as sources of ouabain-induced ROS are in progress.

In the ouabain-initiated signal pathways, as in most ROS-regulated pathways identified to date, the immediate targets of ROS effects are not known. Our data showing that ouabain-induced ROS production is antagonized by NAC and that ouabain-induced inhibition of Na^+/K^+-ATPase and the resulting rise in intracellular Ca^{2+} persist in the presence of NAC clearly show that the generated ROS must affect the ouabain-initiated pathways beyond the point where increased intracellular Ca^{2+} affects all pathways (11). One such potential target is Ras, because direct activation of Ras by ROS has been indicated by studies on cells other than myocytes (18). This, coupled with the likelihood of the Ras-Rac-oxidase cascade being the source of the generated ROS, suggests the possible existence of a signal amplification cycle within the ouabain-initiated and ROS-regulated pathways. AP-1 and NF-κB, the two transcription factors whose activations by ROS have been shown in a large number of studies (13–15, 34), should also be considered as potential downstream targets for any ROS-generating stimulus. Although we have shown before that AP-1 is indeed activated when cardiac myocytes are exposed to H_2O_2 (35), our present findings (Fig. 7) argue against a role of ouabain-generated ROS in ouabain-induced activation of AP-1. NF-κB of the cardiac myocytes, which is also activated by exogenous H_2O_2 (35), is indeed a target of ouabain-generated ROS (Fig. 8). The potential role of this transcription factor in the gene regulatory effects of ouabain needs further exploration.

Perhaps the most intriguing of our present findings is that in the cultured myocytes it is possible to dissociate the effects of ouabain on growth and growth-related genes from the classical effect of the drug on the resting intracellular Ca^{2+}. Because the latter is thought to be responsible for the effects of ouabain and related cardiac glycosides on cardiac contractility (3), this raises the important question of whether or not the redox state of the myocyte or the intact heart can alter the effects of the cardiac glycosides on cardiac hypertrophy without affecting the positive inotropic effects of the drug. As discussed before (6), in the context of the use of these drugs in the management of heart failure, at this time it is not possible to say if the hypertrophic and the gene regulatory effects of the drugs will turn out to be among their beneficial or undesirable effects.

REFERENCES

1. Skou, J. C. (1988) *Methods Enzymol.* **156,** 1–29
2. Lingrel, J. B, and Kuntzweiler, T. (1994) *J. Biol. Chem.* **269,** 19659–19662
3. Eisner, D. A., and Smith, T. W. (1992) in *The Heart and Cardiovascular System* (Fozzard, H. A., Haber, E., Jennigs, R. B., Katz, A. M., and Morgan, H. E., eds) 2nd Ed., Vol. 1, pp. 863–902, Raven Press, New York
4. Kelly, R. A., and Smith, T. W. (1993) *J. Am. Coll. Cardiol.* **22,** 107A–112A
5. The Digitalis Investigational Group (1997) *N. Engl. J. Med.* **336,** 525–533
6. Peng, M., Huang, L., Xie, Z., Huang, W.-H., and Askari, A. (1996) *J. Biol. Chem.* **271,** 10372–10378
7. Huang, L., Li, H., and Xie, Z. (1997) *J. Mol. Cell. Cardiol.* **29,** 429–437
8. Huang, L., Kometiani, P., and Xie, Z. (1997) *J. Mol. Cell. Cardiol.* **29,** 3157–3167
9. Blaustein, M. P. (1993) *Am. J. Physiol.* **264,** C1367–C1387
10. Hefti, M. A., Harder, B. A., Eppenberger, H. M., and Schaub, M. C. (1997) *J. Mol. Cell. Cardiol.* **29,** 2873–2892
11. Kometiani, P., Li, J., Gnudi, L., Kahn, B. B., Askari, A., and Xie, Z. (1998) *J. Biol. Chem.* **273,** 15249–15256
12. Halliwell, B., and Gutteridge, J. M. C. (1990) *Methods Enzymol.* **186,** 1–85
13. Sen, C. K., and Packer, L. (1996) *FASEB J.* **10,** 709–720
14. Lander, H. M. (1997) *FASEB J.* **11,** 118–124
15. Abe, J.-I., and Berk, B. C. (1998) *Trends Cardiovasc. Med.* **8,** 59–64
16. Deora, A. A., Win, T., Vanhaesebroeck, B., and Lander, H. M. (1998) *J. Biol. Chem.* **273,** 29923–29928
17. Irani, K., Xia, Y., Zweier, J. L., Sollott, S. J., Der, C. J., Fearon, E. R., Sundaresan, M., Finkel, T., and Goldschmidt-Clermont, P. J. (1997) *Science* **275,** 1649–1652
18. Lander, H. M., Ogiste, J. S., Teng, K. K., and Novogrodsky, A. (1995) *J. Biol. Chem.* **270,** 21195–21198
19. Royall, J. A., and Ischiropoulos, H. (1993) *Arch. Biochem. Biophys.* **302,** 348–355
20. Sundaresan, M., Yu, Z-X., Ferrans, V. J., Irani, K., and Finkel, T. (1995) *Science* **270,** 296–299
21. Vanden Hoek, T. L., Li, C., Shao, Z., Schumacker, P. T., and Becker, L. B. (1997) *J. Mol. Cell. Cardiol.* **29,** 2571–2583
22. Meyer, M., Schreck, R., and Baeuerle, P. A. (1993) *EMBO J.* **12,** 2005–2015
23. O'Mahoney, J. V., and Adams, T. E. (1994) *DNA Cell Biol.* **13,** 1227–1232
24. Shen, P., Xie, Z., Li, H., and Sanchez, E. R. (1993) *J. Steroid Biochem. Mol. Biol.* **47,** 55–64
25. Stemmer, P., and Akera, T. (1988) *Biochim. Biophys. Acta* **940,** 188–196
26. Dunnmon, P. M., Iwaki, K., Henderson, S. A., Sen, A., and Chien, K. R. (1990) *J. Mol. Cell. Cardiol.* **22,** 901–910
27. Duranteau, J., Chandel, N. S., Kulisz, A., Shao, Z., and Schumacker, P. T. (1998) *J. Biol. Chem.* **273,** 11619–11624
28. Laderante, K. R., and Webster, K. A. (1997) *Circ. Res.* **80,** 336–344
29. Nakamura, K., Fushimi, K., Kouchi, H., Mihara, K., Miyazaki, M., Ohe, T., and Namba, M. (1998) *Circulation* **98,** 794–799
30. Vanden Hoek, T. L., Becker, L. B., Shao, Z., Li, C., and Schumacker, P. T. (1998) *J. Biol. Chem.* **273,** 18092–18098
31. Mohazzab-H, K. M., Kaminski, P. M., and Wolin, M. S. (1997) *Circulation* **96,** 614–620
32. Kim, K-S., Takeda, K., Sethi, R., Pracyk, J. B., Tanaka, K., Zhou, Y. F., Yu, Z-X., Ferrans, V. J., Bruder, J. T., Kovesdi, I., Kaikobad, I., Goldschmidt-Clermont, P., and Finkel, T. (1998) *J. Clin. Invest.* **101,** 1821–1826
33. Pracyk, J. B., Tanaka, K., Hegland, D. D., Kim, K-S., Sethi, R., Rovira, I. I., Blazina, D. R., Lee, L., Bruder, J. T., Kovesdi, I., Goldschmidt-Clermont, P. J., Irani, K., and Finkel, T. (1998) *J. Clin. Invest.* **102,** 929–937
34. Schreck, R., Albermann, K., and Baeuerle, P. A. (1992) *Free Radic. Res. Commun.* **17,** 221–237
35. Peng, M., Huang, L., Xie, Z-J., Huang, W-H., and Askari, A. (1995) *Cell. Mol. Biol. Res.* **41,** 189–197