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Multiple Signal Transduction Pathways Link Na^+/K^+-ATPase to Growth-related Genes in Cardiac Myocytes

THE ROLES OF Ras AND MITOGEN-ACTIVATED PROTEIN KINASES*

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We showed before that in neonatal rat cardiac myocytes partial inhibition of Na^+/K^+-ATPase by nontoxic concentrations of ouabain causes hypertrophic growth and transcriptional regulations of genes that are markers of cardiac hypertrophy. In view of the suggested roles of Ras and p42/44 mitogen-activated protein kinases (MAPKs) as key mediators of cardiac hypertrophy, the aim of this work was to explore their roles in ouabain-initiated signal pathways regulating four growth-related genes of these myocytes, *i.e.* those for c-Fos, skeletal α-actin, atrial natriuretic factor, and the α_3-subunit of Na^+/K^+-ATPase. Ouabain caused rapid activations of Ras and p42/44 MAPKs; the latter was sustained longer than 90 min. Using high efficiency adenoviral-mediated expression of a dominant-negative Ras mutant, and a specific inhibitor of MAPK kinase (MEK), activation of Ras-Raf-MEK-p42/44 MAPK cascade by ouabain was shown. The effects of the mutant Ras, an inhibitor of Ras farnesylation, and the MEK inhibitor on ouabain-induced changes in mRNAs of the four genes indicated that (*a*) skeletal α-actin induction was dependent on Ras but not on p42/44 MAPKs, (*b*) α_3 repression was dependent on the Ras-p42/44 MAPK cascade, and (*c*) induction of c-*fos* or atrial natriuretic factor gene occurred partly through the Ras-p42/44 MAPK cascade, and partly through pathways independent of Ras and p42/44 MAPKs. All ouabain effects required extracellular Ca^{2+}, and were attenuated by a Ca^{2+}/calmodulin antagonist or a protein kinase C inhibitor. The findings show that (*a*) signal pathways linked to sarcolemmal Na^+/K^+-ATPase share early segments involving Ca^{2+} and protein kinase C, but diverge into multiple branches only some of which involve Ras, or p42/44 MAPKs, or both; and (*b*) there are significant differences between this network and the related gene regulatory pathways activated by other hypertrophic stimuli, including those whose responses involve increases in intracellular free Ca^{2+} through different mechanisms.

Na^+/K^+-ATPase catalyzes the coupled active transport of Na^+ and K^+ across the plasma membranes of most mammalian cells (1). In the myocardium, Na^+/K^+-ATPase also regulates contractility; the partial inhibition of the myocardial enzyme by ouabain and related digitalis drugs causes a small increase in intracellular 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 (2). This positive inotropic action is the basis of the continued use of digitalis drugs in the treatment of congestive heart failure (3, 4). Recently, using cultured neonatal rat cardiac myocytes, we showed (5–7) that the same nontoxic concentrations of ouabain that cause partial inhibition of sarcolemmal Na^+/K^+-ATPase and increased intracellular Ca^{2+}, also stimulate the hypertrophic growth of these myocytes, and lead to transcriptional regulation of several genes that have been implicated as markers of cardiac hypertrophy. 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 (8). Cardiac hypertrophy is not only a beneficial adaptive response to increased workload, but also a prelude to the development of heart failure (9). Hence, there is considerable interest in the potential hypertrophic effects of drugs that are widely used in the treatment of heart failure.

Based on studies from both *in vivo* and *in vitro* models of hypertrophy, it is evident that transcriptional regulations of some early-response protooncogenes and late-response fetal genes are associated with myocyte hypertrophy, and that these genes are regulated in distinctively different patterns in response to different hypertrophic stimuli (8–11). While the inductions of these growth-related genes are not sufficient for hypertrophic growth of myocytes (12–15), it is clear that for any hypertrophic stimulus, the mapping of the signal transduction pathways involved in the regulation of growth-related genes is necessary for the definition of the hypertrophic phenotype induced by that stimulus. Therefore, the aim of this work was the continuation of our recent efforts to characterize the molecular mechanisms of the linkage between the sarcolemmal Na^+/K^+-ATPase and the expressions of several growth-related genes of cultured neonatal rat cardiac myocytes. Specifically, in view of the suggested roles of Ras (16–18) and p42/44 MAPKs[1] (19–22) in the development of cardiac hypertrophic phenotype, we

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[1] The abbreviations and trivial names used are: MAPK, mitogen-activated protein kinase; ANF, atrial natriuretic factor; ERK, extracellular signal-regulated protein kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H-7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride; HA1004, *N*-(2-guanidinoethyl)-5-isoquinolinesulfonamide hydrochloride; MEK, MAPK kinase; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; skACT, skeletal α-actin; Raf, Raf-1 kinase; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride.

wished to assess the possible involvements of Ras and these MAPKs in the ouabain-initiated signal transduction pathways of four genes that we had shown to be transcriptionally regulated in the course of ouabain-induced hypertrophy of cardiac myocytes, *i.e.* c-*fos* and the genes for ANF, skACT, and the α_3-subunit of Na^+/K^+-ATPase (5–7). Our findings indicate that while the four ouabain-initiated pathways share an early segment involving Ca^{2+}, calmodulin, and PKC, they subsequently branch out into multiple Ras-dependent and Ras-independent segments only some of which are also dependent on p42/44 MAPKs.

EXPERIMENTAL PROCEDURES

Materials—Chemicals of the highest purity available were purchased from Sigma. TRI reagent for RNA isolation was from Molecular Research Center, Inc. (Cincinnati, OH). Radionucleotides (^{32}P-labeled, about 3,000 Ci/mmol) and [^{32}P]P_i were from NEN Life Science Products. Rabbit Anti-ACTIVE MAPK polyclonal antibody and anti-p42/44 antibodies were obtained from Promega (Madison, WI) and New England Biolabs (Beverly, MA), respectively. An anti-Ha-Ras monoclonal antibody and protein G plus agarose were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All protein kinase inhibitors were purchased from Calbiochem (San Diego, CA).

Cell Preparation and Culture—Neonatal ventricular myocytes were prepared and cultured as described in our previous work (5–7). 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 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 performed after another 48 h of incubation under serum-free conditions. Incubations in Ca^{2+}-free media were performed as described previously (5). These cultures contain more than 95% myocytes as assessed by immunofluorescence staining with a myosin heavy chain antibody.

Northern Blot—Northern blot was performed as described previously (5, 6). 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.

Measurement of Phosphorylation and In-gel Assay of p42/44 MAPKs—Activation of p42/44 MAPKs in cultured myocytes was determined by both in-gel kinase assay and Western blot using a rabbit polyclonal antibody raised against dually phosphorylated p42/44 MAPKs (23, 24). 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). Protein content was measured by the Lowry method (25) using bovine serum albumin as standard. For Western blot analysis, cell lysates (30 μg/lane) were electrophoresized on 10% SDS-polyacrylamide gels, and transferred to a nitrocellulose membrane. The membranes were probed with Anti-ACTIVE MAPK polyclonal antibody, which detects p42/44 MAPKs only when they are activated by phosphorylation at Thr-202 and Tyr-204. To ensure equal loading and protein transfer, duplicate blots were performed for the same samples and probed with a polyclonal antibody recognizing both phosphorylated and non-phosphorylated p42/44 MAPKs. These membranes were developed with a secondary anti-rabbit antibody as we previously described (7). For in-gel assay, cell lysates (30 μg/lane) were resolved in 10% SDS-polyacrylamide gels containing 0.5 mg/ml myelin basic protein. MAPKs in the gels were denatured in 6 M guanidine-HCl and renatured in 50 mM Tris-HCl (pH 8.0) containing 0.05% Triton X-100 and 5 mM 2-mercaptoethanol. The kinase activities were assayed by incubation of these gels with [γ-^{32}P]ATP. The gels were then extensively washed, dried, and subjected to autoradiography. Both Western blots and autoradiograms were scanned with a Bio-Rad densitometer to quantitate MAPK signals.

Analysis of GTP and GDP-bound Ras—Measurement of guanine nucleotide-bound Ras was performed as described previously (26, 27). Cells in 60-mm dishes were prelabeled with 0.4 mCi of $^{32}P_i$ for 18 h in phosphate-free Dulbecco's modified Eagle's medium. After exposure to 100 μM ouabain for various times, cells were washed twice with ice-cold phosphate-buffered saline, and lysed in 0.5 ml of lysing buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 20 mM $MgCl_2$, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). Cell lysates were centrifuged for 5 min at 2,000 × *g*, and the supernatant was incubated with an anti-Ha-Ras monoclonal antibody for 1 h at 4 °C. The immune complexes were then precipitated with protein G plus agarose overnight at 4 °C. The precipitates were washed four times with the lysing buffer and three times with washing buffer (Tris-HCl, pH 7.4, 20 mM $MgCl_2$, and 150 mM NaCl). The Ras-bound GDP and GTP were then eluted in 20 μl of elution buffer (20 mM Tris-HCl, pH 7.4, 20 mM EDTA, 2% SDS, 0.5 mM GDP, and 0.5 mM GTP). Eluted GDP and GTP were separated on a polyethyleneimine-cellulose thin layer plate and developed with 0.75 M KH_2PO_4 (pH 3.4). Labeled GDP and GTP were visualized by autoradiography. GDP and GTP fractions were then cut out from the plate and quantitated by scintillation counting. Ras activity is expressed as a ratio of GTP/(GDP + GTP).

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 described previously (28). 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 (29). 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. In three independent infection experiments with different concentrations of adenovirus-β-galactosidase, the percentages of cardiac myocytes expressing β-galactosidase after 12 h of infection, as determined by histochemical staining, were 81 ± 5% (100 viral particles/cell) and 96 ± 3% (1,000 viral particles/cell).

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

Ouabain Activates p42/44 MAPKs in Cardiac Myocytes—Involvement of p42/44 MAPK isoforms (ERK1 and ERK2) in the development of cardiac myocyte hypertrophy has been suggested, and activations of these MAPKs by several cardiac hypertrophic stimuli such as phenylephrine, endothelin, and PMA have been demonstrated (19–22). Since our previous studies suggested that p42/44 MAPKs may participate in ouabain-induced down-regulation of the α_3 subunit of Na/K-ATPase in cardiac myocytes (7), we wished to determine if these kinases are indeed activated when myocytes are exposed to ouabain concentrations that cause partial inhibition of Na^+/K^+-ATPase. Myocytes were exposed to 100 μM ouabain for various times, and the levels of phosphorylated and activated p42/44 MAPKs were measured by Western blot analysis. As shown in Fig. 1*A*, ouabain increased phosphorylation levels of both p42 and p44 in a time-dependent manner. Because p42 Western blot signals were much stronger than those of p44, ouabain-induced increase in p42 phosphorylation was quantitated as shown in Fig. 1*B*. A significant activation of p42 MAPK was observed after 5 min of exposure, reached maximal value after 15 min, and lasted for at least 90 min. Limited quantitations were also performed for p44 in several overdeveloped membranes, and similar activation patterns were noted (data not shown). When concentration-dependent changes in response to ouabain were measured, significant activations of both p42 and p44 MAPKs were observed with ouabain concentrations as low as 10 μM (Fig. 2). To confirm if ouabain-induced increases in MAPK phosphorylation are correlated with increase in MAPK activity, extracts from myocytes treated with 100 μM ouabain for 15 min were also analyzed using the in-gel kinase assay (see "Experimental Procedures"). In three inde-

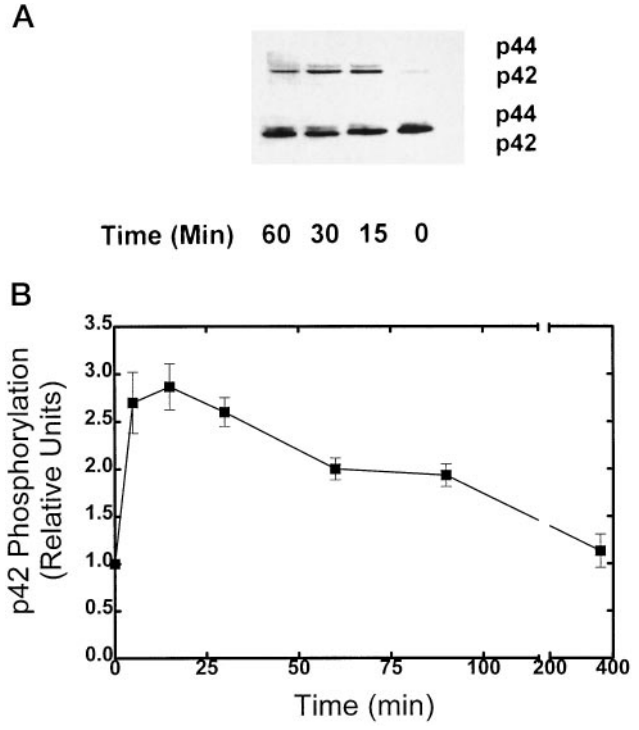

FIG. 1. **Time course of the effects of ouabain on p42/44 MAPKs.** Cells were treated with 100 μM ouabain for various times and assayed for p42/44 MAPKs as described under "Experimental Procedures." *A*, a representative Western blot. The *top panel* was detected by an antibody specific for dual phosphorylated p42/44, and the *bottom panel* was detected by an antibody recognizing total p42/44 MAPKs, showing an equal loading and protein transfer among different lanes. *B*, combined data from four independent experiments. Phosphorylated p42 values were normalized against the total p42 signal and expressed as mean ± S.E.

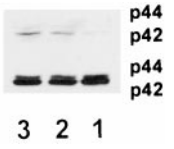

FIG. 2. **Dose-dependent effects of ouabain on p42/44 MAPKs.** Cells were treated with various concentrations of ouabain for 15 min, and assayed for p42/44 MAPK phosphorylation as in Fig. 1. A representative Western blot of three independent experiments is shown. *Lane 1*, control; *lane 2*, 10 μM ouabain; *lane 3*, 100 μM ouabain.

pendent experiments, ouabain caused a 2.6 ± 0.5-fold increase in p44 and a 3.2 ± 0.4-fold increase in p42 activities.

Phosphorylation and activation of p42/44 MAPKs are catalyzed by MEK, and PD98059 has been shown to be a highly specific inhibitor of MEK (30). To verify the role of MEK in ouabain-induced activation of p42/44 MAPKs, in experiments similar to those of Figs. 1 and 2, myocytes pretreated for 15 min with 10 μM PD98059 and control myocytes were exposed to 100 μM ouabain for 15 min, and assayed for MAPK phosphorylation. In three separate experiments, PD98059 caused 93 ± 6% suppression of ouabain-induced phosphorylation of p42/44 MAPKs.

Ouabain Stimulates p42/44 MAPKs through a Ras-dependent Pathway—Activations of MEK and p42/44 MAPKs may occur through the Ras-/Raf-pathway or Ras-independent pathways (31). To test whether Ras is involved in ouabain-induced activation of MAPK, cells were infected with an adenovirus expressing an Asn^{17} dominant-negative mutant of Ras for 12 h, washed, then exposed to either ouabain or PMA for 15 min. Adenovirus β-galactosidase-infected myocytes served as control. As depicted in Fig. 3, expressing dominant negative Ras

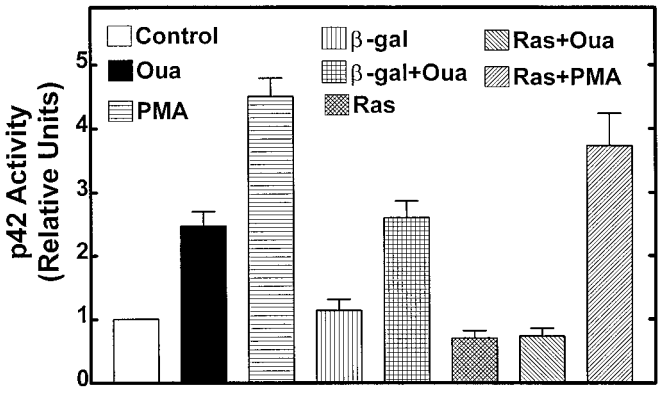

FIG. 3. **Effects of expression of dominant negative Ras on ouabain and PMA-induced p42 MAPK activation.** Cells were transduced either with the adenovirus expressing an Asn^{17} dominant-negative mutant of Ras or with the control virus containing β-galactosidase gene as indicated at a dose of 1,000 particles/cell. After 12 h of incubation, both control and virus-infected cells were washed, then exposed to either 100 μM ouabain or 100 nM PMA for 15 min, and assayed for p42 MAPK phosphorylation as in Fig. 2. Values are mean ± S.E. of three experiments.

blocked ouabain-induced but not PMA-induced, activation of p42 MAPK, indicating that ouabain signals through Ras in contrast to PMA, which is known to use a Ras-independent pathway in activation of MAPKs (32, 33).

That Ras is indeed activated when myocytes are exposed to ouabain was shown in experiments of Fig. 4, indicating that 100 μM ouabain induced a rapid accumulation of GTP-bound Ras, which reached maximum in 5 min and returned to basal levels after 60 min. This time course of ouabain-induced Ras activation correlates well with the time course of MAPK activation by ouabain (Fig. 1).

Different Roles of MAPKs in Ouabain-induced Regulations of the Growth-related Genes—We showed before (7) that the MEK inhibitor PD98059 blocked ouabain-induced repression of α_3 mRNA, but had no effect on ouabain-induced increase in skACT mRNA. To assess the roles of MEK-p42/44 MAPKs in ouabain-activated pathways regulating c-*fos* and ANF gene, myocytes that were pretreated with PD98059 were exposed to 100 μM ouabain and assayed for appropriate mRNAs. The results, along with the effects of PD98059 on α_3 and skACT mRNA, are presented in Fig. 5. Clearly, under the same condition where PD98059 has no influence on ouabain's effect on skACT, the MEK inhibitor causes complete blockade of the ouabain effect on α_3 and only partial blockade of the ouabain effects on ANF and c-*fos*. It is important to note that 10 μM PD98059 used in experiments of Fig. 5 causes near complete inhibition of myocyte MAPK phosphorylation (Ref. 23 and the results presented above); and that concentrations of PD98059 higher than 10 μM caused no further effects than those shown in Fig. 5 (data not shown).

Different Roles of Ras in Ouabain-induced Regulations of the Growth-related Genes—Since ouabain activation of p42/44 MAPKs seems to be through Ras/Raf/MEK/MAPK sequence (Fig. 3), and since some of the ouabain-activated pathways regulating the four ouabain-regulated genes do not involve p42/44 MAPK activations (Fig. 5), it was of interest to explore the role of Ras in the MAPK-dependent and MAPK-independent pathways of the ouabain-regulated genes. Myocytes were infected with the adenovirus containing the dominant-negative Ras, exposed to ouabain, and assayed for the appropriate mRNAs. The results summarized in Fig. 6 showed that the expression of dominant-negative Ras caused (*a*) complete blockade of the ouabain-induced increase in skACT mRNA, (*b*) complete reversal of the ouabain-induced decrease in α_3

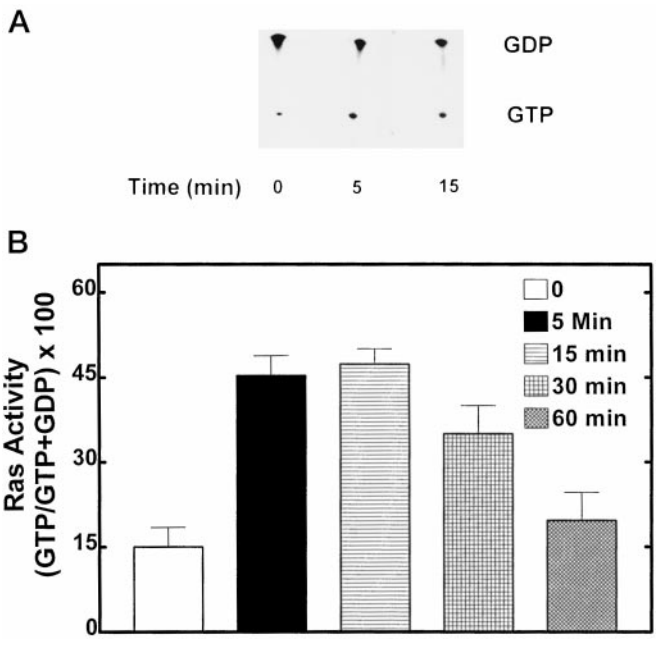

FIG. 4. **Time course of ouabain-induced Ras activation.** Cells were labeled with [^{32}P]P_i for 18 h, then exposed to 100 μM ouabain for various times. Ras was immunoprecipitated with an anti-Ha-Ras monoclonal antibody, and Ras-bound GTP and GDP were separated as described under "Experimental Procedures." *A*, a representative autoradiogram. *B*, combined data from four independent experiments. Values are mean ± S.E.

mRNA, and (*c*) partial blockade of the ouabain-induced increase in ANF mRNA. Quantitation of the results of similar experiments on ouabain-induced increase in c-*fos* mRNA were problematic, since the control β-galactosidase vector had significant effects on c-*fos* expression. However, neither for c-*fos* nor in the case of ANF (Fig. 6*C*) was it possible to approach complete blockade of the ouabain effect by using higher concentrations of the virus containing the dominant-negative Ras (not shown).

To confirm the role of Ras in ouabain-induced regulation of the cardiac genes, we used α-hydroxyfarnesylphosphoric acid, a cell-permeable farnesyltransferase inhibitor that is known to block Ras farnesylation, prevent its association with the plasma membrane, and inhibit Ras signaling (34, 35). As depicted in Fig. 7, when myocytes were pretreated with this inhibitor, a dose-dependent suppression of ouabain-induced skACT expression was noted. This inhibitor also caused significant reversal of ouabain-induced up-regulation of ANF and down-regulation of α_3 mRNAs (data not shown).

Ouabain Effects on p42/44 MAPKs and the Growth-related Genes Are Dependent on Extracellular Ca^{2+}, Calmodulin, and PKC—We demonstrated previously (5–7) that the presence of extracellular Ca^{2+} was required for ouabain-induced regulations of c-*fos*, skACT, and α_3 subunit genes in myocytes. Experiments, the results of which are summarized in Fig. 8, showed that extracellular Ca^{2+} was also necessary for activation of p42/44 MAPKs and for increase of ANF mRNA caused by ouabain.

Our previous studies suggested the involvements of calmodulin and PKC in the ouabain-induced pathways regulating the expressions of skACT, and the α_3 subunit (6, 7). Experiments of Fig. 9 showed that membrane permeable Ca^{2+}/calmodulin antagonist W-7, and PKC inhibitor H-7, also antagonized activation of MAPKs, and increased expressions of c-*fos* and ANF by ouabain.

Because H-7 also inhibits protein kinase A, a more selective inhibitor of this kinase (HA1004) was also used in experiments

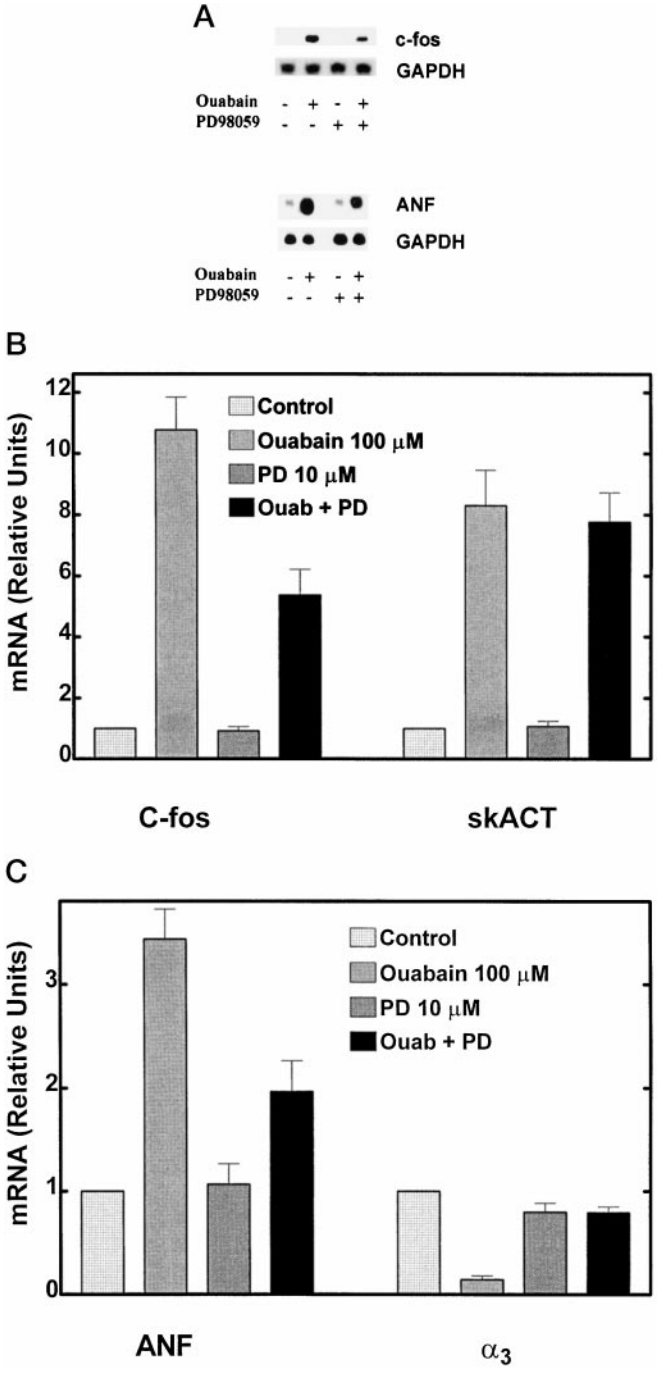

FIG. 5. **Effects of PD98059 on ouabain-induced c-*fos*, skACT, α_3, and ANF expression.** Myocytes were pretreated with 10 μM PD98059. After 15 min of incubation, both control and PD98059-treated cells were exposed to 100 μM ouabain for 45 min, then assayed for c-*fos* mRNA. In another set of experiments, cells were treated with 100 μM ouabain for 12 h, then assayed for skACT, ANF, and α_3 mRNA. Ouabain treatment times were chosen to obtain maximal effects on the mRNAs of the four genes (5–7). Assays were performed as described under "Experimental Procedures." mRNA values were normalized to those of corresponding GAPDH measured on the same blot and expressed relative to a control value of one. The values are mean ± S.E. of three experiments. *A*, representative Northern blots of c-*fos* and ANF mRNAs. *B*, effects of ouabain on c-*fos* and skACT. *C*, effects of ouabain on ANF and α_3.

of Fig. 9, as it was used in our previous studies (6, 7). HA1004 (50 μM) had no significant effect on ouabain-induced increases in c-*fos* and ANF mRNAs, and activations of p42/44 MAPKs (data not shown); supporting the assumption that H-7 effects noted in Fig. 9 are due to PKC inhibition. The combined results of Figs. 8 and 9 and our previous data (5–7) suggest that

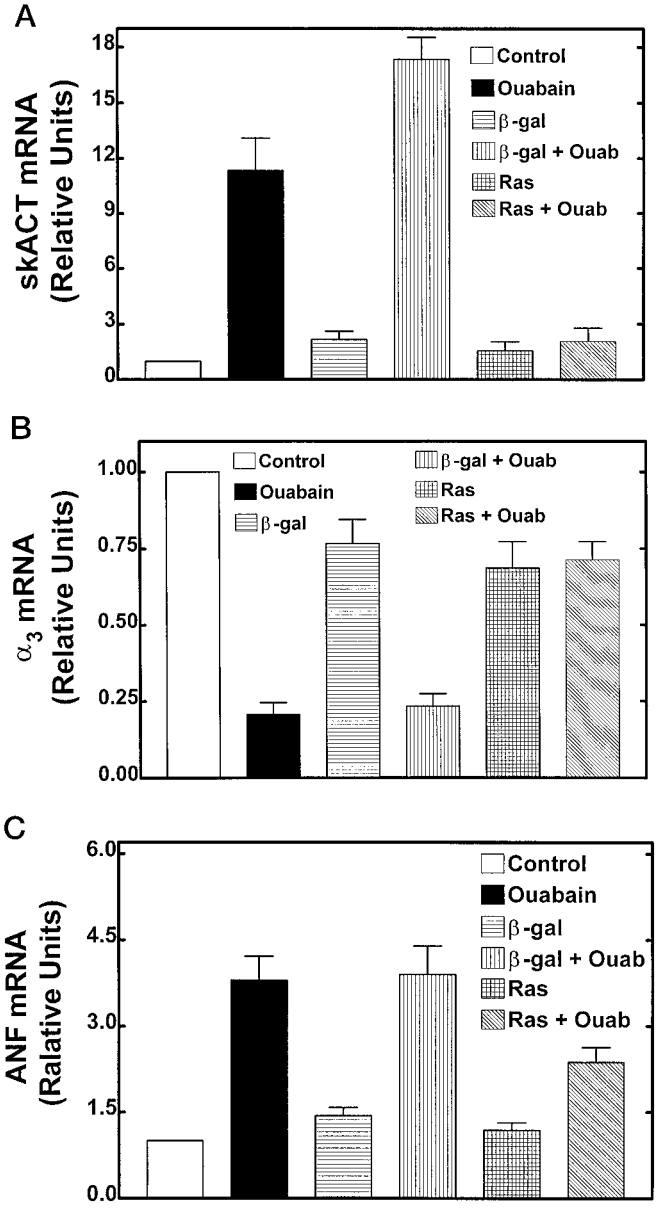

FIG. 6. **Expression of dominant negative Ras suppresses the effects of ouabain on late response genes.** Cells were transduced either with the adenovirus expressing an Asn^{17} dominant-negative mutant of Ras or with the control virus containing β-galactosidase gene as indicated at a dose of 1,000 particles/cell. Both control and virus-infected cells were then washed and treated with 100 μM ouabain for additional 12 h, and assayed for skACT (*A*), α_3 (*B*), ANF (*C*), and GAPDH mRNAs as in Fig. 5. Ouabain treatment times were chosen to obtain maximal effects on the indicated mRNA (6, 7). Values are mean ± S.E. of four experiments.

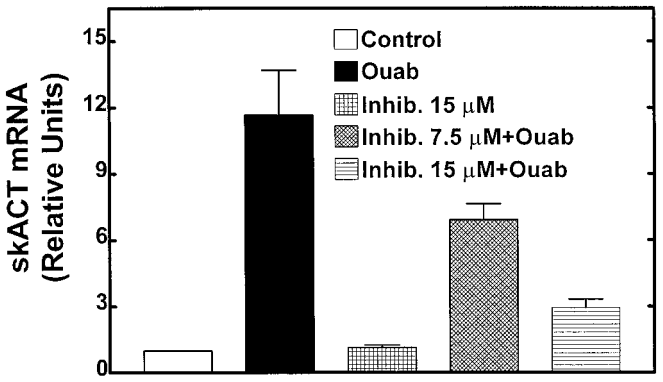

FIG. 7. **Inhibition of Ras farnesylation represses ouabain-induced skACT expression.** Cells were pretreated with different concentrations of α-hydroxyfarnesylphosphoric acid for 18 h. Both control and inhibitor-treated cells were then exposed to 100 μM ouabain for 12 h, and assayed for skACT and GAPDH mRNAs as in Fig. 5. Values are mean ± S.E. of three experiments.

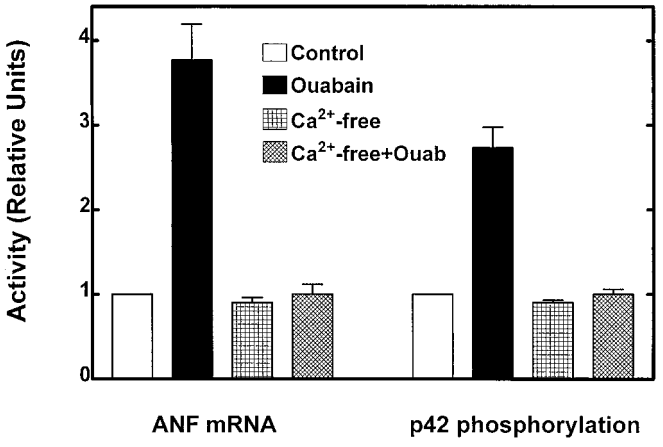

FIG. 8. **Effects of extracellular Ca^{2+} on ouabain-induced ANF expression and p42 MAPK phosphorylation.** Cells were treated with 100 μM ouabain either in the control medium or in the Ca^{2+}-free medium for either 15 min (*MAPK*) or 12 h (*ANF*), then assayed for p42 MAPK phosphorylation and ANF mRNA respectively. Ouabain treatment times were chosen to obtain maximal effects on p42 MAPK (Fig. 1) and on ANF induction (6). The assays were performed as in Fig. 1 and Fig. 5.

increased Ca^{2+} influx, calmodulin, and PKC affect the signal pathways of the four ouabain-regulated genes upstream of the Ras/Raf/MEK/MAPK sequence.

Myocyte Viability—We showed before (5) that myocyte viability, as measured by lactate dehydrogenase release, was not affected by ouabain concentrations used here. In similar experiment, we found that myocyte viability was also not affected significantly when 10 μM PD98059, 10 μM W-7, or 50 μM H-7 was added to the culture medium in addition to ouabain, and incubations were carried out for the longest indicated periods (data not shown). In agreement with others (55), we also found that adenoviral infection of these neonatal myocytes under the indicated conditions had no significant effect on myocyte viability (data not shown).

DISCUSSION

A number of physiological, pathological, and pharmacological stimuli are known to cause cardiac myocyte hypertrophy (8). To determine how these stimuli work in concert to regulate cardiac growth, remodeling, and failure, it is necessary to define the signal transduction pathways that are activated by the various stimuli, and to clarify the mechanisms of cross-talk among these pathways. In this context, and in view of our recent discovery of the hypertrophic effects of ouabain on cardiac myocytes (5, 6), the primary objectives of this work were to determine if partial inhibition of cardiac myocyte Na^+/K^+-ATPase leads to activations of Ras and p42/44 MAPKs, and to assess the roles of any such activations in the signal pathways that link the sarcolemmal Na^+/K^+-ATPase to the expression of several growth-related genes of these myocytes. In all experiments, we used ouabain at concentrations of 100 μM or less, which are known to cause less than 50% inhibition of Na^+/K^+-ATPase, and no overt toxicity, in neonatal rat myocytes (5). Under these conditions that mimic those under which ouabain causes its positive inotropic effect on the heart (2), our findings clearly show that Ras and the MAPKs are rapidly activated, and that these activations are within some, but not all, gene

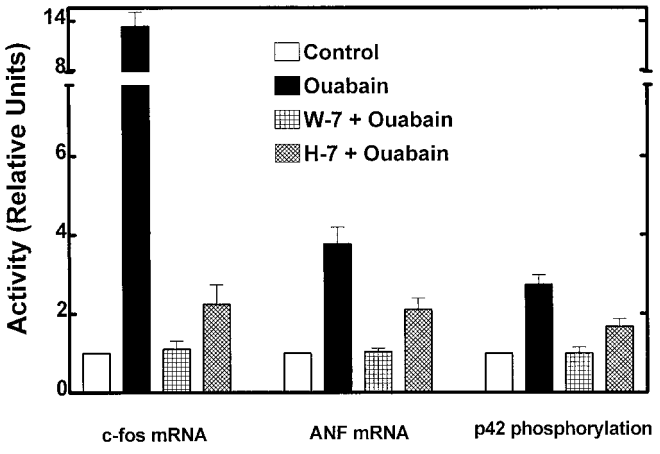

FIG. 9. **Effects of protein kinase inhibitors on ouabain-induced p42 MAPK phosphorylation and expressions of c-*fos* and ANF mRNAs.** Cells were pretreated with different protein kinase inhibitors as indicated for 15 min, then exposed to 100 μM ouabain for 15 min, and assayed for p42 MAPK phosphorylation. In the other two sets of experiments, the inhibitor-pretreated cells were exposed to ouabain for either 45 min or 12 h, then assayed for c-*fos* and ANF mRNAs, respectively. Ouabain treatment times were chosen to obtain maximal effects on the indicated mRNAs (5, 6). Values are mean ± S.E. of three experiments.

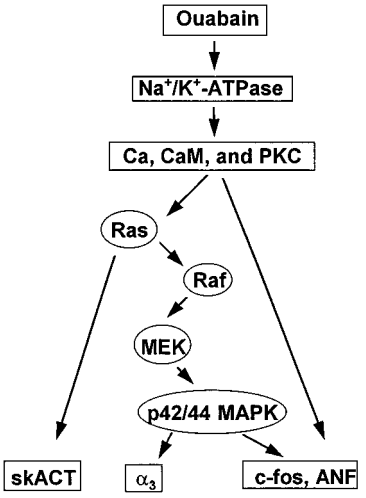

FIG. 10. **Schematic representation showing that multiple signal transduction pathways link Na^+/K^+-ATPase to ouabain-specific regulation of growth-related genes in cardiac myocytes.**

regulatory pathways that are initiated by Na^+/K^+-ATPase inhibition.

Multiplicity of Ouabain-initiated Gene Regulatory Pathways—Based on our data, the following conclusions, as summarized in Fig. 10, emerge about the roles of Ras and p42/44 MAPKs in the pathways that link the activity of cardiac Na^+/K^+-ATPase to the transcriptions of the four genes that we have studied here.

The case of the α_3 subunit of Na^+/K^+-ATPase is the most straightforward. Since ouabain-stimulated activations of the p42/44 MAPKs are entirely dependent on Ras (Fig. 3), and since ouabain-induced repression of α_3 is completely reversed by blockade of Ras (Fig. 6*B*) or by MEK inhibition (Fig. 5*B*), it is clear that repression of α_3 by ouabain must proceed through the well established Ras-Raf-MEK-p42/44 MAPK sequence (Fig. 10). The inductions of c-*fos* and ANF gene by ouabain are similar in that both are partially antagonized by Ras blockade or by MEK inhibition (Figs. 5 (*A* and *B*) and 6*C*; see "Results"), indicating the existence of at least two distinct branches leading to the regulation of each of these two genes: one involving the Ras-Raf-MEK-p42/44 MAPKs sequence, and the other independent of Ras and these MAPKs (Fig. 10). Finally, since the induction of skACT by ouabain is completely prevented by the blockade of Ras (Fig. 6*A*), but not affected at all by MEK inhibition (Fig. 5*A*), it must involve a Ras-dependent pathway that is different from the Ras-Raf-MEK-p42/44 MAPKs sequence (Fig. 10). The existence of such Ras-dependent pathways, *e.g.* through Ras-MEK kinase-c-Jun N-terminal kinase, are well established (8, 27, 31), and their possible involvement in skACT induction by ouabain is being explored.

Further information regarding the signal pathways initiated by the partial inhibition of cardiac Na^+/K^+-ATPase is provided by our findings on the roles of Ca^{2+}, calmodulin, and PKC. The ouabain-induced regulations of the four genes studied here, and the ouabain-induced activations of p42/44 MAPKs, are totally dependent on extracellular Ca^{2+} (Fig. 8 and Refs. 5–7), and all are prevented by a calmodulin antagonist or a PKC inhibitor (Fig. 9 and Refs. 6 and 7). The most economical way of explaining these data is to assume that each of these three factors (increased influx of Ca^{2+}, calmodulin, and PKC) has a single locus of action that is shared by all ouabain-stimulated pathways and, therefore, must be upstream of Ras in the Ras-dependent branches (Fig. 10). The mechanism of Ras activation resulting from the inhibition of Na^+/K^+-ATPase is not known. However, its dependence on Ca^{2+}/calmodulin, as depicted in Fig. 10, provides a lead for the future clarification of this issue; the Ca^{2+}/calmodulin-dependent activation of Ras by signals that do not directly interact with receptor tyrosine kinases, but activate cytosolic tyrosine kinases, has been noted in cells other than cardiac myocytes (24, 36).

If Ca^{2+}/calmodulin and PKC act upstream of Ras, as indicated in Fig. 10, the question may arise as to whether ouabain-induced increase in intracellular free Ca^{2+}, and the postulated ouabain-induced PKC activation, are fast enough to be consistent with the noted rapid activations of Ras and p42/44 MAPKs (Figs. 1 and 4). There is ample prior evidence to show that, in cultured chick cardiac myocytes, inotropic concentrations of ouabain increase intracellular free Ca^{2+}, reaching steady state maximal levels within 5–6 min (Ref. 56 and references therein); we have confirmed this using the myocyte preparation used here.[2] We know of no published data on ouabain-induced activation of PKC, and its time course, in cultured cardiac myocytes; however, in rabbit papillary muscle, activation of PKC has been suggested to occur within 0.5–7 min after the addition of ouabain (57). Regarding the roles of Ca^{2+}, calmodulin, and PKC, it is also important to note that their additional downstream effects within the branched pathways of Fig. 10 cannot be ruled out. For example, c-*fos* transcription in cells other than myocytes is known to be regulated by a number of downstream mechanisms involving Ca^{2+} or PKC (37, 38). The existence of similar downstream effects within Ras-dependent and -independent branches of Fig. 10 needs to be explored.

Specificities of the Ouabain-initiated Gene Regulatory Pathways—The growth-related genes of the neonatal cardiac myocytes, including the four we have studied here, are regulated by a number of hypertrophic stimuli. Extensive previous studies on the signal pathways that are activated in cardiac myocytes by well established hypertrophic stimuli (*e.g.* phenylephrine, angiotensin II, and mechanical stretch) have clearly shown the existence of a complex network of pathways with stimulus-specific segments and segments that are shared by different stimuli (8, 27, 33, 39, 40). The emerging information on the pathways that are activated by ouabain (Fig. 10) also point to ouabain-specific effects within the same over-all network. Although it is not practical to do a thorough analysis of the

[2] P. Kometiani, J. Li, A. Askari, and Z. Xie, unpublished observations.

interrelation of the ouabain-regulated pathways and those of other stimuli, it is instructive to consider similarities and differences between some of the ouabain effects noted here and the related effects of other stimuli.

The p42/44 MAPKs of cardiac myocytes have been suggested to be key mediators of cardiac hypertrophy by several studies (19–22); as is the case for ouabain-induced pathways, recent work has established the role of these kinases in some, but not all, signal transduction pathways that are activated by other stimuli (33, 40–42). It is appropriate, therefore, to see how ouabain effects on cardiac p42/44 MAPKs compare with those of other stimuli. Ouabain causes rapid activation of p42/44 MAPKs, which is sustained for at least 90 min (Fig. 1), and is totally dependent on extracellular Ca^{2+} (Fig. 8) and on Ras (Fig. 3). In similar myocyte preparations, activations of p42/44 MAPKs have been noted by hypertrophic stimuli phenylephrine (23, 43), isoproterenol (43), phorbol esters (43, 44), fibroblast growth factors (44), endothelin (23, 44), angiotensin II (33, 45), mechanical stretch (26, 42, 46), and by a number of receptor agonists that do not cause hypertrophy (23). Unlike ouabain, however, all but one of the above stimuli cause rapid but transient activation levels that return to basal or lower than basal levels within 30–60 min (23, 26, 43–46). Activation by phorbol esters has a time course similar to that of ouabain (43–45); however, unlike ouabain activation, activation by phorbol esters in these myocytes is not dependent on Ras (Fig. 3). The importance of sustained activations of MAPKs in relation to their gene regulatory actions has been noted (47, 48). The potential significance of differences between the activation time courses of ouabain and the other stimuli remains to be determined.

As in the case of ouabain-induced activation of p42/44 MAPKs, extracellular Ca^{2+} is required for activations by isoproterenol and angiotensin II (43, 45); however, activation by phorbol esters, endothelin, fibroblast growth factor, and phenylephrine are not dependent on extracellular Ca^{2+} (43). Additions of Ca^{2+} ionophores to myocytes in Ca^{2+}-containing media also cause rapid activations of p42/44 MAPKs (43, 45); however, these activations are also quite short-lived (45). Most interestingly, electrical pacing which also causes cardiac myocyte hypertrophy and increase in intracellular Ca^{2+}, does not cause activations of p42/44 MAPKs (49, 50). Taken together, the above clearly suggest that increases in intracellular Ca^{2+} achieved by ouabain and by other means have different effects on p42/44 MAPKs, lending further support to our previous proposal (7) that in cardiac myocytes, as in other cells (51), different intracellular pools of Ca^{2+} may have different effects within the signal pathways.

Other noteworthy differences between the ouabain effects presented here and those of other stimuli are related to the skACT gene. Although this fetal gene is induced by a large number of cardiac hypertrophic stimuli (8), the signal pathways that lead to its induction have not been studied as extensively as those of ANF induction. Nevertheless, the available data point to significant ouabain selectivity within multiple pathways of skACT induction. Our data clearly show that induction of skACT by ouabain is totally Ras-dependent, but independent of MEK-p42/44 MAPK activations (Figs. 5, 6, and 10). In similar preparations of cardiac myocytes, however, stimulation of skACT promoter activity by MEK and p42-MAPK has been shown in one study (21), and other studies have failed to demonstrate the involvement of Ras in transforming growth factor-β-induced regulation of skACT gene (52, 53). Interestingly, these latter studies indicated a "global" role of Ras in the basal expressions of a variety of cardiac myocyte genes. With the use of the adenoviral vector for the high efficiency expression of the Ras mutant, and under the indicated experimental conditions, we see no evidence of such a global effect of Ras. The phenylephrine-induced regulation of skACT is dependent on PKC (54), as is the ouabain-induced regulation of skACT (6). Other similarities or differences between the effects of ouabain and other stimuli on the pathways regulating skACT expression remain to be explored.

In summary, our present and earlier findings (5–7) show that partial inhibition of cardiac sarcolemmal Na^+/K^+-ATPase, within the limits that are achieved by the positive inotropic concentrations of digitalis drugs, induces a distinct pattern of hypertrophy and gene regulation through multiple pathways that are related but not identical to the network of pathways that are activated by other hypertrophic stimuli. Studies on the further characterization of the gene regulatory mechanisms that are linked to cardiac Na^+/K^+-ATPase, and on the mechanisms of interactions between these and the related pathways activated by other physiological and pathological hypertrophic stimuli are in progress.

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