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Involvement of Src and Epidermal Growth Factor Receptor in the Signal-transducing Function of Na^+/K^+-ATPase*

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Nontoxic concentrations of ouabain, causing partial inhibition of the cardiac myocyte Na^+/K^+-ATPase, induce hypertrophy and several growth-related genes through signal pathways that include the activation of Ras and p42/44 mitogen-activated protein kinase (MAPK). The aim of this work was to examine the ouabain-induced events upstream of the Ras/MAPK cascade. Treatment of myocytes with genistein antagonized ouabain-induced activation of the MAPK, suggesting that protein tyrosine phosphorylation has a role. Tyrosine phosphorylation of several myocyte proteins was increased rapidly upon cell exposure to ouabain. Lowering of extracellular K^+ had a similar ouabain-like effect. Ouabain also increased protein tyrosine phosphorylation in A7r5, HeLa, and L929 cells. In cardiac myocytes and A7r5 cells, herbimycin A antagonized the ouabain-induced increase in protein tyrosine phosphorylation and MAPK activation. In both cell types, ouabain stimulated Src kinase activity, Src translocation to the Triton-insoluble fraction, Src association with the epidermal growth factor receptor, and the tyrosine phosphorylation of this receptor on site(s) other than its major autophosphorylation site, Tyr^{1173}. The findings suggest that (*a*) the ouabain-induced activation of Src and the Src-induced phosphorylation of the growth factor receptor provide the scaffolding for the recruitment of adaptor proteins and Ras and the activation of Ras/MAPK cascade; and (*b*) the activation of such pathways may be a common feature of the signal-transducing function of Na^+/K^+-ATPase in most cells.

Na^+/K^+-ATPase catalyzes the active transport of Na^+ and K^+ across the plasma membrane of most mammalian cells (1, 2). In the heart, partial inhibition of the Na^+/K^+-ATPase by ouabain and other cardiac glycosides produces a modest increase in intracellular Na^+, which in turn causes significant increases in intracellular Ca^{2+} and cardiac contractility (3). This is the basis for the continued use of cardiac glycosides in the management of congestive heart failure (4, 5). Recently, we have shown that the same nontoxic concentrations of ouabain that partially inhibit the Na^+/K^+-ATPase and increase intracellular Ca^{2+} also stimulate hypertrophic growth of rat neonatal cardiac myocytes and regulate the transcription of the genes that are known to be markers of cardiac hypertrophy (6–10). We have also shown that these gene regulatory actions of ouabain involve the activation of multiple interrelated signal transduction pathways, including the Ras/Raf/MEK/MAPK[1] cascade (9–10). With the long range goal of clarifying the mechanisms through which the plasma membrane Na^+/K^+-ATPase is linked to cardiac growth-related genes, the aim of this work was to explore the events upstream of the Ras/MAPK cascade and close to ouabain interaction with the Na^+/K^+-ATPase. The pathway for Ras/MAPK activation by the classical growth factor receptor tyrosine kinases, involving the tyrosine phosphorylation and recruitment of complexes of adaptor proteins and Ras to the plasma membrane, are well characterized (11). It is evident now that a number of membrane receptors that lack intrinsic tyrosine kinase activity also use similar mechanisms for the activation of the Ras/MAPK cascade through either nonreceptor tyrosine kinases and/or transactivation of receptor tyrosine kinases (12–14). In view of this, it seemed appropriate to determine whether ouabain-induced activation of the Ras/MAPK cascade is associated with increased protein tyrosine phosphorylation; and if so, to assess the role of specific protein-tyrosine kinases in this process. A preliminary account of portions of this work has been presented (15).

EXPERIMENTAL PROCEDURES

Materials—Chemicals of the highest purity were purchased from Sigma. Genistein and herbimycin A were obtained from Calbiochem (San Diego, CA). The antibodies used and their sources are as follows: Anti-phosphotyrosine monoclonal antibody (PY99), the MAPK polyclonal antibodies, and the goat anti-rabbit secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The affinity-purified anti-dually phosphorylated MAPK antibodies were purchased from Promega (Madison, WI). The goat anti-mouse secondary antibody was purchased from Pierce (Rockford, IL). Monoclonal anti-Src (clone GD 11) antibody, both polyclonal anti-EGFR antibodies, and the monoclonal anti-phospho-EGFR (Tyr^{1173}) antibody (clone 9H2) as well as the Src kinase assay kit were obtained from Upstate Biotechnology (Lake Placid, NY). Protein A/G Plus-Agarose was obtained from Santa Cruz Biotechnology. The Optitran nitrocellulose membranes used for Western blotting were obtained from Schleicher and Schuell (Keene, NH).

Cell Preparation and Culture—The same protocol was used to prepare and culture neonatal ventricular myocytes as described in our previous work (6). In short, myocytes from 1-day-old Harlan Sprague-Dawley rats were isolated and purified on Percoll gradients. In a medium containing four parts of DMEM and one part Medium 199 (Life Technologies, Inc.), penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% fetal bovine serum, myocytes were cultured for 24 h at 37 °C in humidified air with 5% CO_2. After 24 h, the myocytes were serum-starved for 48 h at which time all experiments were performed. Some experiments were done on cells incubated in DMEM with altered salt composition as indicated. As determined by immunofluorescence stain-

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[1] The abbreviations used are: MAPK, mitogen-activated protein kinase; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; EGFR, EGF receptor; FBS, fetal bovine serum; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; PAGE, polyacrylamide gel electrophoresis.

ing with a myosin heavy chain antibody, the cultures contained more than 95% myocytes. Rat A7r5 cells, mouse L929 cells, and HeLa cells were obtained from American Type Culture Collection, and cultured in DMEM medium containing 10% FBS, and penicillin (100 units/ml)/streptomycin (100 μg/ml). When cell cultures reached about 90% confluence, cells were serum-starved for 24 h and used for the experiments.

Measurement of Protein Tyrosine Phosphorylation and p42/44 MAPK Activity—Immunoblotting was performed to identify increases in tyrosine phosphorylation and the activation of p42/44 MAPK using the antibodies described under "Experimental Procedures." Dilutions of these antibodies were done as recommended by the manufacturer. Following the indicated treatment, the incubation medium was rapidly replaced with 5 ml of ice-cold phosphate-buffered saline. The washed cells were then lysed in 200 μl of ice-cold radioimmune precipitation buffer containing 1% Nonidet P-40, 1% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 50 mM Tris-HCl (pH 7.4). Cell lysates were centrifuged at 16,000 $\times$ *g* for 10 min, and supernatants were used for Western blot analysis. Samples were separated by SDS-PAGE (60 μg/lane) and transferred to an Optitran membrane as we previously described (9). The membranes were then probed with an anti-phosphotyrosine monoclonal antibody or anti-active MAPK polyclonal antibody. The anti-active MAPK polyclonal antibody was then stripped, and the membrane was reprobed with a polyclonal antibody that recognizes the total amount of MAPK to account for equal loading as we previously reported (9). The secondary antibodies were conjugated to horseradish peroxidase. The immunoreactive bands were developed using chemiluminescence (Pierce) and detected by exposure to x-ray film. Images were scanned with a Bio-Rad densitometer to quantitate the relative intensities of the bands. When necessary, different dilutions of the samples were subjected to immunoblotting, and multiple exposures of the films were used to assure that quantitative comparisons were made within the linear range of the assay.

Assay for Src Activation—Src activation was measured by both Src translocation and Src kinase activity (16–18). To measure Src translocation, cells were lysed in Triton lysis buffer containing 50 mM NaCl, 1 mM NaF, 1 mM Na_3VO_4, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 50 mM tetrasodium pyrophosphate, 10% glycerol, 10 nM okadaic acid, 1% Triton X-100, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 50 mM HEPES (pH 7.6). After centrifugation at 16,000 $\times$ *g* for 10 min, the supernatant was removed and the Triton-insoluble material was extracted with radioimmune precipitation buffer described in the previous section. Both Triton-soluble and -insoluble materials were then dissolved in sample buffer, separated on 10% SDS-PAGE, and immunoblotted with a monoclonal anti-Src antibody specific to $pp60^{Src}$ (19). To assay Src kinase activity, cells were lysed in radioimmune precipitation buffer at 4 °C. The insoluble material was removed by centrifugation at 16,000 $\times$ *g* for 10 min, and the cell lysates were immunoprecipitated using the same Src monoclonal antibody and protein A/G Plus-Agarose. The immunoprecipitate was washed once with radioimmune precipitation buffer and three times with ice-cold phosphate-buffered saline, then placed in the Src kinase reaction buffer containing 125 mM $MgCl_2$, 25 mM $MnCl_2$, 2 mM EGTA, 250 μM sodium orthovanadate, 2 mM dithiothreitol, and 100 mM Tris-HCl (pH 7.2). The Src kinase reaction was then carried out using a commercially available kit according to the manufacturer's instructions (Upstate Biotechnology Inc., Lake Placid, NY), which is based on the phosphorylation of a specific synthetic peptide (KVEKIGEGTYGVVYK) corresponding to amino acids 6–20 of $p34^{cdc2}$ (16).

Assay for EGFR Tyrosine Phosphorylation and Src Association—Cell lysates made in radioimmune precipitation buffer were immunoprecipitated using a polyclonal anti-EGFR antibody made against recombinant human EGFR, having established reactivity with the rat receptor. The immunoprecipitate was dissolved in sample buffer, separated on 10% SDS-PAGE, and blotted with the monoclonal anti-phosphotyrosine antibody. To determine if Src binds to the EGFR, the same blots were stripped and reprobed with the monoclonal anti-Src antibody. To determine if the EGFR is autophosphorylated at its major phosphorylation site (Tyr^{1173}), the radioimmune precipitation buffer cell lysates were separated on 10% SDS-PAGE and immunoblotted with a monoclonal anti-phospho-EGFR antibody raised against the peptide NAE(pY)LRV, which corresponds to the protein sequence around Tyr^{1173} of the EGFR.

Analysis of Data—Data are given as the mean ± S.E. Statistical analysis was performed using the Student's *t* test, and significance was accepted at $p < 0.05$. Each presented immunoblot is representative of the similar results of at least three separate experiments.

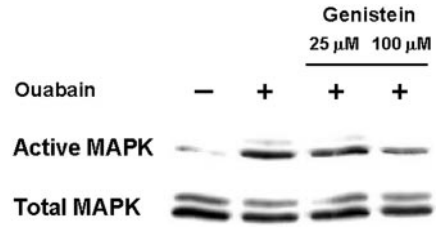

FIG. 1. **Effect of genistein on ouabain-induced activation of p42/44 MAPK.** Myocytes were pretreated with different concentrations of genistein for 30 min then exposed to 100 μM ouabain for 15 min. Activation of p42/44 MAPK was assayed as described under "Experimental Procedures."

RESULTS

Relation of the Na^+/K^+-ATPase Inhibition to Protein Tyrosine Phosphorylation and p42/44 MAPK Activation in Cardiac Myocytes—We showed previously that exposure of rat neonatal myocytes to nontoxic concentrations of ouabain causes rapid activation of Ras and p42/44 MAPK and that ouabain-induced activation of p42/44 MAPK is Ras-dependent (9). In experiments shown in Fig. 1 we examined the effect of the tyrosine kinase inhibitor genistein on ouabain-induced activation of p42/44 MAPK. The results showed that genistein blocked the effect of ouabain, suggesting that ouabain-induced protein tyrosine phosphorylation is involved in the activation of the Ras/MAPK cascade. To see if ouabain does in fact induce increased protein tyrosine phosphorylation, serum-starved myocytes were exposed to ouabain or FBS and cell lysates were subjected to SDS-PAGE and immunoblotted using an anti-phosphotyrosine antibody. As depicted in Fig. 2, 100 μM ouabain caused a rapid increase in tyrosine phosphorylation of several proteins in the range of 30–170 kDa; and the pattern of this increased phosphorylation was, at least superficially, similar to that induced by FBS. This is in keeping with the known presence of growth factors in FBS, the established hypertrophic effects of these on cardiac myocytes, and the protein-tyrosine kinase activities of the growth factor receptors (20, 21). Note that 100 μM ouabain causes hypertrophy and partial inhibition of the Na^+/K^+-ATPase in these myocytes without having significant effects on myocyte viability (6, 7). When additional experiments similar to those of Fig. 2 were done, and time-dependent changes in the intensities of some of the tyrosine-phosphorylated bands were appropriately compared, it became evident that some increased tyrosine phosphorylation occurred as early as 30 s after exposure to ouabain, but that there were also significant differences in the time-courses of the ouabain-induced changes (Fig. 3). Of particular interest was that the increased phosphorylation of a band, which could also be detected with an antibody specific for p42/44 MAPK and had the same mobility as p42/44 MAPK, lagged behind those of several other phosphorylated proteins (Fig. 3). This supports the proposition that ouabain-induced activation of the Ras/MAPK cascade is preceded by more rapid increases of other protein tyrosine phosphorylations. For the remaining experiments on protein tyrosine phosphorylation that are presented below (Figs. 4–8), only the upper portions of the blots are shown. With the exceptions noted, no significant differences between the patterns of the phosphorylated bands of these gels and those of Fig. 2 were observed.

Experiments shown in Fig. 4 on the dose dependence of the ouabain effect showed significant increases in protein tyrosine phosphorylation at concentrations as low as 10 μM ouabain. Concentrations equal or lower than 1 μM were ineffective.

To determine if inhibition of the Na^+/K^+-ATPase of the intact cardiac myocytes by means other than exposure to ouabain also leads to increased tyrosine phosphorylation, the effect of

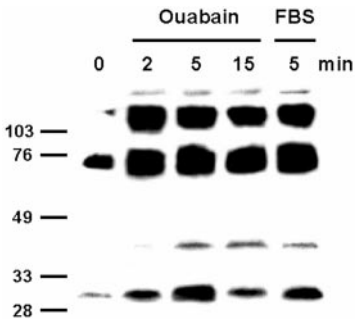

FIG. 2. **Effects of ouabain on protein tyrosine phosphorylation.** Myocytes were exposed to 100 μM ouabain or 10% FBS for the indicated times. Cell lysates were subjected to 10% SDS-PAGE and immunoblotted with a monoclonal anti-phosphotyrosine antibody as described under "Experimental Procedures." In the absence of ouabain and FBS, there were no changes in the intensities of the bands as a function of incubation time.

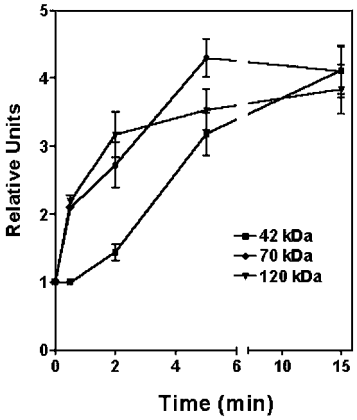

FIG. 3. **Time-dependent effects of ouabain on protein tyrosine phosphorylation.** The experiments of Fig. 2 were repeated with additional early time points. The densities of three tyrosine-phosphorylated bands (42, 70, and 120 kDa) were quantitated and expressed relative to a control value of one. Different exposures were used for the quantitations of the various bands.

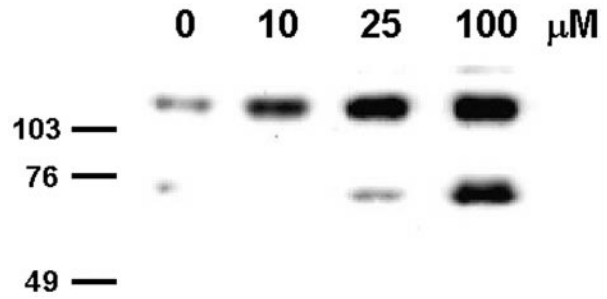

FIG. 4. **Concentration-dependent effects of ouabain on protein tyrosine phosphorylation.** Myocytes were treated with different concentrations of ouabain for 5 min then assayed for protein tyrosine phosphorylation as in Fig. 2. Ouabain concentrations equal or lower than 1 μM were ineffective (not shown).

lowering of extracellular K^+ concentration was compared with that of ouabain. As shown in Fig. 5, a change in extracellular K^+ from 4 to 1.2 mM also increased protein tyrosine phosphorylation. In separate experiments, the same change in medium K^+ also led to rapid activation of p42/44 MAPK (data not shown). The $K_{0.5}$ of extracellular K^+ for ouabain-sensitive K^+ influx in neonatal rat cardiac myocytes has been determined to be about 1.2 mM (22).

Ouabain-induced Increase of Tyrosine-phosphorylated Proteins in Various Cell Types—To assess the cell type specificity of the ouabain-induced tyrosine phosphorylation, ouabain effects on cardiac myocytes were compared with those on three cell lines. In A7r5 cells, which are derived from rat smooth muscle cells, the effects of 100 μM ouabain were similar to those observed in rat cardiac myocytes, causing rapid time-dependent increases in tyrosine phosphorylation (Fig. 6*A*) and activation of p42/44 MAPK (Fig. 6*B*). Ouabain also induced increased tyrosine phosphorylation in human HeLa cells (Fig. 7) and in mouse L929 cells (data not shown). Significantly, in HeLa cells, ouabain concentrations as low as 0.1 μM caused increases in protein tyrosine phosphorylation, whereas the effective ouabain concentrations in A7r5 and L929 cells were more similar to those of rat cardiac myocytes (Fig. 4); *i.e.* 10 μM ouabain or higher were required for an increase in tyrosine phosphorylation. This is consistent with the established higher ouabain sensitivities of the human Na^+/K^+-ATPase isoforms than that of the α_1 rodent isoform (2), which is the predominant isoform in rat cardiac myocytes (23, 24), A7r5 cells (25), and L929 cells.[2]

FIG. 5. **Effects of low extracellular K^+ on protein tyrosine phosphorylation.** Myocytes cultured in a medium with 4 mM K^+ were rapidly transferred to a medium containing 1.2 mM K^+ for different times and assayed for protein tyrosine phosphorylation as in Fig. 2. There were no changes in band intensities without a change in medium K^+.

Role of Src Kinase in Ouabain-induced Increase in Tyrosine Phosphorylation and Activation of p42/44 MAPK—Nonreceptor Src family kinases have been implicated in the development of the hypertrophic myocardium and in the regulation of cardiac growth-related genes by several agonists of G protein-coupled receptors that induce hypertrophy in cultured cardiac myocytes (18, 26, 27). To explore the possibility of the involvement of Src in the above ouabain-induced increases in tyrosine phosphorylation, myocytes were pretreated with herbimycin A, a Src family kinase inhibitor, and then exposed to ouabain. As shown in Fig. 8*A*, herbimycin A antagonized ouabain-induced increases in most of the tyrosine-phosphorylated protein bands with the exception of the band of about 120 kDa. To date, we have not pursued the possible significance of these effects of herbimycin A and ouabain on the unidentified 120-kDa band. Ouabain-induced activation of cardiac myocyte p42/44 MAPK was also blocked by herbimycin A (Fig. 8*B*). When similar experiments were done with A7r5 cells, herbimycin A again antagonized ouabain-induced increase in tyrosine phosphorylation (not shown) and p42/44 MAPK activation (Fig. 8*B*).

To test the role of Src more directly, cardiac myocytes and A7r5 cells were exposed to 100 μM ouabain for various times and Src was immunoprecipitated from cell lysates with an Src-specific antibody and assayed for Src kinase activity by established procedures. Ouabain treatment increased total Src kinase activity in both cardiac myocytes and A7r5 cells (Fig. 9*A*). Activated Src is known to be associated with the cytoskeleton in many cell types other than cardiac myocytes (28), and its cytoskeletal association has also been shown in the heart that is undergoing hypertrophy (26). To explore the effect of ouabain treatment on Src localization, the relative amounts of Src in Triton-soluble and -insoluble cytoskeletal fractions were determined by Western blots. Src clearly translocated to the Triton-insoluble fraction in ouabain-treated cardiac myocytes

[2] Z. Xie, unpublished observations.

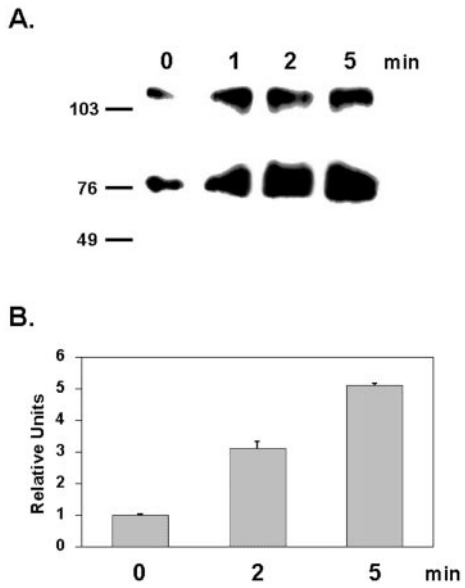

FIG. 6. **Effects of ouabain on protein tyrosine phosphorylation and p42 MAPK in A7r5 cells.** Cells were treated with 100 μM ouabain for various times and assayed for protein tyrosine phosphorylation and activation of p42/44 MAPK as in Figs. 1 and 2. *A*, a representative Western blot on protein tyrosine phosphorylation. *B*, ouabain-induced activation of p42 MAPK. Similar stimulation was observed when p44 MAPK was quantitated (data not shown).

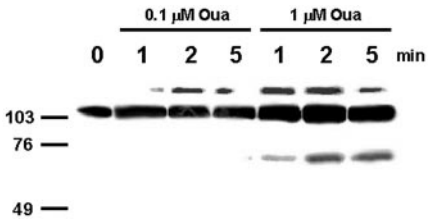

FIG. 7. **Ouabain-induced protein tyrosine phosphorylation in HeLa cells.** Cells were treated with different concentrations of ouabain for different times and assayed for tyrosine phosphorylation as in Fig. 2.

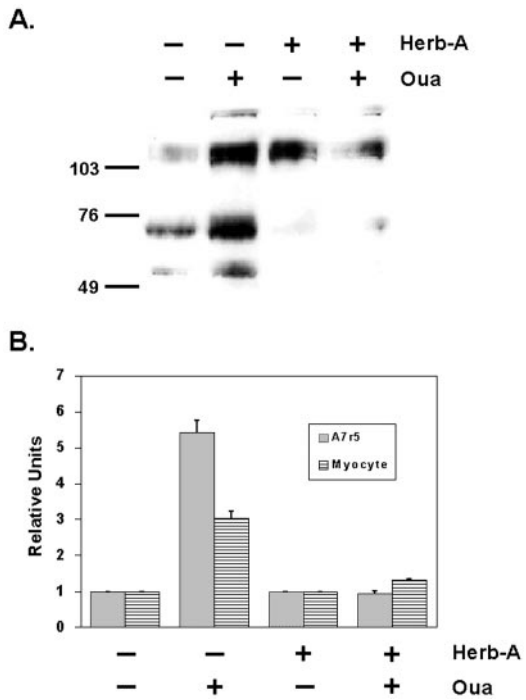

FIG. 8. **Effects of herbimycin A on ouabain-induced tyrosine phosphorylation and p42 MAPK activation.** Cells were preincubated with 1 μM herbimycin A for 2 h then exposed to 100 μM ouabain for 5 min and assayed for protein tyrosine phosphorylation and p42 MAPK activity as in Figs. 1 and 2. *A*, a representative Western blot showing the effect of herbimycin A on ouabain-induced tyrosine phosphorylation in myocytes. *B*, effects of herbimycin A on ouabain-induced p42 MAPK activation in myocytes and A7r5 cells.

(Fig. 9*B*) and A7r5 cells (Fig. 9*C*).

Ouabain-induced Src Interaction with EGFR—In cells other than cardiac myocytes, Src acts as a cotransducer of the signals initiated at the growth factor receptor tyrosine kinases (29, 30, 31). Src is also involved in the transactivation of receptor tyrosine kinases by events that begin at plasma membrane receptors that do not have intrinsic tyrosine kinase activity (12–14). Because the EGFR has been implicated in such mechanisms by several studies (13, 29–32) and because a prominent tyrosine-phosphorylated band having about the same size as the EGFR (170 kDa) was consistently noted in the lysates of ouabain-treated cells (*e.g.* Fig. 2), we explored the possibility of ouabain-induced interaction of Src with the EGFR. The following experiments were done in cardiac myocytes and A7r5 cells with nearly identical results. Only the data with A7r5 cells are presented.

When cell lysates were immunoprecipitated with an anti-EGFR antibody and the precipitate was probed with an anti-Src antibody, Src was detected and the coprecipitated Src was increased in a time-dependent manner upon exposure of the cells to ouabain (Fig. 10*A*). Reprobing of the blots with the anti-EGFR antibody showed that ouabain treatment did not increase the amount of the immunoprecipitated EGFR (Fig. 10*B*). Reprobing with a phosphotyrosine antibody showed, however, that ouabain increased the level of phosphorylated EGFR (Fig. 10*C*). When experiments similar to those of Fig. 10 were done, and the immunoprecipitate was probed with antibodies known to be reactive with the α-subunits of the Na^+/K^+-ATPase of these cells, the presence of such subunits in the Src-EGFR precipitate could not be demonstrated.

To determine if ouabain-induced tyrosine phosphorylation of the EGFR occurs at a major autophosphorylation site (Tyr^{1173}), an antibody specific for the detection of this phosphotyrosine was used to do Western blots on lysates of the cells exposed to EGF or ouabain (Fig. 11). Although EGF increased phosphorylation at the autophosphorylation site, ouabain did not (Fig. 11*B*), suggesting that the ouabain-induced tyrosine phosphorylation of the EGFR (Figs. 10*C* and 11*A*) is at site(s) different from Tyr^{1173}. This result and data of Figs. 8–10 suggest that activated Src obtained in response to ouabain interacts with the EGFR causing receptor tyrosine phosphorylation at sites other than the major autophosphorylation site of this receptor.

DISCUSSION

Ouabain and related cardiac glycosides inhibit the plasma membrane Na^+/K^+-ATPase by binding to the extracellular domains of the enzyme (1, 2). In recent studies (6–10) we have demonstrated that the Na^+/K^+-ATPase of the cardiac myocyte acts as a signal transducer by relaying the message of its interaction with extracellular ouabain to the nucleus through multiple interconnected gene regulatory pathways, some of which we have identified. In the studies reported here, we have explored the nature of the events proximal to the interaction of the Na^+/K^+-ATPase with the extracellular stimulus. Focusing on the previously demonstrated activation of the Ras/Raf/MEK/MAPK cascade by ouabain (9), our findings provide the outline of a pathway that connects the Na^+/K^+-ATPase to this cascade.

Na^+/K^+-ATPase Is Linked to the Ras/MAPK Cascade through Src and EGFR—Our data suggest the following conclusions, which are summarized in Fig. 12.

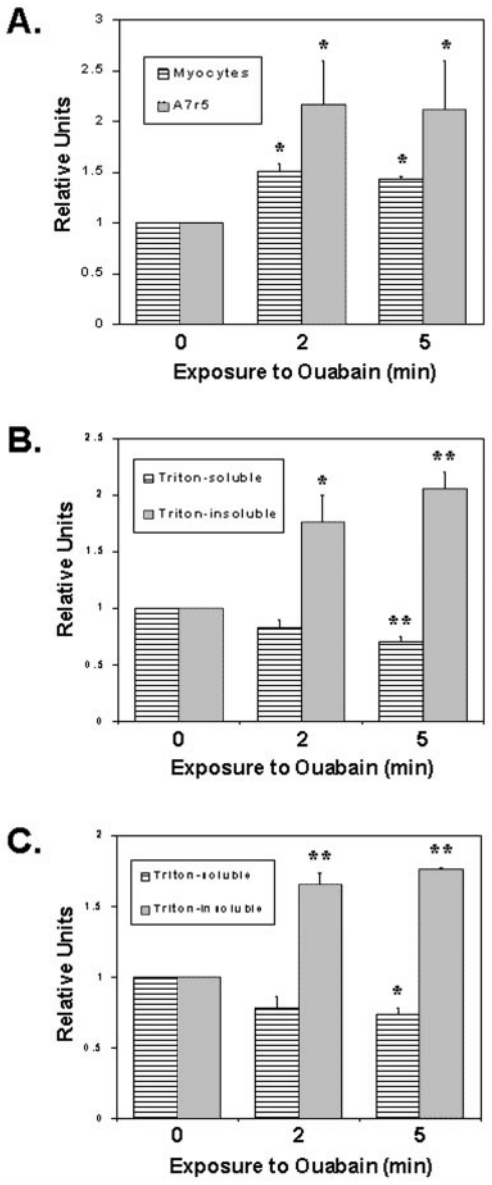

FIG. 9. **Effects of ouabain on Src kinase activity and translocation from Triton-soluble fraction to Triton-insoluble fraction in cardiac myocytes and A7r5 cells.** *A*, cells were exposed to 100 μM ouabain for different times, lysed, immunoprecipitated with anti-Src antibody, and assayed for Src kinase activity as described under "Experimental Procedures." It was established experimentally, using Western blots, that total Src protein immunoprecipitated from ouabain-treated cells did not differ significantly from that of the control cells (treated = 1.04 ± 0.05 relative to control value of one; $p > 0.05$). *B* and *C*, myocytes (*B*) and A7r5 cells (*C*) were exposed to 100 μM ouabain for the indicated times and assayed for Src translocation as described under "Experimental Procedures." The indicated values are mean ± S.E. of four experiments. *, $p < 0.05$; **, $p < 0.01$.

Significant increases in tyrosine phosphorylation of a number of cellular proteins are detected as early as 30 s after exposure of the intact cells to ouabain, and some of these increases precede the tyrosine phosphorylation and activation of p42/44 MAPK (Figs. 2 and 3, and "Results"). The fact that ouabain-induced protein tyrosine phosphorylation and MAPK activation are prevented by two protein-tyrosine kinase inhibitors (Figs. 1 and 8) and that one of these (herbimycin A) exhibits some selectivity for Src family kinases (33) suggest that Src is involved. This was confirmed by the direct demonstration of ouabain-induced activation of Src kinase and ouabain-induced Src translocation (Fig. 9). In turn, the activated

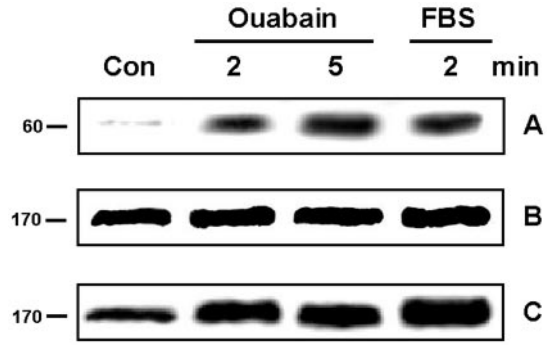

FIG. 10. **Effects of ouabain on Src binding to EGFR and on the tyrosine phosphorylation of EGFR.** A7r5 cells were treated with 100 μM ouabain or FBS (10%) for the indicated times. Cell lysates from control and treated cells were immunoprecipitated with a polyclonal anti-EGFR antibody as described under "Experimental Procedures." Immunoprecipitates were separated on SDS-PAGE and immunoblotted with anti-Src antibody (*A*), anti-EGFR antibody (*B*), and anti-phosphotyrosine antibody (*C*).

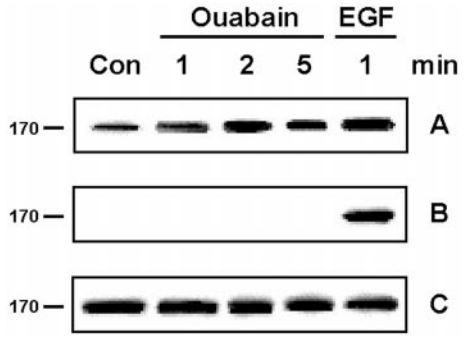

FIG. 11. **Effects of ouabain and EGF on tyrosine phosphorylation of Tyr^{1173} of the EGFR.** A7r5 cells were exposed to 100 μM ouabain or 10 nM EGF for the indicated times. Cell lysates from control and treated cells were separated on 10% SDS-PAGE and immunoblotted with anti-phosphotyrosine antibody (*A*), anti-EGFR antibody (*C*), and anti-phosphotyrosine (Tyr^{1173}) EGFR antibody (*B*).

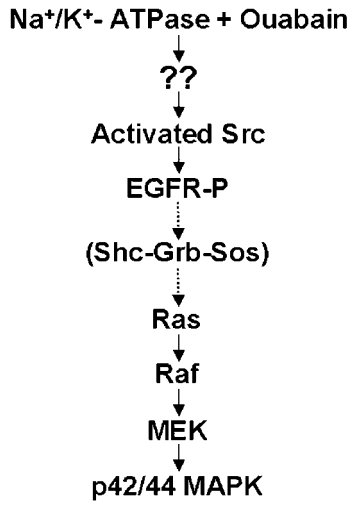

FIG. 12. **Schematic representation showing pathways that link the Na/K-ATPase to the Ras/MAPK cascade.**

Src interacts with EGFR and phosphorylates it on site(s) different from the receptor's major autophosphorylation site (Figs. 10 and 11). Because in cells other than cardiac myocytes such Src-mediated transphosphorylation of the EGFR is capable of providing the scaffolding for the phosphorylation of docking proteins such as Shc, followed by the recruiting of growth factor receptor-bound protein 2, son of sevenless, and Ras to the

plasma membrane (12, 13, 30), it is reasonable to assume that this course of events transpires for the ouabain-induced activation of Ras and the Ras/MAPK cascade (Fig. 12). Studies to test the assumed participation of the adaptor proteins in this scheme are in progress.

Although our data clearly support the existence of the pathway in Fig. 12, it is important to emphasize that additional mechanisms for the linkage of Na^+/K^+-ATPase to the Ras/MAPK cascade are also possible. In cells other than cardiac myocytes, Ras/MAPK activation that is linked to G protein-coupled receptors has been shown to involve not only Src and EGFR but also the nonreceptor tyrosine kinases of the focal adhesion complex (12, 32). The possibility of the ouabain-induced activation of such kinases remains open. Also to be explored is the possibility that ouabain-induced Src activation may transactivate receptor tyrosine kinases other than EGFR.

Increased Protein Tyrosine Phosphorylation Is a Common Feature of Ouabain Binding to the Na^+/K^+-ATPases of Different Cell Types—Our previous studies (6–10) leading to the present work were focused on ouabain-induced hypertrophy in cardiac myocytes, and the role of the Na^+/K^+-ATPase as a signal transducer in this process. We have pointed out previously (6) that the gene regulatory actions of ouabain (*e.g.* the induction of c-*fos*) occur with distinctly different characteristics in myocytes and other cell types. The results presented here (Figs. 6–11) and in the accompanying paper (34) show, however, that the signaling events close to ouabain's interaction with the Na^+/K^+-ATPase may indeed be similar in various cell types. This suggests that the cellular specificity of the ouabain's gene regulatory effects are due to the divergence of the pathways downstream of the shared proximal segments.

Our data on the dose dependence of ouabain-induced tyrosine phosphorylation in different cells also help resolve another important issue. Although the assumption that any effect of a reasonable concentration of ouabain on a mammalian cell must be due to interaction with the Na^+/K^+-ATPase is supported by a large body of cumulative evidence on the specificity of this interaction (1, 2), there is always the possibility that a newly observed effect of ouabain may be due to its interaction with a previously unknown receptor. The correlation between the known differences in the sensitivities of the predominant Na^+/K^+-ATPase isoforms of the different cell types and the different ouabain concentrations required to stimulate tyrosine phosphorylation in these cells (Figs. 4 and 7; and "Results") clearly indicate that the signal-transducing effects of ouabain are indeed through the Na^+/K^+-ATPase. This is also reinforced by the similar effects of ouabain and low extracellular K^+ on protein tyrosine phosphorylation (Fig. 5).

How Does Inhibition of the Na^+/K^+-ATPase by Ouabain Cause Src Activation?—Because Src kinase activity may be regulated by a variety of different mechanisms (28), the simple answer is that the mechanism of Src activation resulting from the inhibition of the Na^+/K^+-ATPase remains to be established. Consideration of some broad alternatives, however, is appropriate. In some cells other than cardiac myocytes, an increased intracellular concentration of free Ca^{2+} has been shown to be sufficient for the activation of Src (17). Because a significant rise in intracellular Ca^{2+} is the well established consequence of the partial inhibition of the cardiac Na^+/K^+-ATPase (3), it is reasonable to begin by asking if this rise may account for Src activation. In fact, as we show in the accompanying paper (34), the altered intracellular ionic concentrations resulting from the partial inhibition of the ion-pumping function of the Na^+/K^+-ATPase are not likely to be involved in increased tyrosine phosphorylation leading to Ras-dependent generation of intracellular reactive oxygen species. This suggests that the primary signaling events emanating from the Na^+/K^+-ATPase in response to ouabain are altered protein-protein interactions; *i.e.* changes in the interactions of the Na^+/K^+-ATPase subunits with neighboring proteins at or within the plasma membrane. In this context, Src activation must be due to altered interaction(s) of the Na^+/K^+-ATPase with Src, either directly or through intermediary proteins. Although our initial attempts failed to demonstrate the existence of a Na^+/K^+-ATPase·Src·EGFR complex ("Results"), this is not sufficient to rule out the possibility of the interaction of Src, directly or otherwise, with one or more of the different conformational states of the Na^+/K^+-ATPase. Studies aimed at the resolution of these issues are in progress.

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