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Src-mediated Inter-receptor Cross-talk between the Na^+/K^+-ATPase and the Epidermal Growth Factor Receptor Relays the Signal from Ouabain to Mitogen-activated Protein Kinases*

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Binding of ouabain to Na^+/K^+-ATPase activates tyrosine phosphorylation of the epidermal growth factor receptor (EGFR), Src, and p42/44 mitogen-activated protein kinases (MAPKs) in both cardiac myocytes and A7r5 cells. Here, we explored the roles of Src and the EGFR in the ouabain-invoked pathways that lead to the activation of MAPKs. Exposure of A7r5 and LLC-PK1 cells to ouabain caused a dose-dependent inhibition of Na^+/K^+-ATPase activity, which correlated well with ouabain-induced activation of Src and MAPKs in these cells. Immunoprecipitation experiments showed that ouabain stimulated Src binding to Na^+/K^+-ATPase in a dose- and time-dependent manner and increased phosphorylation of Src at Tyr^{418} but had no effect on Tyr^{529} phosphorylation. Ouabain failed to activate MAPKs in A7r5 cells that were pretreated with the Src inhibitor PP2 and in SYF cells in which Src family kinases are knocked out. Preincubation with AG1478, but not AG1295, also blocked the effects of ouabain on p42/44 MAPKs in A7r5 cells. Significantly, both herbimycin A and PP2 abrogated ouabain-induced but not epidermal growth factor-induced Src binding to the EGFR and the subsequent EGFR tyrosine phosphorylation. Ouabain also failed to affect tyrosine phosphorylation of the EGFR in SYF cells. In addition, unlike epidermal growth factor, ouabain did not increase EGFR autophosphorylation at Tyr^{1173}. These findings clearly indicate that ouabain transactivates the EGFR by activation of Src and stimulation of Src binding to the EGFR. Furthermore, we found that the transactivated EGFR was capable of recruiting and phosphorylating the adaptor protein Shc. This resulted in increased binding of another adaptor protein Grb2 to the Src-EGFR complex and the subsequent activation of Ras and MAPKs. Taken together, these new findings suggest that Src mediates the inter-receptor cross-talk between Na^+/K^+-ATPase and the EGFR to transduce the signals from ouabain to the Ras/MAPK cascade.

Na^+/K^+-ATPase, or the sodium pump, is a ubiquitous transmembrane enzyme that has long been ascribed the function of transporting Na^+ and K^+ across the plasma membrane (1–3). By specific binding to the extracellular surface of the α-subunit of Na^+/K^+-ATPase, cardiotonic steroids such as ouabain inhibit the ion pumping function of the enzyme. In recent years, we have demonstrated that the binding of ouabain to cardiac Na^+/K^+-ATPase can also activate multiple signal transduction pathways including activation of Ras and p42/44 MAPKs[1] and increased production of reactive oxygen species (4–8). Interestingly, the activation of some of the signal transduction pathways by ouabain is independent of the ouabain-induced changes in intracellular ion concentrations as well as the contractility of cardiac myocytes (9, 10). Significantly, we have recently shown that the classic effects of ouabain on intracellular calcium also depend on the signal transducing function of Na^+/K^+-ATPase (11). Inhibition of either protein tyrosine phosphorylation or Ras/p42/44 MAPKs diminishes ouabain-induced increases in intracellular calcium. Furthermore, interplays among different pathways (*e.g.* MAPKs, calcium, and reactive oxygen species) in cardiac myocytes eventually lead to hypertrophic growth and changes in the expression of multiple growth-related genes (4).

Because there is sufficient evidence that activation of the Ras/MAPKs is important for regulating cell function and growth, we have recently initiated studies to explore the upstream signaling events that emanate from the binding of ouabain to Na^+/K^+-ATPase. Traditionally, binding of the growth factor to its receptor results in receptor dimerization and autophosphorylation (12, 13). The tyrosine-phosphorylated receptor can then serve as a scaffold to recruit Src and adaptor proteins, resulting in activation of the Ras/MAPK cascade. More recently, the EGFR and Src have been identified as critical elements that relay the signals from G protein-coupled receptors, cytokines, and cellular stress to a variety of cellular responses, which include the activation of MAPKs (14–19). Our previous work demonstrated that ouabain caused a rapid activation of Src and stimulated tyrosine phosphorylation of the EGFR in both cardiac myocytes and A7r5 cells (9). Furthermore, inhibition of tyrosine kinases by either genistein or herbimycin A antagonized the effects of ouabain on MAPKs. These findings led us to postulate that the activation of Src by ouabain was sufficient to transactivate the EGFR, resulting in the recruitment of Shc and Grb2 to the receptor and subsequent activation of the Ras/MAPK cascade (9). The present studies were performed to test this hypothesis and delineate the molecular mechanisms of ouabain-induced activation of Src and transactivation of the EGFR.

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[1] The abbreviations used are: MAPK, mitogen-activated protein kinase; EGF, epidermal growth factor; EGFR, EGF receptor; PDGF, platelet-derived growth factor; PDGFR, PDGF receptor; RIPA, radioimmune precipitation buffer; SH, Src homology.

EXPERIMENTAL PROCEDURES

Materials—Chemicals of the highest purity were used. Herbimycin A, PP2, tyrphostin AG1295, and tyrphostin AG1478 were obtained from Calbiochem. Sodium binding benzofuran isophthalate acetoxymethyl ester was from Molecular Probes (Eugene, OR). [γ-^{32}P]ATP was obtained from PerkinElmer Life Sciences. The following antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA): monoclonal anti-phosphotyrosine antibody (PY99), polyclonal anti-MAPK antibody, monoclonal anti-phospho-MAPK antibody, and goat anti-rabbit and goat anti-mouse secondary antibodies. The following antibodies and kits were obtained from Upstate Biotechnology (Lake Placid, NY): monoclonal anti-Src (clone GD11), anti-Ras (clone RAS10), anti-Grb2, and anti-phospho-EGFR (Tyr^{1173}) (clone 9H2) antibodies, polyclonal anti-Shc and anti-EGFR antibodies, Src kinase activity and Ras activation kits, and protein G-agarose. The polyclonal anti-Src (pY^{418}) and anti-Src (pY^{529}) antibodies were from BIOSOURCE. The monoclonal anti-α_1 antibody (α6F) was obtained from the Developmental Studies Hybridoma Bank at the University of Iowa (Iowa City, IA). The Optitran nitrocellulose membranes used for Western blotting were obtained from Schleicher and Schuell.

Cell Preparation and Culture—Rat A7r5, pig LLC-PK1, and mouse SYF and SYF + c-Src cells were obtained from American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). When cell cultures reached about 80% confluence, A7r5 and LLC-PK1 cells were serum-starved for 24 h and used for the experiments, whereas SYF and SYF + c-Src cells were cultured in medium containing 0.5% fetal bovine serum for 24 h before being used for the experiments.

Measurement of Protein Tyrosine Phosphorylation and p42/44 MAPK Activity—Immunoblotting was performed to identify increases in tyrosine phosphorylation and activation of p42/44 MAPK using the antibodies described under "Materials" (9). Dilutions of these antibodies were done as recommended by the manufacturer. After 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 RIPA containing 1% Nonidet P-40, 0.25% 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 the supernatants were used for Western blot analysis. Samples were separated by SDS-PAGE (60 μg/lane) and transferred to an Optitran membrane as described previously (9). The membranes were then probed with an anti-phosphotyrosine monoclonal antibody or an anti-phospho-MAPK monoclonal antibody. The anti-phospho-MAPK monoclonal 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 reported previously (9). Autoradiograms were scanned with a Bio-Rad densitometer to quantitate MAPK signals as described previously (9).

Assay for Src Kinase Activity—To assay Src kinase activity, cells were lysed in RIPA 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 Src monoclonal antibody and protein G-agarose. The immunoprecipitate was washed twice with RIPA and twice with ice-cold phosphate-buffered saline. Src kinase activity was assayed as described previously (9).

Assay for EGFR Tyrosine Phosphorylation and Src, Shc, and Grb2 Association—Cell lysates made in RIPA were immunoprecipitated using a polyclonal anti-EGFR antibody that has 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 whether EGFR binds to Src, Shc, and Grb2, the same blots were stripped and reprobed with the monoclonal anti-Src antibody, the polyclonal anti-Shc antibody, and the monoclonal anti-Grb2 antibody. To determine whether the EGFR is autophosphorylated at its major phosphorylation site (Tyr^{1173}), the lysates made in RIPA were immunoprecipitated using a polyclonal anti-EGFR antibody. The immunoprecipitate was dissolved in SDS sample buffer, separated by 10% SDS-PAGE, and blotted with a monoclonal anti-phospho-EGFR antibody raised against peptide NA-E[pY]LRV that corresponds to the protein sequence around Tyr^{1173} of the EGFR.

Assay for Src Binding to Na^+/K^+-ATPase—RIPA cell lysates were immunoprecipitated with either a polyclonal anti-α_1 antibody or a monoclonal anti-Src antibody as described in the preceding paragraph. Immunoprecipitates were then separated by SDS-PAGE and immunoblotted for α_1 and Src.

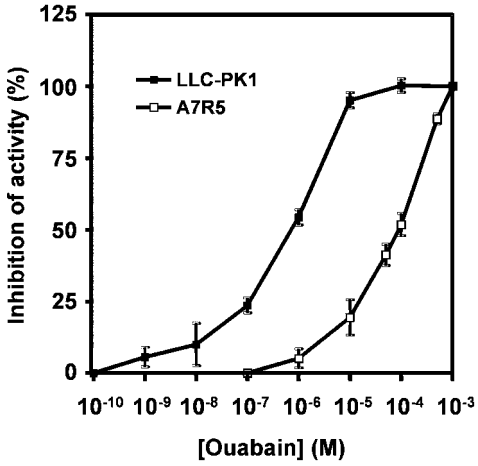

FIG. 1. **Dose-dependent effect of ouabain on Na^+/K^+-ATPase activity in both A7r5 and LLC-PK1 cells.** A7r5 and LLC-PK1 cells were treated with various concentrations of ouabain, and Na^+/K^+-ATPase activity was measured as described under "Experimental Procedures." The values are the mean ± S.E. of three independent experiments.

Assay for Ras Activation—To assay for Ras activation, cells were collected in a Mg^{2+}-containing lysis buffer (MLB) containing 150 mM NaCl, 1% Igepal CA-630, 0.25% sodium deoxycholate, 10% glycerol, 25 mM NaF, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM sodium orthovanadate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 25 mM HEPES (pH 7.5). Active Ras was then affinity-precipitated by adding 20 μl of the Raf-1 Ras binding domain-agarose conjugate to the cell lysates and gently rocking them for 30 min at 4 °C. The agarose beads were washed three times with MLB, dissolved in 50 μl of 2$\times$ SDS sample buffer, and boiled for 5 min. The samples were separated by 10% SDS-PAGE, transferred to a supported nitrocellulose membrane, and blotted with a monoclonal anti-Ras antibody.

Assay for Na^+/K^+-ATPase Activity and Intracellular Na^+ Concentration—Whole cell total Na^+/K^+-ATPase activity was assayed in the presence of alamethicin as described previously (20). Briefly, cells were collected from the cultures and treated with alamethicin at a concentration of 0.1 mg/mg protein. Na^+/K^+-ATPase activity was measured by the determination of the initial rate of release of P_i from ATP. The reaction was carried out in a final volume of 1 ml of reaction mixture containing 100 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$, 1 mM EGTA, 5 mM NaN_3, and 2 mM [γ-^{32}P]ATP. To determine the ouabain concentration curves, whole cell lysates were preincubated with different concentrations of ouabain for 15 min. The reaction was started by the addition of an aliquot of cell lysate (50 μg). Intracellular Na^+ concentration was measured using sodium binding benzofuran isophthalate as a probe as described previously (10).

Analysis of Data—Data are given as the mean ± SE. Statistical analysis was performed using 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.

RESULTS

The Dose-dependent Effects of Ouabain on Src and MAPKs Correlate with the Ouabain Sensitivities of the Cells—We have shown previously that ouabain activates Src and increases tyrosine phosphorylation of the EGFR in several cell types (9). Although it is well established that Na^+/K^+-ATPase is the specific receptor for cardiotonic steroids such as ouabain, it is always possible that some of the novel effects of ouabain may be due to its binding to other cellular proteins, especially at higher ouabain concentrations. To address this issue, we compared the dose-dependent effects of ouabain on Na^+/K^+-ATPase activity and MAPK activation. When Na^+/K^+-ATPase activity was measured in A7r5 and LLC-PK1 cells as a function of ouabain concentration, it is clear that LLC-PK1 cells express a highly ouabain-sensitive isoform of Na^+/K^+-ATPase (Fig. 1). The en-

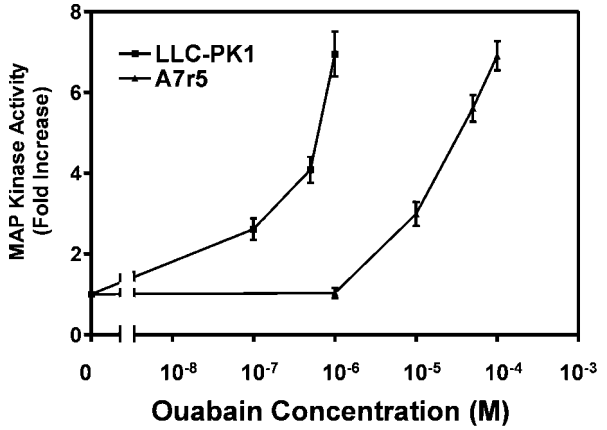

FIG. 2. **Dose-dependent effects of ouabain on MAPKs in both A7r5 and LLC-PK1 cells.** Cells were treated with different concentrations of ouabain for 5 min and assayed for MAPKs as described under "Experimental Procedures." The values are the mean ± S.E. of three independent experiments.

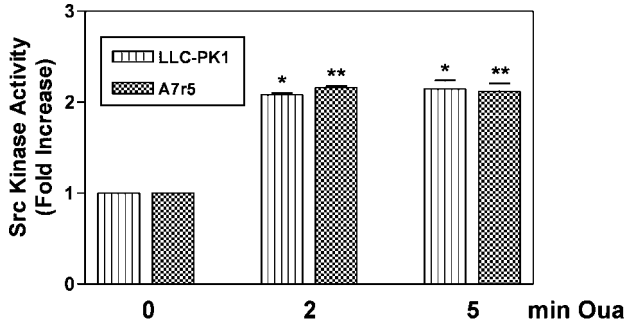

FIG. 3. **Effect of ouabain on Src kinase activity in both A7r5 and LLC-PK1 cells.** A7r5 and LLC-PK1 cells were exposed to 100 and 1 μM ouabain, respectively, for the indicated times. The cells were lysed, immunoprecipitated with a monoclonal anti-Src antibody, and assayed for Src kinase activity as described under "Experimental Procedures." The values are the mean ± S.E. of at least three independent experiments. Data are expressed relative to a control value of 1. *, $p < 0.05$; **, $p < 0.01$.

zyme was inhibited 50% in LLC-PK1 cells when the cells were exposed to about 1 μM ouabain. In contrast, 100 μM ouabain was required to cause the same degree of inhibition of Na^+/K^+-ATPase activity in A7r5 cells. Interestingly, when Src and MAPKs were measured in these cells as a function of ouabain concentration, we found that the effects of ouabain on both MAPKs and Src correlated well with the inhibition curves of ouabain on Na^+/K^+-ATPase in these cells (Figs. 2 and 3). To ensure that ouabain-induced activation of MAPKs and Src is not a result of significant changes in intracellular Na^+ or depolarization of the membrane potential due to inhibition of the ion pumping function of Na^+/K^+-ATPase, we measured the effects of ouabain on intracellular Na^+ as a function of time in A7r5 cells loaded with sodium binding benzofuran isophthalate. Consistent with our prior studies (10), exposure of A7r5 cells to 100 μM ouabain for 20 min did not change the intracellular Na^+ concentration (17.1 ± 1.2 *versus* 17.2 ± 1.3 mM). Therefore, it is unlikely that the effects of ouabain on MAPKs are due to changes in either intracellular Na^+ or membrane potential. This notion is further supported by the following observations. First, although the addition of monensin caused a large increase in intracellular Na^+, it failed to activate MAPKs (99.7 ± 9.6% of control; $n = 3$). Second, increasing extracellular K^+ from 5 to 20 mM, which should cause partial depolarization of the plasma membrane, also showed no significant effect on MAPKs (114.8 ± 5.4% of control; $n = 3$).

Both Src and EGFR Are Required for Ouabain-induced Activation of MAPK—We showed that inhibition of protein tyrosine kinases by a nonspecific tyrosine kinase inhibitor (genistein) or a relatively selective Src inhibitor (herbimycin A) abolished ouabain-induced stimulation of p42/44 MAPKs (9). To obtain additional evidence that Src is involved in ouabain-induced activation of MAPKs, A7r5 cells were pretreated with another Src-specific inhibitor, PP2, and then exposed to 100 μM ouabain and assayed for MAPK activation. As depicted in Fig. 4, inhibition of Src completely abrogated ouabain-induced but not EGF-induced activation of MAPKs. The data supported our previous proposition that Src plays a key role in ouabain-activated signaling pathways. They also indicate that ouabain and EGF signal differently in A7r5 cells. Because both PP2 and herbimycin A might have nonspecific effects on other kinases, we employed both SYF and SYF + c-Src cells in the experiments of Fig. 5 to further test the hypothesis that Src relays the signal from ouabain to MAPKs. The SYF cells are derived from mouse embryos harboring functional null mutations in both alleles of the Src family kinases Src, Yes, and Fyn. The SYF + c-Src cells are the stable transfectants of SYF cells that express c-Src. As shown in Fig. 5, ouabain activated p42/44 MAPKs in a dose-dependent manner in the SYF + c-Src cells. However, under the same experimental conditions, ouabain failed to stimulate MAPKs in the SYF cells. Clearly, activation of Src is critical for ouabain to stimulate MAPKs.

Because receptor tyrosine kinases are involved in transmitting signals from H_2O_2 and angiotensin II to MAPKs, the effects of either AG1478 or AG1295 on ouabain-induced activation of MAPKs were studied in the experiments of Fig. 6. Both AG1478 and AG1295 belong to the tyrphostin family of receptor tyrosine kinase inhibitors. They are ATP analogues and specific toward the ATP binding sites of receptor tyrosine kinases. The selectivity and dose-dependent effects of these inhibitors on MAPK activation induced by either EGF or PDGF had been assessed previously in rat vascular smooth muscle cells (25). These studies showed that AG1478 completely blocked the effects of EGF (50 ng/ml) on MAPKs at 250 nM, whereas AG1295 abolished the effects of PDGF (30 ng/ml) at 25 μM. Control experiments showed that both AG1478 (250 nM) and AG1295 (25 μM) had no effect on cell morphology and viability (assessed by trypan blue exclusion) when A7r5 cells were exposed to them for 1 h. Therefore, A7r5 cells were exposed to either 250 nM AG1478 or 25 μM AG1295 and then treated with 100 μM ouabain. Interestingly, whereas AG1478 blocked the effects of both EGF and ouabain on MAPKs (Fig. 6), AG1295 inhibited the effects of PDGF but not ouabain on MAPKs. To ensure that the effects of AG1478 on ouabain-induced activation of MAPKs are specifically mediated through inhibition of the EGFR, the effects of 10 nM AG1478 on MAPK activation were also assessed. Previous studies on rat vascular smooth muscle cells indicated that 10 nM AG1478 caused about 50% inhibition of EGF-induced MAPK activation (25). We found that AG1478 at the same concentration inhibited about 37% of both EGF- and ouabain-induced activation of MAPKs. Taken together, the above findings indicate that ouabain signals differently than both EGF and PDGF and demonstrate an involvement of the EGFR, but not the PDGFR, in ouabain-induced activation of MAPKs.

Ouabain Increases Src Phosphorylation at Tyr^{418} and Src Binding to the Na^+/K^+-ATPase—The above findings firmly established the role of Src and the EGFR in ouabain-induced activation of MAPKs. To understand how ouabain transduces signals through Src and the EGFR, we first determined the effects of ouabain on Src tyrosine phosphorylation. It is well established that Src kinase activity is regulated by the phosphorylation of Tyr^{418} and Tyr^{529}. Either decreases in phospho-

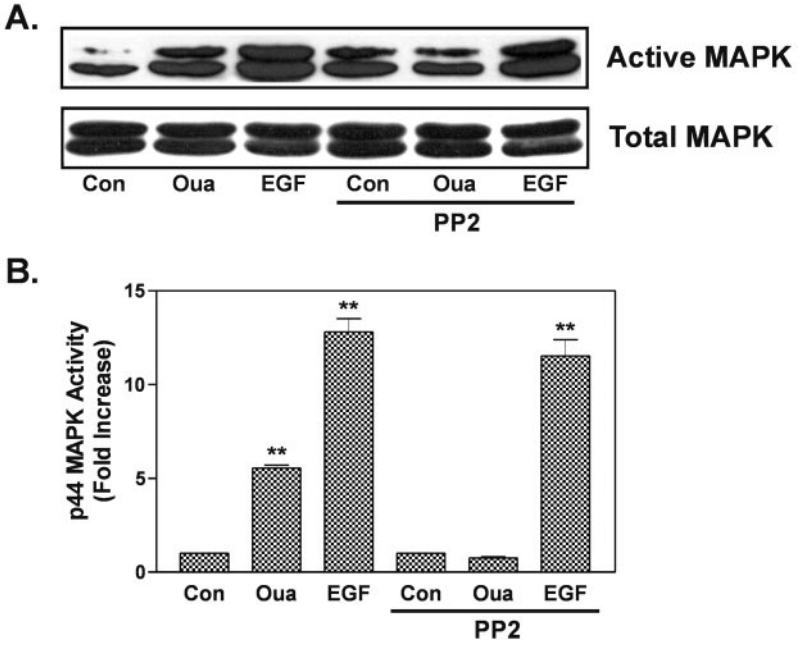

FIG. 4. **Effects of PP2 on ouabain- and EGF-induced MAPK activation in A7r5 cells.** A7r5 cells were preincubated with 1.0 μM PP2 for 15 min and then exposed to either 100 μM ouabain for 5 min or 50 ng/ml EGF for 2 min. MAPK activity was assayed as described in the Fig. 2 legend. Control experiments showed that maximal stimulation of MAPKs was obtained when the cells were exposed to ouabain for 5 min or EGF for 2 min. *A*, a representative Western blot showing that PP2 blocks ouabain-induced but not EGF-induced MAPK activation in A7r5 cells. *B*, quantitation of the effects of PP2 on p44 MAPK measured in three independent experiments. Data are expressed relative to a control value of one. **, $p < 0.01$.

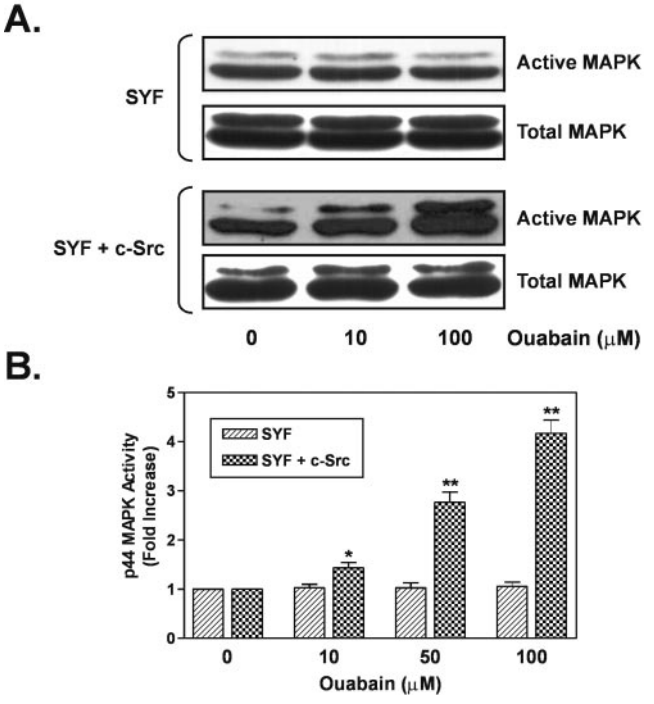

FIG. 5. **Dose-dependent effects of ouabain on MAPKs in SYF and SYF + c-Src cells.** Cells were treated with different concentrations of ouabain for 5 min and assayed for MAPKs as described in the Fig. 2 legend. Control experiments showed that ouabain-induced activation of MAPKs reached maximal levels after the cells were exposed to ouabain for 5 min. *A*, a representative Western blot is shown. *B*, quantitation of the effects of ouabain on p44 MAPK measured in three independent experiments. Data are expressed relative to a control value of 1. *, $p < 0.05$; **, $p < 0.01$.

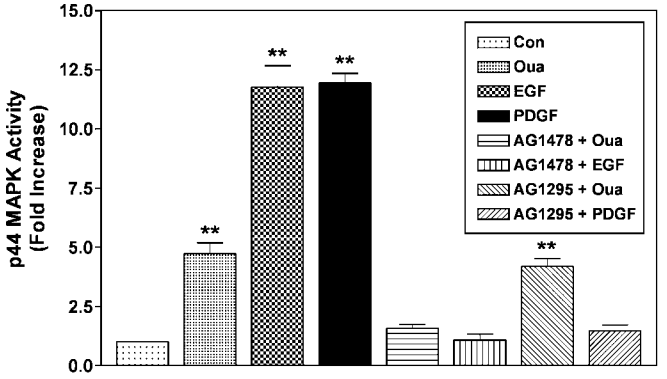

FIG. 6. **Effects of AG1478 and AG1295 on ouabain-, EGF-, and PDGF-induced MAPK activation in A7r5 cells.** A7r5 cells were preincubated with either 250 nM AG1478 or 25 μM AG1295 for 10 min and then exposed to different stimuli as indicated. MAPK activity was assayed as described in the Fig. 2 legend. The values are the mean ± S.E. of four independent experiments. Data are expressed relative to a control value of 1. **, $p < 0.01$.

rylation of Tyr^{529} by activation of a phosphotyrosine phosphatase or increases in phosphorylation of Tyr^{418} by binding of Src to a regulatory protein stimulates Src kinase activity. As shown in Fig. 7, ouabain caused a significant increase in Tyr^{418} phosphorylation but had no effect on Tyr^{529} phosphorylation. Because there is evidence that binding of a regulatory protein to either the SH2, SH3, or kinase domain of Src can activate kinase activity by stimulation of Tyr^{418} phosphorylation, we tested whether ouabain regulates Src by increasing its interaction with Na^+/K^+-ATPase. In the experiments of Fig. 8, we immunoprecipitated either α_1 or Src from both control and ouabain-treated LLC-PK1 cells. LLC-PK1 cells were chosen for the immunoprecipitations because these cells express high levels of ouabain-sensitive Na^+/K^+-ATPase. Initial experiments with A7r5 cells proved to be difficult because each immunoprecipitation needed more than 2 mg of cell lysates to give a detectable signal of α_1 by Western blot. As shown in Figs. 8 and 9, ouabain increased Src binding to Na^+/K^+-ATPase in a dose- and time-dependent manner. The maximal effects of ouabain on Src binding were observed after the cells were incubated with ouabain for 2–5 min and decreased thereafter.

Inhibition of Src Blocks Ouabain-induced Src Binding to and Tyrosine Phosphorylation of the EGFR—Because Src can mediate inter-receptor communications, the above findings prompted us to hypothesize that the Src-mediated inter-receptor cross-talk between Na^+/K^+-ATPase and the EGFR is essential for ouabain-induced activation of MAPKs. To test this hypothesis, we assessed the role of Src in ouabain-induced transactivation of the EGFR. In the experiments of Fig. 10, A7r5 cells were pretreated with herbimycin A for 30 min and then exposed to either 100 μM ouabain or 50 ng/ml EGF for 5

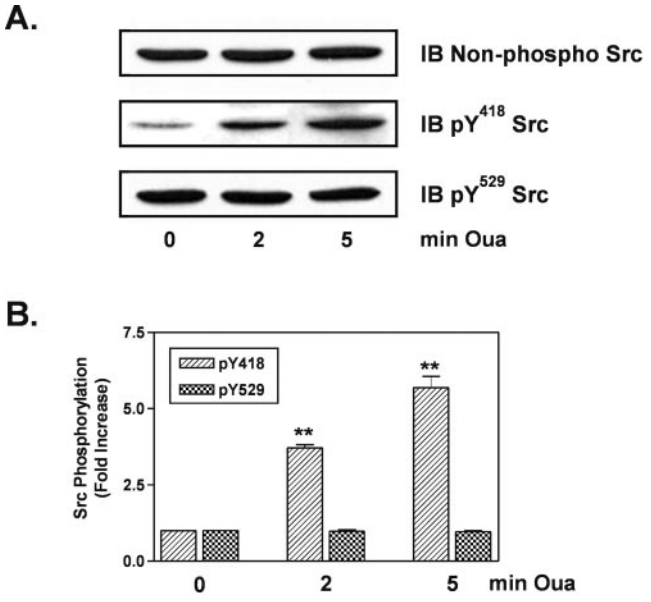

FIG. 7. **Effects of ouabain on Src tyrosine phosphorylation.** Cells were treated with 100 μM ouabain for different times and assayed for Src tyrosine phosphorylation by Western blot using either Tyr^{418}- or Tyr^{529}-specific polyclonal antibodies. *A*, a representative Western blot is shown. *B*, quantitation data from at least three independent experiments. Data are expressed relative to a control value of 1. **, $p < 0.01$.

min. After cells lysates were cleared by centrifugation and immunoprecipitated with an anti-EGFR antibody, immunoprecipitates were resolved by SDS-PAGE and probed for protein tyrosine phosphorylation and Src. As reported previously (9), ouabain increased Src binding to the EGFR and stimulated tyrosine phosphorylation of the receptor (Fig. 10). EGF also increased tyrosine phosphorylation of the receptor and increased Src binding to the receptor. However, preincubation with herbimycin A diminished the above-mentioned effects of ouabain, but not EGF, on the EGFR (Fig. 10). In addition, preincubation of A7r5 cells with another Src inhibitor, PP2, also blocked Src binding to the EGFR and increased tyrosine phosphorylation of the receptor in response to ouabain (data not shown). Consistent with these findings and those of Fig. 5, we found that ouabain stimulated tyrosine phosphorylation of the EGFR in SYF + c-Src cells, but not in SYF cells (Fig. 11). In contrast, EGF stimulated the receptor in both cells (Fig. 11). Taken together, these findings support the notion that binding of ouabain to Na^+/K^+-ATPase activates Src kinase, which in turn increases its association with the EGFR and subsequently transactivates the receptor.

After binding to its cognate ligand, the EGFR monomers dimerize, resulting in activation of tyrosine kinase activity and trans-(auto)-phosphorylation of the receptor at the multiple tyrosine residues in the carboxyl-terminal tail. We showed previously by Western blot analysis of cell lysates that ouabain increased tyrosine phosphorylation of the EGFR at sites other than the major autophosphorylation site (Tyr^{1173}). Because the experiments in Fig. 6 indicated that EGFR tyrosine kinase activity was required for ouabain-induced activation of MAPKs, we reasoned that Western blot analysis of the cell lysates might not be sensitive enough to detect modest increases in phosphorylation of Tyr^{1173}. Therefore, we re-examined the issue of whether ouabain stimulates tyrosine phosphorylation of the EGFR at Tyr^{1173} by first immunoprecipitating the EGFR from the cell lysates and then probing for Tyr^{1173} phosphorylation. As depicted in Fig. 12, ouabain had no effect on the phosphorylation of Tyr^{1173}, whereas EGF did (Fig. 12).

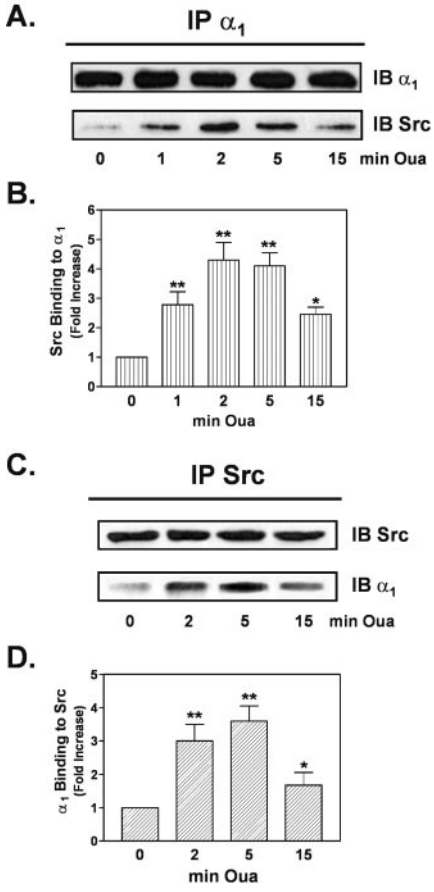

FIG. 8. **Time-dependent effects of ouabain on Src binding to Na^+/K^+-ATPase.** *A*, LLC-PK1 cells were treated with 1 μM ouabain for the times indicated. The cells were then lysed in RIPA, immunoprecipitated with an anti-α_1 antibody, and assayed for Src binding by Western blot using an anti-Src antibody. A representative immunoblot is shown. *B*, quantitation data from four independent experiments. Data are expressed relative to a control value of 1. *C*, LLC-PK1 cells were treated as described in *A*. The cells were then lysed in RIPA, immunoprecipitated with an anti-Src antibody, and assayed for α_1 binding by Western blot using the α6F antibody. A representative immunoblot is shown. *D*, quantitation data from four independent experiments. Data are expressed relative to a control value of 1. *, $p < 0.05$; **, $p < 0.01$.

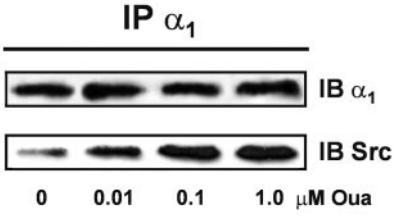

FIG. 9. **Dose-dependent effects of ouabain on Src binding to the Na^+/K^+-ATPase.** LLC-PK1 cells were treated with various concentrations of ouabain for 5 min. The cells were then lysed in RIPA, immunoprecipitated with an anti-α_1 antibody, and assayed for Src binding by Western blot using an anti-Src antibody. A representative immunoblot from four independent experiments is shown.

Clearly, as reported previously (9), the ouabain-induced phosphorylation of the EGFR seen in Fig. 10 is likely at a site or sites other than Tyr^{1173}.

Activation of the EGFR Is Sufficient for the Recruitment of Adaptor Proteins and Activation of Ras in Response to Ouabain—The above experiments clearly demonstrated that ouabain-activated Src kinase results in transactivation of the EGFR in A7r5 cells. However, the question remained as to whether the transactivated EGFR is capable of recruiting adaptor proteins, leading to Ras activation in response to ouabain, as is the case

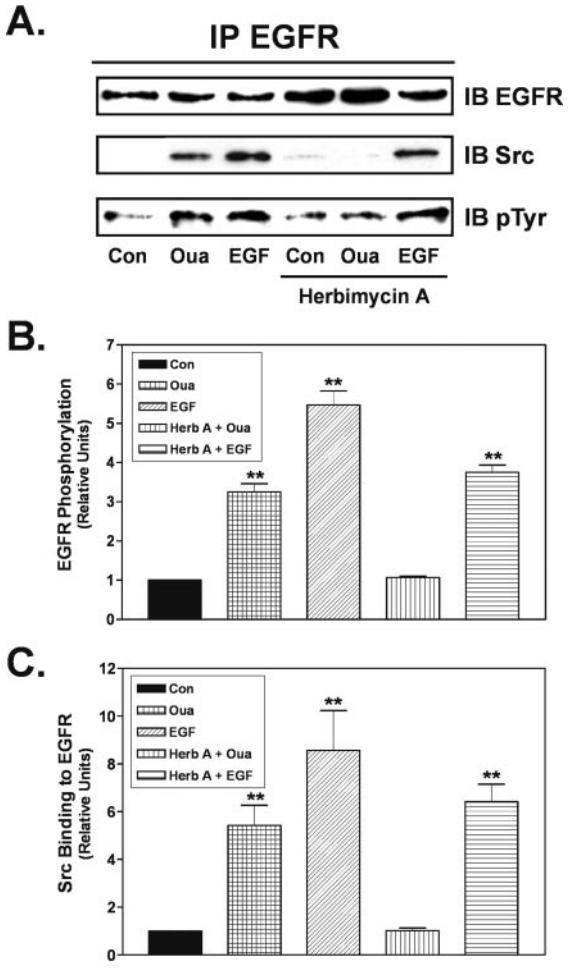

FIG. 10. **Herbimycin A blocks ouabain-induced but not EGF-induced Src binding to the EGFR and the increases in tyrosine phosphorylation of the receptor.** A7r5 cells were preincubated with 1 μM herbimycin A for 2 h and then exposed to 100 μM ouabain for 5 min or 50 ng/ml EGF for 2 min. Cell lysates from both control and treated cells were immunoprecipitated with a polyclonal anti-EGFR antibody as described under "Experimental Procedures." Immunoprecipitates were separated by SDS-PAGE and immunoblotted with either an anti-EGFR antibody, an anti-Src antibody, or an anti-phosphotyrosine antibody. *A*, a representative Western blot is shown. *B*, quantitation of the EGFR phosphorylation. The values are the mean ± S.E. of four independent experiments. *C*, quantitation of Src binding to the EGFR. The values are the mean ± S.E. of four independent experiments. Data are expressed relative to a control value of 1. **, $p < 0.01$.

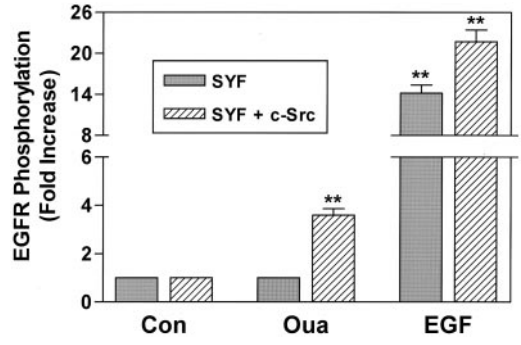

FIG. 11. **Effects of ouabain and EGF on EGFR tyrosine phosphorylation in both SYF and SYF + c-Src cells.** Both SYF and SYF + c-Src cells were exposed to either 100 μM ouabain for 5 min or 50 ng/ml EGF for 2 min. Cell lysates from control and treated cells were immunoprecipitated with a polyclonal anti-EGFR antibody as described under "Experimental Procedures." Immunoprecipitates were separated by SDS-PAGE and immunoblotted with an anti-EGFR antibody and an anti-phospho-tyrosine antibody. The values are the mean ± S.E. of three independent experiments from the SYF cells and seven independent experiments from the SYF + c-Src cells. Data are expressed relative to a control value of 1. **, $p < 0.01$.

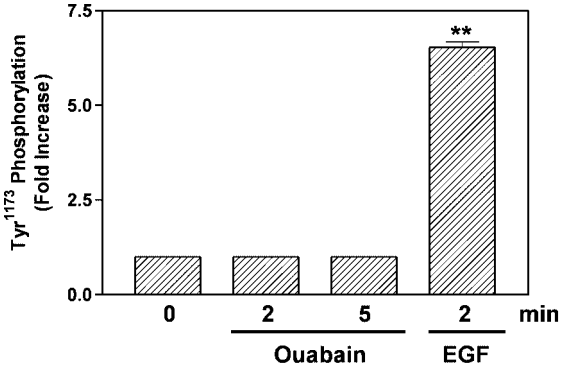

FIG. 12. **Effects of ouabain and EGF on tyrosine phosphorylation of Tyr^{1173} of the EGFR in A7r5 cells.** A7r5 cells were exposed to 100 μM ouabain or 50 ng/ml EGF for the times indicated. Cell lysates from control and treated cells were immunoprecipitated with a polyclonal anti-EGFR antibody as described under "Experimental Procedures." Immunoprecipitates were separated by SDS-PAGE and immunoblotted with an anti-EGFR antibody and an anti-phospho-tyrosine (Tyr^{1173}) antibody. The values are the mean ± S.E. of at least three independent experiments. Data are expressed relative to a control value of 1. **, $p < 0.01$.

with EGF stimulation. Therefore, we assessed whether ouabain increased the binding of Shc to the activated EGFR. As in the experiments of Fig. 10, anti-EGFR immunoprecipitates were resolved by SDS-PAGE and blotted with either an anti-Shc or an anti-phosphotyrosine antibody. EGF was again used as a positive control in these experiments. As depicted in Fig. 13*A*, both ouabain and EGF increased the association of Shc to the EGFR. Interestingly, although all three isoforms of Shc are expressed in A7r5 cells, ouabain increased the binding of $p46^{Shc}$ and $p52^{Shc}$, but not $p66^{Shc}$, to the EGFR. In addition, because binding of Shc to the activated EGFR can stimulate Shc tyrosine phosphorylation, thus serving as binding sites for other adaptor proteins such as Grb2, we measured the effects of ouabain on tyrosine phosphorylation of Shc in A7r5 cells. We found that ouabain increased tyrosine phosphorylation of $p52^{Shc}$ (Fig. 13*B*) and $p46^{Shc}$, but not $p66^{Shc}$ (data not shown), in A7r5 cells. As expected, pretreating A7r5 cells with the Src inhibitor PP2 blocked ouabain-induced increases in Shc phosphorylation (Fig. 13*B*). Interestingly, inhibition of the EGFR by AG1478 also abolished the effects of ouabain on Shc tyrosine phosphorylation. These findings indicate that the Src-transactivated EGFR is capable of recruiting and phosphorylating Shc in response to ouabain. Because binding of Grb2/Sos to the transactivated EGFR is important for the subsequent recruitment and activation of Ras, we performed the experiments of Fig. 13*A*, showing that ouabain did indeed cause a significant increase in Grb2 binding to the transactivated EGFR-Shc complex.

To further investigate the signaling cascade following the binding of Shc and Grb2 to the EGFR, the effects of ouabain on Ras and Ras binding to Raf were also measured. Active or GTP-bound Ras was first affinity-precipitated from A7r5 cell lysates using the glutathione *S*-transferase-fused Ras-binding domain of Raf after treatment with either ouabain or EGF. The amount of Raf-bound Ras, or active Ras, was then determined by using an anti-Ras antibody. In cells treated with either ouabain or EGF, there was approximately a 3.5- and 10-fold increase in Ras binding to Raf, respectively (Fig. 14). These findings indicated that ouabain activated Ras and increased

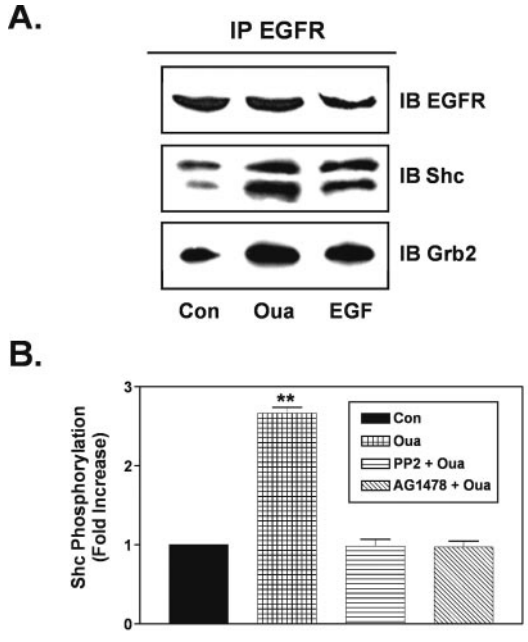

FIG. 13. *A*, effects of ouabain and EGF on Shc and Grb2 binding to the EGFR. Cells were treated with either ouabain or EGF as described in the Fig. 4 legend. Cell lysates from both control and treated cells were immunoprecipitated with a polyclonal anti-EGFR antibody. Western blots were done to measure the binding of Shc and Grb2 to the EGFR. A representative blot of four independent experiments is shown. *B*, effects of PP2 and AG1478 on ouabain-induced Shc tyrosine phosphorylation. Cells were treated as described in the Fig. 12*A* legend, and cell lysates were immunoprecipitated with a monoclonal anti-Shc antibody. Tyrosine-phosphorylated Shc was then detected by Western blot using an anti-phosphotyrosine antibody. The values are mean ± S.E. of four independent experiments. Data are expressed relative to a control value of 1. **, $p < 0.01$.

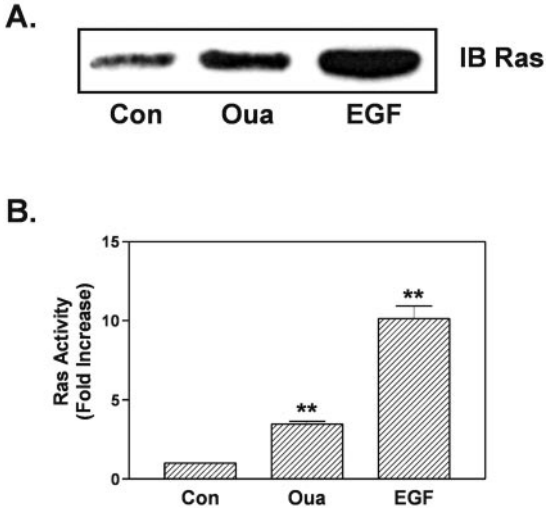

FIG. 14. **Effects of ouabain and EGF on Ras activation in A7r5 cells.** Cells were treated as described in the Fig. 13 legend. Affinity precipitation of activated Ras from control and treated cell lysates was performed as described under "Experimental Procedures." The affinity-precipitated active Ras was then separated by SDS-PAGE and immunoblotted with an anti-Ras antibody. *A*, a representative Western blot of affinity-precipitated active Ras. *B*, quantitation of the effects of ouabain and EGF on Ras activation. The values are the mean ± S.E. of three independent experiments. Data are expressed relative to a control value of 1. **, $p < 0.01$.

Ras binding to Raf. Clearly, the formation of a Src-EGFR complex in response to ouabain is sufficient to recruit other proteins to the plasma membrane, which results in the activation of the Ras/MAPK cascade.

DISCUSSION

Na^+/K^+-ATPase is well known for its essential function in maintaining the Na^+ and K^+ ion concentrations across the plasma membrane in most mammalian cells. Cardiac glycosides such as ouabain are known to bind to plasma membrane Na^+/K^+-ATPase and inhibit its ion transporting ability (1–3). Previously, we have shown that ouabain binding to Na^+/K^+-ATPase not only inhibits the ion pumping function of the enzyme but also converts the enzyme to a signal transducer. Subsequently, this results in the activation of several signaling cascades that ultimately lead to changes in the expression of multiple genes (4–10). In the present study, we have elucidated the mechanisms by which ouabain-bound Na^+/K^+-ATPase uses tyrosine kinases to assemble a membrane-associated protein complex for the regulation of the Ras/MAPK cascade in A7r5 and LLC-PK1 cells.

Interaction of Ouabain with Na^+/K^+-ATPase Initiates the Signal Transducing Function of the Enzyme—The earlier work in our laboratory focusing on MAPK and Src activation in response to ouabain was done in both neonatal cardiac myocytes and A7r5 cells (7–9). These cells as well as mouse SYF + c-Src cells are known to express a relatively ouabain-insensitive Na^+/K^+-ATPase (Fig. 1; Ref. 20), and significant stimulation of MAPKs occurs when 10–20% of the enzyme is inhibited by ouabain. Although the effects of ouabain on MAPKs correlate well with its binding to Na^+/K^+-ATPase in these cells, there is always a possibility that 10–100 μM ouabain may affect other cellular proteins in addition to Na^+/K^+-ATPase. As shown in Fig. 1, LLC-PK1 cells express a highly ouabain-sensitive Na^+/K^+-ATPase α_1 isoform. Fifty percent inhibition of the enzyme activity was observed when the cells were treated with 1 μM ouabain. Concomitantly, these cells showed a high sensitivity to ouabain with respect to MAPK activation. Significant stimulation of MAPKs was observed when LLC-PK1 cells were exposed to 100 nM ouabain that bound to and inhibited about 20% of the enzyme in these cells (Figs. 1 and 2). In addition, when Src kinase activity was measured, 1 μM ouabain caused the same degree of stimulation of Src kinase in LLC-PK1 cells as that induced by 100 μM ouabain in A7r5 cells (Fig. 3). Furthermore, our previous work also demonstrated that ouabain-induced increases in reactive oxygen species production in HeLa cells and rat cardiac myocytes correlated well with the ouabain sensitivities of the α_1 isoforms expressed in these cells (10). It was also reported that in canine smooth muscle cells, MAPKs were activated by 1 nM ouabain that would bind to about 5% of the enzyme in these cells (21). Clearly, the signal transducing effects of ouabain are indeed due to its interaction with Na^+/K^+-ATPase.

Activation of Src by Ouabain Leads to the Transactivation of the EGFR and Assembly of the Proteins Involved in the Regulation of MAPKs—Receptor tyrosine kinases are central elements for cellular signal transduction. Binding of a ligand to its receptor induces the formation of either homo- or heterodimers that subsequently trigger the autophosphorylation of cytoplasmic tyrosine residues of the receptor. These phosphorylated amino acid residues then function as docking sites for a variety of adaptor proteins that regulate membrane-proximal events of a signaling network, ultimately defining the biological response to a given signal (12). In recent years, there has been a growing body of evidence that receptor tyrosine kinases such as EGFR and PDGFR cross-communicate with other signaling systems to integrate the variety of extracellular stimuli into a limited number of signaling pathways. The activated EGFR, for example, has been identified as a critical element in the signal transduction networks of cytokines, H_2O_2, and those using G protein-coupled receptors (14–19). To distinguish this

nonclassical mode of stimulation from receptor activation by its cognate ligands, this process has been termed "EGFR transactivation" (14). We previously demonstrated that binding of ouabain to Na^+/K^+-ATPase stimulated Src kinase activity and transactivated the EGFR. These findings prompted us to test whether the EGFR cross-talks to ouabain-bound Na^+/K^+-ATPase, resulting in activation of multiple downstream signaling processes. In the experiments of Figs. 4 and 6, where the cells were pretreated with different tyrosine kinase inhibitors, we showed that inhibition of the EGFR abrogated the effects of ouabain on MAPKs. Interestingly, the EGFR was also shown to be involved in ouabain-induced activation of MAPKs in canine smooth muscle cells (21). These findings convincingly demonstrated a cross-talk between Na^+/K^+-ATPase and the EGFR and identified the EGFR as a potential molecular scaffold for the assembly of a membrane-associated signaling complex for regulation of MAPKs. Because the PDGFR can also be transactivated by a variety of stimuli (22), AG1295, a specific inhibitor of PDGFR, was used in the experiments of Fig. 6 to test its role in ouabain-induced activation of MAPKs. The results showed that AG1295 blocked PDGF-induced but not ouabain-induced activation of MAPKs. Thus, it is unlikely that the PDGFR plays a role in ouabain-induced activation of MAPKs. Furthermore, the findings of the experiments of Figs. 13 and 14 confirmed that the activated EGFR could function as a molecular scaffold to recruit and assemble a signaling complex on the cell membrane in response to ouabain stimulation because of the following observations. First, the tyrosine-phosphorylated EGFR was able to recruit both $p46^{Shc}$ and $p52^{Shc}$, but not $p66^{Shc}$, in response to ouabain (Fig. 13*A*). This is consistent with the notion that both $p46^{Shc}$ and $p52^{Shc}$, but not $p66^{Shc}$, are involved in transmitting growth signals from receptor tyrosine kinases to downstream effectors such as MAPKs (14). Significantly, AG1478 suppressed ouabain-induced tyrosine phosphorylation of Shc (Fig. 13*B*), indicating that a functional EGFR is required for binding and phosphorylating Shc in response to ouabain. Because tyrosine phosphorylation of Shc is considered a critical step for the subsequent activation of the Ras/MAPK cascade (12–14), these findings are consistent with those of Fig. 6 showing that AG1478 blocked ouabain-induced activation of MAPKs. Second, after the stimulation of Shc tyrosine phosphorylation, ouabain increased the binding of the Grb2-Sos complex to the activated EGFR (Fig. 13*A*). This resulted in the recruitment of Ras and the subsequent activation of the Raf/MEK/MAPK cascade in A7r5 cells (Fig. 14). Clearly, ouabain stimulates MAPK activity in A7r5 cells via the transactivated EGFR.

At least two distinct mechanisms have been revealed for the transactivation of the EGFR by stimuli other than its cognate ligand. One of them involves the increased release of the EGFR ligand heparin-binding EGF (14). For example, stimulation of G-protein-coupled receptors by thrombin induces the proteolytic processing of the pro-heparin-binding EGF, and inhibition of either protease activity or heparin-binding EGF significantly reduces the stimulus-induced EGFR transactivation (14). The other mechanism involves Src kinases in which stimulation of Src increases its binding to the EGFR and subsequent tyrosine phosphorylation of the receptor (14). Furthermore, the Src-mediated tyrosine phosphorylation occurs at sites (Tyr^{845} and Tyr^{1101}) other than those induced by autophosphorylation of the receptor upon binding its cognate ligand (23). Such Src-mediated EGFR transactivation has been observed in different types of cells stimulated by either angiotensin II or H_2O_2 (17–19). In the experiments of Figs. 4 and 5, we showed that activation of Src was essential for ouabain-induced activation of MAPKs. We further demonstrated that inhibition of Src by both herbimycin A and PP2 completely blocked ouabain-induced but not EGF-induced Src association to the EGFR (Fig. 10). These inhibitors also abolished ouabain-induced but not EGF-induced tyrosine phosphorylation of the EGFR (Fig. 10). In addition, ouabain stimulated tyrosine phosphorylation of the EGFR in SYF + c-Src cells, but not in SYF cells. These findings support the proposal that ouabain is most likely to transactivate the EGFR by increasing the association of the activated Src to the receptor. Furthermore, because ouabain acted differently than EGF in the regulation of MAPKs (Fig. 4) and failed to stimulate autophosphorylation of Tyr^{1173} of the EGFR (Fig. 12; Ref. 9), it is unlikely that increases in heparin-binding EGF release play an important role in the activation of the EGFR in response to ouabain in A7r5 cells. Taken together, the new findings support the proposal that the activation of Src is the initial critical step that relays the signal emanated from the interaction of ouabain with Na^+/K^+-ATPase to the EGFR. However, the molecular mechanisms by which ouabain-activated Src transactivates the EGFR remain to be resolved.

Can the Ouabain-Na^+/K^+-ATPase Complex Function as a Molecular Scaffold for Assembly of Signaling Complexes?—The assembly of a signaling module is essential for efficient and specific transmission of a signal from the receptor to the downstream effector (24). Because the new findings above indicate that ouabain-bound Na^+/K^+-ATPase cross-communicates with the EGFR through activated Src, we are tempted to speculate that a pool of Na^+/K^+-ATPase is compartmentalized to a specific membrane domain that is enriched with multiple signaling proteins. The binding of ouabain to Na^+/K^+-ATPase increases the interaction of the enzyme with several proteins and brings them together so that the activated Src can transactivate the EGFR (24). This hypothesis appears to be supported by the findings of Figs. 7 and 9. It is well established that Src kinase activity can be regulated by two different mechanisms. Activation of Src by angiotensin II is likely due to stimulation of a phosphotyrosine phosphatase because it decreases Src phosphorylation at Tyr^{529} (25). On the other hand, many stimuli can increase the competitive binding of a regulatory protein to either the SH2, SH3, or kinase domain of Src, resulting in increased phosphorylation at Tyr^{418} and a subsequent increase in kinase activity (13, 17). Because ouabain stimulated Src phosphorylation at Tyr^{418} without affecting Tyr^{529} phosphorylation (Fig. 7), it is likely that the ouabain-induced Src activation is due to the increased interaction between Na^+/K^+-ATPase and Src. Consistent with this notion, immunoprecipitation experiments showed that ouabain stimulated the binding of Src to Na^+/K^+-ATPase in a dose- and time-dependent manner (Figs. 8 and 9). Interestingly, the α_1 subunit of Na^+/K^+-ATPase contains a conserved functional proline-rich domain that has been shown to interact with the SH3 domain of phosphatidylinositol 3-kinase upon dopamine stimulation (26). Therefore, we suggest that ouabain may increase the interaction of Na^+/K^+-ATPase with the SH3 domain of Src, which results in Src activation and the subsequent transactivation of the EGFR that resides by the Na^+/K^+-ATPase-Src complex. We are currently in the process of investigating such a possibility.

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