In addition to inhibition of the Na-K ATPase, ouabain activates a signal transduction function, triggering growth and proliferation of cultured cells even at nanomolar concentrations. An isomer of ouabain (EO) circulates in mammals at subnanomolar concentrations, and increased levels are associated with cardiac hypertrophy and hypertension. We present here a study of cardiac and renal hypertrophy induced by ouabain infused into rats for prolonged periods and relate this effect to the recently described ouabain-induced activation of the Src-EGFr-ERK signaling pathway. Ouabain infusion into rats (15 μg/kg/day for 18 weeks) doubled plasma ouabain levels from 0.3 to 0.7 nm and increased blood pressure by 20 mm Hg (p < 0.001), cardiac left ventricle (+11%, p < 0.05), and kidney weight (+9%, p < 0.01). These effects in vivo are associated with significant enrichment of α1, β1, γ1 Na-K ATPase subunits together with Src and EGFr in isolated renal caveolae membranes and activation of ERK1/2. In caveolae, direct Na-K ATPase/Src interactions can be demonstrated by co-immunoprecipitation. The interaction is amplified by ouabain, at a high affinity binding site, detectable in caveolae but not in total rat renal membranes. The high affinity site for ouabain is associated with Src-dependent tyrosine phosphorylation of rat α1 Na-K ATPase. The antihypertensive compound, PST 2238, antagonized all ouabain-induced effects at 10 μg/kg/day in vivo or 10–12–10–9 M in vitro. These findings provide a molecular mechanism for the in vivo pro-hypertrophic and hypertensinogenic activity of ouabain, or by analogy those of EO in humans. They also explain the pharmacological basis for PST 2238 treatment.

Until recently, the main, if not unique, function ascribed to the integral membrane protein Na-K ATPase is the maintenance and regulation of the electrochemical gradient across the cell membrane in all tissues (1). Ouabain and other steroidal cardenolides (2) or bufadienolides (3) are considered to be the specific inhibitors of the Na-K ATPase activity. However, in recent years, several studies have indicated that Na-K ATPase can also act as a signal transducer in response to the interaction with its natural ligand ouabain (4). This finding originates mainly from studies carried out on cultured rat cardiomyocytes or renal tubular cells based on effects on cell growth and hypertrophy of ouabain in the micromolar range. At these rather high concentrations, which, however, do not seem to affect the bulk intracellular Na+ and Ca2+ concentrations (5), ouabain activates (a) tyrosine phosphorylation of the epidermal growth factor receptor (EGFr) (6), Src, and p42/44 mitogen-activated protein kinase (MAPKs) in both neonatal rat cardiomyocytes and A7r5 cells (4, 6); (b) the same signaling pathway within the cellular membrane microdomain of caveolae in isolated perfused rat heart (7); and (c) slow intracellular Ca2+ oscillations in rat tubular cells that favor the association of Na-K ATPase with the inositol 1,4,5-trisphosphate receptor (InsP₃R) in a signaling microdomain (8). Recently, it has been shown that even subnanomolar ouabain concentrations stimulate growth of cultured rat tubular cells, via activation of an extracellular signal-regulated kinase (ERK)-dependent pathway and, surprisingly, in view of the fact that these rat cells contain only the α1 isoform of Na-K ATPase, ouabain inhibits a component of active ⁸⁶Rb uptake with a high affinity (9).

The endogenous ouabain (EO), or a close related isomer (10), circulates in mammals at concentrations in the subnanomolar range (11–13) and has been implicated in the development of hypertension (12, 14), alterations of renal sodium reabsorption and cardiac and renal complications (12–16). Furthermore, the hypertensinogenic activity of low ouabain concentrations might also be ascribed to a specific vasotonic effect as demonstrated in rodent vessels (17, 18).

However, a direct demonstration that the ouabain/Na-K ATPase signaling effects are also relevant to the cardiovascular effects of EO in vivo is still lacking and the following questions have to be elucidated, at least in the experimental rat model. 1) Do in vivo variations of rat plasma ouabain, within the subnanomolar concentrations range, produce cardiac and renal hypertrophy and activate an ERK-dependent transduction pathway mediated by the interaction of α1 Na-K ATPase with signaling proteins in defined membrane microdomains, such as caveolae? 2) Is it possible to reproduce in vitro the in vivo signaling effects by demonstrating that subnanomolar concentrations of ouabain trigger interactions among Na-K ATPase, Src, and EGFr?

The new ouabain-antagonist, PST 2238 (19, 20) is an important tool in this study. We have demonstrated previously that PST 2238 selectively binds to Na-K ATPase and not to other...
PST 2238 Reverts Ouabain-induced Hypertrophy

Na-K ATPase Activity Assay—Na-K ATPase activity was measured by [3H]PiATP hydrolysis method (23) in sucrose fractions washed in: (mm) 250 sucrose, 30 histidine, pH 7.2, and in purified Na-K ATPase obtained from rat kidney medulla, as described (24).

To investigate the effect of Src on Na-K ATPase activity, recombinant Src kinase, or its medium, was incubated with purified rat renal α1 Na-K ATPase (protein ratio 1:50) for 30 min at 3°C in a buffer containing (mm) 150 NaCl, 3 MgCl2, 3 ATP, and 70 Hepes, pH 7.4, supplemented with 3 mM MnCl2. The effect of increasing concentrations of ouabain was evaluated. Na-K ATPase activity was measured as above (23).

\[^{3}H\]Ouabain Binding and Scatchard Analysis—Washed renal caveolae were incubated for 37°C for 1 h with increasing concentrations of \[^{3}H\]Ouabain in a medium containing 3 mM MgCl2,5 mM phosphoric acid, pH 7.4. Concentrations of labeled ouabain higher than 10^{-6} M were isolated by isotopic dilution with cold ouabain. Nonspecific binding was determined in the presence of 20 mM ouabain. The separation of bound from free-labeled ouabain was achieved by a rapid filtration method on Whatman GF/F filters, which were washed three times and counted for radioactivity.

Co-immunoprecipitation Experiments—Washed renal caveolae were incubated for 30 min at 30°C in the absence or presence of ouabain in: (mm) 10 Tris, 10 MgCl2, 5 MnCl2, 0.25 EGTA, 0.025 sodium orthovanadate, 80 NaCl, 2 ATP, 100 mg/liter Pefabloc, pH 7.4. When specified, PST 2238 or amloidipine were added. The samples were incubated with 0.2% CHAPS for 15 min at 4°C and then with anti-Src antibody (GD11 clone), conjugated to protein G-Sepharose beads (Sigma) for 4 h. A sample of caveolae, incubated with a non-immune IgG (Sigma), instead of the anti-Src antibody, was used as control. The immunocomplexes were precipitated, washed three times with the immunoprecipitation buffer containing CHAPS, and boiled with Laemmli sample buffer. Supernatants were subjected to SDS-polyacrylamide gel electrophoresis and Western blotting and probed with specific antibodies.

In Vitro Src Phosphorylation of Na-K ATPase—Na-K ATPase phosphorylation by Src was measured in vitro by \[^{3}P\]incorporation method (A) and Western blotting with an anti-phosphotyrosine antibody (PY99) (B). Method A: 3 α of purified rat renal α1 Na-K ATPase was incubated for 10 min at 30°C in the absence, or presence, of 50 ng of recombinant Src protein kinase in a medium containing: (mm) 10 Tris, pH 7.4, 10 MgCl2, 5 MnCl2, 0.25 EGTA, 0.025 sodium orthovanadate, 80 NaCl, 0.1 ATP, and \[^{3}H\]PiATP (1500 cpm/mml, specific activity 3000 Ci/mmol). The reaction was stopped by addition of Laemmli sample buffer. The samples were subjected to SDS-polyacrylamide gel electrophoresis, followed by autoradiography. Method B: Na-K ATPase was incubated with Src kinase, as in method A, in a medium supplemented with 2 μM cold ATP in the absence of \[^{3}H\]PiATP. When specified, before the addition of Na-K ATPase, Src kinase was preincubated with its specific inhibitor, PP2 (5 μM), for 30 min at 4°C or with a specific Src substrate peptide (500 μM), instead of Na-K ATPase. The effect of 10^{-10} to 10^{-8} M ouabain was tested. The reaction was stopped by addition of Laemmli sample buffer. The samples were subjected to SDS-polyacrylamide gel electrophoresis and Western blotting and probed with specific antibodies to verify the tyrosine phosphorylation of Na-K ATPase (PY99) and Src kinase (Src-Tyr^{418} and Src-Tyr^{529}).

Western Blotting—Samples were separated by SDS-polyacrylamide gel electrophoresis (7–15% acrylamide in glycine or 12% acrylamide in Tricine gels), blotted and overnight incubated at 4°C with specific primary antibodies, followed by 1 h incubation with horseradish peroxidase-linked secondary antibody and chemiluminescent reaction (LumiGlo, Promega). Cell Signaling Autoradiography was performed, bands were scanned with Bio-Rad GS710 densitometer and quantified by Bio-Rad Quantity One Software. Blots were stripped (Re-Blot plus, Chemicon) and reprobed no more than three times.

Statistical Analysis—Data are reported as mean ± S.E. The difference among groups was analyzed by one-way analysis of variance, followed by Fisher’s least squares difference test. p < 0.05 was considered statistically significant. Ouabain dose-response curves and Scatchard plots were analyzed by a nonlinear regression program (GraphPad Prism Software, version 3).

RESULTS

Ouabain Effects on Blood Pressure and Organ Weight—Ouabain infusion doubled plasma ouabain concentration in OS (0.7 ± 0.07 nm, n = 10, p < 0.001) as compared with CS rats (0.3 ± 0.04 nm, n = 10), reaching a value close to that of plasma EO found in hypertensive patients with cardiac hypertrophy.
PST 2238 Reverts Ouabain-induced Hypertrophy

**A. Systolic Blood Pressure**

![Graph](image1)

**B. Left Ventricle Weight**

![Graph](image2)

**C. Kidney Weight**

![Graph](image3)

Fig. 2. Effect of ouabain infusion (15 μg/kg/day, OS rats) and PST 2238 oral treatment (10 μg/kg/day) on SBP and organ hypertrophy in rats. A. SBP at the end of treatment. B. left ventricle free-wall (LVFW) and kidney weight (KW), normalized for tibia length, in CS, OS, and PST 2238-treated OS rats. Mean ± S.E. of 10 rats for each group. *, p < 0.05; **, p < 0.01; ###, p < 0.001 versus CS; #, p < 0.05; ##, p < 0.01 versus OS.

(15, 16) and in hypertensive rat models (11). Tissue ouabain content (ng/g tissue) was increased 3-fold in OS LVFW (0.96 ± 0.091, n = 10, p < 0.01) and kidney (10.9 ± 1.4, n = 10, p < 0.01) as compared with CS (LVFW: 0.31 ± 0.033, n = 10; kidney: 3.93 ± 0.3, n = 10). Before starting with ouabain infusion, all rats had a similar SBP (130–135 mmHg). It was significant increase in SBP observed in OS as compared with CS controls (+20 mmHg, p < 0.001) (Fig. 2A). The oral treatment with PST 2238 (10 μg/kg/day) significantly reduced SBP in OS rats (−20 mmHg, n = 10, p < 0.001 versus OS, Fig. 2A) within 3 weeks. Neither ouabain nor PST 2238 affected HR (beats/min: CS, 360 ± 8.3; OS, 381 ± 9.5; OS + PST 2238: 358 ± 9) and body weight (g, CS, 459 ± 7; OS, 466 ± 8; OS + PST 2238: 456 ± 6). Ouabain infusion increased cardiac LVFW (+11%, p < 0.05) and kidney weight (+9%, p < 0.01) in OS rats as compared with CS controls (Fig. 2, B and C). PST 2238 treatment prevented the ouabain-induced cardiac and kidney hypertrophy (Fig. 2, B and C).

The Ca\(^{2+}\) antagonist amlodipine (oral dose 5 mg/kg/day), taken as an antihypertensive reference compound, significantly reduced SBP in OS rats (−20 mmHg, p < 0.01) but did not affect cardiac and kidney weight (data not shown).

These data indicate that in vivo variations of ouabain concentrations within the subnanomolar range, similar to those reported for EO in humans, cause organ hypertrophy in rats besides raising blood pressure. The demonstration that amlodipine prevents the ouabain-induced increase of blood pressure without affecting in vivo organ hypertrophy excludes that the prohypertrophic effect of ouabain might be secondary to pressure overload.

**Expression of Na-K-ATPase and Signaling Molecules in Total Renal Membranes and Caveolae—Preliminary Western blotting analysis of Na-K ATPase, EGFr, and Src in total renal membranes (MT) from CS, OS, and PST 2238-treated OS rats (n = 6) indicated that ouabain infusion significantly increased β1 Na-K ATPase subunit (+16%, p < 0.01) paralleled by an increase, although not statistically significant, of the α1 Na-K ATPase subunit (+11%), EGFr (+14%), and Src (+10%) compared with CS. In the PST 2238-treated OS rats all these differences disappeared.

In order to investigate the possibility that the ouabain-induced effects on the protein expression observed in MT are amplified in restricted membrane subdomains, we isolated and characterized renal caveolae from CS, OS, and PST 2238-treated OS rats. A typical protein distribution of the renal membrane fractionation on the sucrose density gradient is depicted in Fig. 3. The low density fractions (Fig. 3A, fractions 5–8, containing 5% of the total proteins) contained the specific markers of caveolae (25, 26), such as caveolin 1, EGFr, and Src, which were enriched 30-, 40-, and 22-fold, respectively, compared with renal MT (Fig. 3B) and were therefore referred to as caveolae. Interestingly, caveolae were enriched 4-fold in α1, β1, and the γ splice variant of the Na-K ATPase subunits as compared with MT (Fig. 3B). The γ variant was not detected. The absence of clathrin, the marker for coated pits (Fig. 3B), excludes contamination by these vesicles. Mannosidase and glucosidase activities were also absent (not shown) in caveolae, thus excluding possible Golgi or endoplasmic reticulum membrane contaminations.

The high buoyant density fractions 11–15 (Fig. 3A), positive for all these markers, including clathrin and γ and γ splice variants of Na-K ATPase (not shown), were referred to as plasma membrane.

**Effect of Ouabain on the Expression of Na-K-ATPase and Signaling Molecules in Renal Caveolae—A representative immunoblotting of the Na-K ATPase subunits and signaling mol-
percent increase of CS. Increased in OS over CS control rats (p < 0.01). Moreover, the content of the Na-K ATPase subunits was significantly increased in OS caveolae as compared with CS controls (Fig. 4B). Mean optical densities ± S.E. (n = 6), normalized for protein content, expressed as percent increase of CS. **, p < 0.01 versus CS; ##, p < 0.01 versus OS.

cavity in caveolae isolated from one CS, OS, and PST 2238-treated OS rat is shown in Fig. 4A. The densitometric analysis performed on caveolae from six rats for each group, expressed as percent increase over CS, is shown in Fig. 4B and C. Caveolin 1 content was similar in the three groups of rats. Ouabain significantly increased the recruitment of EGFr, Src, and phosphorylated Src at Tyr418, the Src active form (26) (Fig. 4A) and p44 (ERK1) and p42 (ERK2) levels as compared with CS controls and PST 2238-treated OS rats. Mean optical densities ± S.E. (n = 6), normalized for protein content: *, p < 0.05; **, p < 0.01 versus CS; #, p < 0.05; ##, p < 0.01 versus OS.

PST 2238 largely reverted all the ouabain-induced effects in caveolae of OS rats (Fig. 4). It reduced the ouabain-induced targeting into caveolae of EGFr, Src, Src-Tyr418 (Fig. 4, A and B), normalized the phospho/non-phospho Src ratio to the level of CS controls and reduced the Na-K ATPase subunit content, particularly the α and β subunits (Fig. 4C).

Effect of Ouabain on ERK1/2 Activation—We further investigated whether the effect of ouabain infusion on Na-K ATPase, Src, EGFr expression into caveolae might result in the activation of the ERK pathway and whether PST 2238 might reverse this effect. Fig. 5 shows the results obtained by probing kidney extracts from CS rats, OS, and PST 2238-treated OS rats (n = 6) with anti-total and anti-dual phosphorylated ERK1/2 antibodies. Ouabain caused a significant increase of total p44 (ERK1) and p42 (ERK2) levels as compared with CS controls and PST 2238 reverted this effect (Fig. 5A). Interestingly, ouabain induced an increase of dual-phosphorylated forms of ERK1/2 versus CS controls that was abolished by PST 2238 (Fig. 5B).

These data demonstrate, for the first time, that the in vivo variation of circulating ouabain within the subnanomolar concentration range raises the expression levels of defined Na-K ATPase subunits and signaling molecules into caveolae and the concomitant activation of the ERK1/2 pathway.

Na-K ATPase Activity and Ouabain Affinity in Rat Renal Caveolae—Since previous work indicated that ouabain signaling depends upon its interaction with Na-K ATPase (5, 6), we investigated whether rat α1 Na-K ATPase localized in caveolae retains its catalytic activity and affinity for ouabain. This latter aspect is crucial when assessing the physiological relevance of nanomolar concentrations of ouabain in rat kidney where only the ouabain-resistant α1 isoform has been detected (27).

The enzymatic activity of renal MT and caveolae, obtained from CS rats (n = 4), was respectively 3.6 and 2.4 μmol of Pi/min/mg protein as compared with 6 μmol of Pi/min/mg protein for a partially purified rat renal α1 Na-K ATPase. The inhibitory ouabain dose-response curves measured in rat renal caveolae showed the presence of a predominant component with low affinity for ouabain (IC50 = 1.3 × 10^{-6} M), close to that of the purified renal Na-K ATPase (Fig. 6A) and MT (not shown). However, only in caveolae, the ouabain dose-response curve was best fitted according to a two-binding site model (nonlinear regression program, GraphPad Prism Software: one-binding site model, R^2 = 0.99; two-binding site model, R^2 = 0.99, p < 0.0001, F = 84.09) showing a second minor compo-
nent (around 25%) of Na-K ATPase having a higher affinity for ouabain (IC_{50} = 1.2 \times 10^{-7} \text{ M}) (Fig. 6A, inset) and activity of 0.6 \mu\text{mol of P/ min/mg protein}.

**Ouabain Binding to Na-K ATPase in Rat Renal Caveolae: Effect of PST 2238**—The presence of a ouabain high affinity Na-K ATPase component in rat renal caveolae was further investigated by measuring the [3H]ouabain binding in preparations obtained from CS rats. The ouabain dose-response curve (Scatchard plot, Fig. 6B) was analyzed by a nonlinear regression program comparing one ($R^2 = 0.76$) or two classes ($R^2 = 0.97$) of binding sites (GraphPad Prism Software). Data were best fitted by a two-site model ($p < 0.0001, F = 27.02$) revealing the presence of the predominant ouabain-resistant Na-K ATPase isoform ($K_r = 1 \times 10^{-8} \text{ M}$; $B_{max} = 260 \text{ pmol of ouabain bound/mg of protein}$) and a minor component with higher affinity for ouabain ($K_r = 2 \times 10^{-7} \text{ M}$; $B_{max} = 20 \text{ pmol of ouabain bound/mg of protein}$). PST 2238, added simultaneously to ouabain at $10^{-8} \text{ M}$, antagonized ouabain binding to the high affinity site without affecting the binding of ouabain to the low affinity site (Fig. 6B). The measurements of Na-K ATPase activity and ouabain binding capacity in caveolae permitted the calculation of the turnover number that was 9500 min^{-1}, which is comparable to that estimated in rat renal microsomes (23). In order to test whether the caveolar high affinity component of Na-K ATPase might be indicative of the presence of the α2 or α3 Na-K ATPase isoforms, caveolae were probed with the anti-α2 and anti-α3 Na-K ATPase specific antibodies and analyzed by immunoblotting (Fig. 6C) in comparison to renal MT. Neither α2 nor α3 isoforms were detected in caveolae or MT while, as expected, a positive immunoreactivity against the two isoforms was observed in rat brain Na-K ATPase (enzymatic activity = 4 \mu\text{mol of P/ min/mg of protein}). These data (Fig. 6, A and B) indicate that a pool of Na-K ATPase having high affinity for ouabain is present in rat kidney caveolae.

**Co-immunoprecipitation of α1 Na-K ATPase and Src in Rat Renal Caveolae: Effect of Ouabain and PST 2238**—To further investigate the role of subnanomolar ouabain concentrations in determining the interaction between Na-K ATPase and signaling molecules in caveolae, co-immunoprecipitation experiments between Na-K ATPase and Src were performed in the
The efficiency of the Na-K ATPase/Src ratio induced by 10^{-10}M amlodipine at 10^{-10}M ouabain alone resulted inactive (Fig. 7A). The result is a bell-shaped curve of the efficiency of the α1 Na-K ATPase co-immunoprecipitated with Src (control sample, Fig. 7A). In the presence of ouabain (10^{-12} to 10^{-5}M), the amount of α1 Na-K ATPase co-immunoprecipitated with Src increased progressively to a maximum as the ouabain concentrations were increased from 10^{-12} to 10^{-10} or 10^{-9}M and decreased further to 10^{-8}M ouabain (Fig. 7A). The probing of the immunoprecipitate from rat renal caveolae with either a phosphotyrosine or with a Src-Tyr418 antibody showed a ouabain-induced increase of some tyrosine-phosphorylated proteins with a maximal effect attained at 10^{-8}M ouabain as compared with controls. After stripping and reprobing the immunoprecipitate with specific antibodies, these proteins were identified as α1 Na-K ATPase (±28%), Src (±33%), and EGFr (±19%). PST 2238, at 10^{-8} or 10^{-10}M, but not amlodipine at 10^{-8}M, antagonized the ouabain-induced increase in tyrosine phosphorylation of the immunoprecipitated proteins (±20%).

In the co-immunoprecipitation experiments, ouabain appeared to be effective at concentrations lower than those for the high affinity ouabain binding site obtained in the Scatchard analysis and Na-K ATPase inhibitory activity. A possible explanation for these apparent discrepant values may reside both in the low specific activity of [3H]ouabain (15 Ci/mmol), that does not allow to measure an appreciable binding below 4×10^{-9}M of [3H]ouabain, and in the presence of 10 mM KCl, needed for the enzymatic activity measurement, that may affect ouabain binding either to the high or low affinity site (28).

In conclusion, these findings further support the evidence that subnanomolar concentrations of ouabain bind to the rat renal α1 Na-K ATPase in the caveolar membranes and favor the interaction of Na-K ATPase with signaling molecules in this membrane subdomain. PST 2238 is able to antagonize this ouabain effect presumably by antagonizing its binding to the high-affinity component of α1 Na-K ATPase localized in this district.

Src Phosphorylation of Na-K ATPase in an in Vitro Assay: Effect of Ouabain—In order to test whether the binding of low concentrations of ouabain to rat α1 Na-K ATPase may be favored by a Src-induced phosphorylation of the enzyme, purified rat renal α1 Na-K ATPase was incubated in vitro with recombinant Src kinase in the presence or absence of low concentrations of ouabain, and Na-K ATPase phosphorylation and activity was measured.

The results showed that rat α1 Na-K ATPase was phosphorylated only in the presence of Src, as demonstrated by two independent methodologies: [32P] incorporation (Fig. 8A, left) and Western blotting with an anti-phosphotyrosine antibody (Fig. 8, A, right and B, lane 4 versus lanes 1 and 2). After stripping and reprobing, it was confirmed that the phosphorylated band was immunopositive for α1 Na-K ATPase (not shown). The presence of 10^{-8} to 10^{-10}M ouabain caused, respectively, a statistically significant 28 and 22% increase of α1 Na-K ATPase.
Na-K ATPase tyrosine phosphorylation as compared with the absence of ouabain (Fig. 8B, lanes 5 and 6 versus lane 4 and C, left) and a parallel 21% (p < 0.05) and 15% increase of Src phosphorylation at Tyr418 (Fig. 8B, lanes 5 and 6 versus lane 4 and C, right), and not at Tyr529 (not shown). In the absence of Src, ouabain did not induce α1 Na-K ATPase phosphorylation (Fig. 8B, lane 3 versus 2). The pretreatment of Src with its specific inhibitor, PP2, abolished the Src-induced phosphorylation of Na-K ATPase either in the absence or presence of ouabain (Fig. 8B, lanes 7 and 8). When a Src substrate peptide was added to Src, instead of Na-K ATPase, the Tyr418 phosphorylation of Src did not change either in the absence or presence of ouabain (Fig. 8D).

Finally, the Src-induced phosphorylation of Na-K ATPase was associated with a 28% increase of Na-K ATPase activity (μmol of P/min/mg: −Src: 4.5 ± 0.44, n = 3; + Src: 5.8 ± 0.56, p < 0.05) and with the appearance of a second, although minor, binding site with high affinity for ouabain (Fig. 8E), as demonstrated by the best fitting according to a two-binding site model (R^2 = 0.97, p < 0.032, F = 5.14; GraphPad Prism Software) of the ouabain-dependent inhibitory activity. The apparent discrepancy between the concentrations of ouabain effective on the Na-K ATPase inhibitory activity (Fig. 8E) and those favoring the Src-dependent Na-K ATPase phosphorylation (Fig. 8, B and C) may be caused by the presence of 10 mM KCl in the medium for the Na-K ATPase activity measurement (28).

These results indicate that, even in vitro, Src kinase causes a tyrosine phosphorylation of purified rat renal α1 Na-K ATPase that is enhanced by low concentrations of ouabain and is associated with the appearance of a high affinity binding site for ouabain.

**DISCUSSION**

This study demonstrates that, in rats, chronic variations of plasma ouabain concentrations within the subnanomolar range, which are close to those of EO in rats and humans (11–16), induce cardiac and kidney hypertrophy in addition to hypertension. Furthermore, the present data indicate that these organ changes are associated with the accumulation of Na-K ATPase subunits, unphosphorylated and phosphorylated Src, and EGFr signaling molecules into caveolae and to the concomitant activation of the ERK1/2 kinase pathway. These in vivo ouabain effects are paralleled with in vitro experiments performed on isolated rat renal caveolae showing that: (a) a component of the rat α1 Na-K ATPase with high affinity for ouabain is enriched in caveolae and (b) subnanomolar ouabain concentrations appear to favor the interaction between Na-K ATPase and Src and increase the amount of tyrosine-phosphorylated proteins such as Na-K ATPase, Src, and EGFr. Furthermore, all these in vivo and in vitro effects induced by ouabain are reversed by a new compound, PST 2238, developed as a specific ouabain antagonist (19, 20), but not by the Ca^{2+} antagonist amiodipine that reduces only the pressor effect of ouabain.

Admittedly, the present data do not provide any direct support to the existence of the endogenous ouabain (EO) that is crucial for postulating a pathophysiologic role of the new ouabain/Na-K ATPase signaling system. However, the clear organ and molecular pathophysiologic changes caused by an ouabain infusion in rats, that only doubles its plasma concentration, may support the biological relevance of EO variations for cardiovascular complications in humans (12–16).

**Signaling Mechanism of Ouabain and Na-K ATPase within Membrane Caveolae**—The original hypothesis that EO may have a pathophysiological role has been often questioned on the basis of the very low circulating concentrations of EO, which should not be enough to produce a biologically relevant interaction with Na-K ATPase. This criticism is even more pointed when the role of EO is investigated in rat models in which the α1 Na-K ATPase isoform displays a low affinity for ouabain (27). In order to reconcile clinical and experimental evidence of the EO pathophysiological role with the biochemical criteria, it has been suggested that the ouabain-EO/Na-K ATPase complex may play a role in signal transduction.

Studies mainly performed on cultured rat cells indicate that ouabain may control cell growth through the activation of two main intracellular pathways: (i) the Ras/ERK1/2 cascade, mediated by Src-induced EGFr transactivation (6); (ii) the Ca^{2+}-activated intracellular signaling that, through slow Ca^{2+} oscillations and NF-κB nuclear translocation, controls gene expression induction and cell proliferation (8). However, all these data have been obtained by using ouabain concentrations higher than those of the circulating EO, thus weakening the biological relevance of the results. Only recently, one group demonstrated that ouabain is a potent agent in promoting cell growth and activating the ERK1/2 pathway in proximal rat tubular cells at 10⁻¹⁰ M (9). This study also revealed a minor component of the α1 Na-K ATPase with a high affinity for ouabain. Moreover, it has been recently demonstrated that many of the proteins involved in the receptor-effector coupling and signaling activation can be compartmentalized within restricted membrane microdomains, the caveolae (25, 26). Among these proteins, Na-K ATPase, Src, and EGFr have been found in cardiac caveolae (7).

These data confirm the presence of Na-K ATPase together with Src and EGFr signaling molecules in a low density membrane fraction from rat renal kidney with the predicted properties of caveolae. Rat kidneys were chosen since our previous studies indicated that ouabain specifically modulates the expression and activity of renal α1 Na-K ATPase causing hypertension in OS rats, effects normalized by the ouabain antagonist PST 2238 (20). Furthermore, kidney is particularly enriched in caveolin 1, the specific marker of caveolae (29). In caveolae, we demonstrated the presence of a pool of Na-K ATPase composed by α1, β1, and the γα splice variant subunit. The localization of the γα variant of Na-K ATPPase, independently from γb, is in line with published data (30) that exclude the existence of mixed complexes of α1β1γa/γb in kidney membranes. The different distribution of the γa and γb variants of Na-K ATPase into caveolae, however, may be related to the difference at the N-terminal residues between the two spliced variants that, as proposed (30), might affect Na-K ATPase membrane targeting, basolateral signaling, and extra-cellular matrix interactions.

We show here that infusion of subnanomolar concentrations of ouabain in rats, which causes hypertension and organ hypertrophy, is associated with an increased amount of Na-K ATPase subunits and signaling molecules, including EGFr, Src, and Tyr418-phosphorylated Src, within renal caveolae. This is compatible with the notion that ouabain triggers a Src-EGFr-dependent signaling cascade that culminates in the activation of ERK. Although these data support the original hypothesis that ouabain may confer a signaling transduction function to Na-K ATPase, even at nanomolar concentrations, the question remains as to how subnanomolar concentrations of ouabain might interact with the high ouabain-resistant rat renal α1 Na-K ATPase. Several independent observations suggest that ouabain can indeed interact with rat α1 Na-K ATPase with high affinity. 1) Ouabain concentration-dependent inhibition of the Na-K ATPase activity and 2) Scatchard plot analysis of the [3H]ouabain binding demonstrated that rat renal caveolae, besides the predominant low affinity component, contain a...
less prominent component with a high ouabain affinity. 3) Co-immunoprecipitation experiments in rat renal caveolae further reinforce the evidence that ouabain, at concentrations as low as $10^{-9}$–$10^{-10} \text{ M}$, interacts with the $\alpha 1$ Na-K ATPase causing an increase in Na-K ATPase/Src complex formation. 4) Src-dependent Na-K ATPase phosphorylation on isolated rat $\alpha 1$ Na-K ATPase is selectively enhanced in the presence of $10^{-12}$–$10^{-8} \text{ M}$ ouabain. Because the presence of the high ouabain affinity $\alpha 2$ and $\alpha 3$ isoforms appears to be excluded, the surprising, but necessary conclusion must be that the high affinity component is associated with the $\alpha 1$ isoform in rat caveolae. Support to this conclusion comes from two other studies performed on rat renal cells (9) and isolated rat nephrons (31), which display a bimodal ouabain dose-response curve, suggestive of the presence of both a high and a low affinity component of Na-K ATPase. It is important to note that although these studies provide data qualitatively similar to those reported here, quantitative differences between the $K_i$ values for the high affinity ouabain binding site can be observed between the former (9, 31) and the present data. In particular, Dmitrieva et al. (9) reported a high affinity $K_i$ around 0.8 $\text{mM}$ by measuring the Na-K pump activity in cultured rat proximal tubular cells as ouabain-dependent $^{45}$K uptake, while Ferraille et al. (31) found a high affinity $IC_{50}$ of 4.6 $\mu \text{M}$ when measuring the ouabain-dependent Na-K ATPase inhibition curve in isolated rat PCT by $[^3P]\text{ATP}$ hydrolysis method. The different tissues examined and the different experimental procedures adopted for measuring the ouabain-dependent Na-K ATPase affinity cannot allow any quantitative comparison among these data. Moreover, in the present study both the low and the high affinity $IC_{50}$ values of ouabain for the Na-K ATPase may be underestimated because of the presence of 10 mM KCl in the medium for the ATPase activity measurement (28).

At present the cause of the high affinity ouabain effects on rat $\alpha 1$ Na-K ATPase in renal caveolae is unknown and should be investigated. Several factors might be involved. First, the high content of cholesterol and sphingolipids of caveolae (26) may influence either the activity or the affinity of ouabain for the rat $\alpha 1$ Na-K ATPase pool localized within this membrane district (32, 33). The contribution of caveolin 1 to the formation of a high affinity Src/Na-K ATPase complex can also be postulated. Second, the selective presence of the $\gamma_2$ but not $\gamma_3$ Na-K ATPase subunit, together with the $\alpha 1$ and $\beta 1$ Na-K ATPase in rat renal caveolae, may participate in the modulation of ouabain affinity in this subdomain. Third, a phosphorylation state of $\alpha 1$ Na-K ATPase within caveolae might induce a conformational modification of the enzyme favoring its interaction with ouabain. In this respect, here we demonstrate that, even in vitro, partially purified rat renal $\alpha 1$ Na-K ATPase is phosphorylated on tyrosine by recombinant Src. This effect is enhanced by $10^{-10}$–$10^{-8} \text{ M}$ ouabain and is associated with the appearance of a high affinity ouabain binding site, undetectable in the absence of Src. By contrast, ouabain does not affect the phosphorylation of another Src substrate. The result seems consistent with the hypothesis that the process of phosphorylation of Na-K ATPase induced by non-receptor tyrosine kinases, such as Src and Lyn (34), or in general by protein kinases (35), may be of physiological relevance in the regulation of Na-K ATPase function. Finally, the ouabain/Na-K ATPase interaction might also be favored by the process of concentration of ouabain into the kidney that produces tissue concentrations higher than those found in plasma, as shown in the present study and by others (36).

The specificity of the ouabain-induced effects in vitro and in vivo is further provided by the selective agonistic operated by PST 2328. Treatment of OS rats with PST 2328 prevents the ouabain-induced organ hypertrophy and antagonizes the binding of ouabain to the high affinity site of Na-K ATPase in rat renal caveolae, without affecting the low affinity component. Accordingly, in the immunoprecipitation experiments, PST 2328 reduces the formation of the Na-K ATPase/Src complex and the tyrosine phosphorylation of the co-immunoprecipitated proteins. Furthermore, the demonstration that the Cu$^{2+}$ antagonist amlodipine prevents the ouabain-induced increase of blood pressure without affecting in vitro organ hypertrophy and in vitro ouabain effects further reinforces the specificity of ouabain hypertrophic signaling.

Conclusions—The present study provides evidence that subnanomolar concentrations of ouabain, close to those of the circulating EO in humans, activate a Src kinase-dependent signaling pathway correlated with organ hypertrophy in vitro. This appears to work via an interaction with a high-affinity component of $\alpha 1$ Na-K ATPase localized within caveolae. Although a high-affinity ouabain binding component on a rat $\alpha 1 \beta 1$ Na-K ATPase is a surprising finding, evidence for this phenomenon has been obtained in several different types of experiment. These data thus fill the gap between in vitro experiments and cell culture findings and in vivo studies. They support the hypothesis of the pathological role of EO in human hypertension and related cardiovascular complications and provide one possible physiological mechanism. These findings are also relevant to the treatment of human hypertension and related cardiovascular complications since the antihypertensive compound, PST 2328, which is able to antagonize the pressor effect of ouabain, is also effective in preventing the ouabain hypertrophic effect by interfering, at the molecular level, with its dependent signaling pathway.

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Organ Hypertrophic Signaling within Caveolae Membrane Subdomains Triggered by Ouabain and Antagonized by PST 2238

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