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Angiotensin II-dependent phosphorylation at Ser¹¹/Ser¹⁸ and Ser⁹³⁸ shifts the E₂ conformations of rat kidney Na⁺/K⁺-ATPase

Katherine J. Massey^{*}, Quanwen Li[†], Noreen F. Rossi^{*‡}, Raymond R. Mattingly[†], and Douglas R. Yingst^{*.1}

^{*}Department of Physiology, Wayne State University, School of Medicine, Detroit, MI 48201, U.S.A.

[†]Department of Pharmacology, Wayne State University, School of Medicine, Detroit, MI 48201, U.S.A.

[‡]Department of Internal Medicine, Wayne State University, School of Medicine, Detroit, MI 48201, U.S.A.

Abstract

Kidney plasma membranes, which contain a single α -1 isoform of Na⁺/K⁺-ATPase, simultaneously contain two sub-conformations of E₂P, differing in their rate of digoxin release in response to Na⁺ and ATP. Treating cells with Ang II (angiotensin II) somehow changes the conformation of both, because it differentially inhibits the rate of digoxin release. In the present study we tested whether Ang II regulates release by increasing phosphorylation at Ser¹¹/Ser¹⁸ and Ser⁹³⁸. Opossum kidney cells co-expressing the AT_{1a} receptor and either α -1.wild-type, α -1.S11A/S18A or α -1.S938A were treated with or without 10 nM Ang II for 5 min, increasing phosphorylation at the three sites. Na⁺/K⁺-ATPase was bound to digoxin-affinity columns in the presence of Na⁺, ATP and Mg²⁺. A solution containing 30 mM NaCl and 3 mM ATP eluted ~ 20 % of bound untreated Na⁺/K⁺-ATPase (Population #1). Pre-treating cells with Ang II slowed the elution of Population #1 in α -1.wild-type and α -1.S938A, but not α -1.S11A/S18A cells. Another 50 % of bound Na⁺/K⁺-ATPase (Population #2) was subsequently eluted in two phases by a solution containing 150 mM NaCl and 3 mM ATP. Ang II increased the initial rate and slowed the second phase in α -1.wild-type, but not α -1.S938A, cells. Thus Ang II changes the conformation of two forms of EP₂ via differential phosphorylation.

Keywords

hypertension; kidney; ouabain; proximal tubule; sodium

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¹To whom correspondence should be addressed (dyingst@med.wayne.edu).

AUTHOR CONTRIBUTION

Katherine Massey characterized the phosphospecific antibodies, developed the experimental approach for measuring changes in phosphorylation, did experimental work with the digoxin-affinity columns and made the measurements of ouabain-sensitive ⁸⁶Rb uptake. Quanwen Li mutated the phosphorylation sites on the Na⁺/K⁺-ATPase. Noreen Rossi provided biochemical reagents and made critical comments on the physiological significance of the data. Raymond Mattingly performed the transfections and selection of the stable cell lines and supervised the testing of phosphospecific antibodies. Douglas Yingst supervised the overall project and wrote the paper. All authors contributed to the experimental design, the analysis of data and editing of the paper prior to submission.

INTRODUCTION

Ang II (angiotensin II) plays a key role in regulating sodium reabsorption in the proximal tubule, which reabsorbs two-thirds of the filtered sodium, and thereby affects blood pressure and may contribute to the development of hypertension and congestive heart failure. One of the major mechanisms by which Ang II regulates sodium reabsorption is to control both the short- and long-term activity of Na^+/K^+ -ATPase, the active transport mechanism that drives sodium transport across proximal tubules. These control mechanisms, which include both inhibitory and stimulatory effects [1–6], are only partially understood. One mechanism of acute stimulation is mediated by the AT_1 receptor, which activates PKC (protein kinase C) resulting in phosphorylation of the rat kidney Na^+/K^+ -ATPase at Ser¹¹ and Ser¹⁸, which increases trafficking of rat kidney Na^+/K^+ -ATPase to the plasma membrane [4]. Ang II also stimulates the activity of the rat kidney Na^+/K^+ -ATPase by increasing its affinity for intracellular sodium [1], the pump's rate-limiting substrate. This increase in affinity for sodium can occur in less than 2 min after Ang II binds to rat proximal tubules, and is associated with both increases and decreases in the phosphorylation of the Na^+/K^+ -ATPase at multiple sites on the α -subunit [6]. It has, however, never been demonstrated that Ang II-dependent phosphorylation of the Na^+/K^+ -ATPase can change any of the basic biochemical properties of the Na^+/K^+ -ATPase. We were, therefore, intrigued by the evidence that exposing rat proximal tubule cells to Ang II changes the rate at which the Na^+/K^+ -ATPase is released from a digoxin-affinity column in response to Na^+ and ATP [7], because this response means that Ang II must have changed the conformation of Na^+/K^+ -ATPase before it bound to digoxin. Furthermore, the Na^+/K^+ -ATPase in the plasma membranes from rat kidney elutes in two distinct peaks in response to different concentrations of Na^+ and each peak is differentially regulated by Ang II [7]. Thus Ang II-dependent cell signalling is capable of changing the basic biochemical properties of the rat kidney Na^+/K^+ -ATPase and this regulation involves two distinct populations of Na^+/K^+ -ATPase in plasma membranes that contain only a single isoform of Na^+/K^+ -ATPase [8].

In evaluating these experiments it is helpful to appreciate that digoxin and other cardiac glycosides bind to Na^+/K^+ -ATPase with high affinity when it is in either of two sub-conformations of E_2P [9,10]. In our experiments rat kidney Na^+/K^+ -ATPase was bound to the digoxin-affinity column in the type of E_2P conformation formed in the presence of Na^+ , ATP and Mg^{2+} [7]. After the binding of E_2P , the phosphate would have been quickly lost [9] to form what has historically been called a type I complex [9,10]. This is a stable dead-end complex with a single high-affinity binding site for cardiac glycosides that no longer participates in the Na^+/K^+ -ATPase reaction mechanism [10]. This complex, however, still contains both high- and low-affinity binding sites for Na^+ and ATP, which interact to change the conformation of Na^+/K^+ -ATPase and cause the subsequent release of bound cardiac glycoside [10]. Another sub-conformation of E_2P formed in the presence of $\text{Mg} + \text{P}_i$ also rapidly loses its phosphate to form what has historically been called a type II complex [9,10]. Complex II also has a single binding site for cardiac glycosides, but has different affinities for Na^+ and ATP than complex I, and releases bound cardiac glycoside at rates significantly different than complex I [9,10]. Thus these two sub-conformations of E_2P have different biochemical properties, as demonstrated by differences in which their immediate products subsequently release bound cardiac glycosides in response to Na^+ and ATP. On the basis of this information, we reasoned that if all the Na^+/K^+ -ATPase in the plasma membrane was in a single conformation of E_2P at the time it bound to digoxin, one would expect all of the Na^+/K^+ -ATPase to be eluted from the digoxin-affinity column in a single peak in response to the appropriate combination of Na^+ and ATP. Instead, we observed that rat kidney Na^+/K^+ -ATPase was eluted in two separate peaks in response to Na^+ and ATP, and that these differences were not just due to the ability of these ligands to reach their binding sites on the Na^+/K^+ -ATPase [7].

To explain how a single gene product [8] can be in different sub-conformations of E₂P at the time it bound to digoxin, we suggest that there are differences in the extent to which the Na⁺/K⁺-ATPase in rat kidney plasma membranes are post-translationally modified at multiple sites [11,12] and that phosphorylation can affect how E₂P is formed. On the basis of our previous work with minimally disrupted preparations of plasma membranes [7], we define Population #1 as molecules of Na⁺/K⁺-ATPase that release digoxin in the presence of low NaCl (30 mM) and physiological concentrations of ATP (3 mM) present in Solution #1 (fully defined below). Population #2 is defined as Na⁺/K⁺-ATPase that remains bound to digoxin in the presence of Solution #1 and subsequently releases digoxin in response to higher concentrations of NaCl (150 mM) and 3 mM ATP present in Solution #2 (see below).

In the present study we test the hypothesis that Ang II regulates the release of digoxin from Populations #1 and #2 by increasing phosphorylation at Ser¹¹/Ser¹⁸ and/or Ser⁹³⁸. Ser¹¹ and Ser¹⁸ were examined as a pair because both are phosphorylated by PKC when Ang II activates the AT₁ receptor and increases trafficking of rat kidney Na⁺/K⁺-ATPase to the plasma membrane [3]. Ser⁹³⁸ was tested because pertussis toxin blocks the ability of Ang II to regulate Na⁺/K⁺-ATPase activity [2] and Ser⁹³⁸ is phosphorylated by PKA (protein kinase A) [13–15]. The results of the present study support our hypothesis and show that Ang II inhibits the release of digoxin from Population #1 by increasing phosphorylation at Ser¹¹/Ser¹⁸ and inhibits the release of digoxin from Population #2, primarily by increasing phosphorylation at Ser⁹³⁸.

MATERIALS AND METHODS

Plasmids, OK (opossum kidney) cell lines and site-directed mutagenesis

The coding sequence for the rat α -1 subunit of the Na⁺/K⁺-ATPase subcloned into the pRC/CMV (cytomegalovirus) plasmid was a gift from Dr J. Lingrel (Vontz Center for Molecular Studies, University of Cincinnati Medical Center, Cincinnati, OH, U.S.A.) [16]. To mutate the phosphorylation sites, a pair of complementary and mutagenic primers was designed for each site: S11A, S18A and S938A. PCR was performed using PfuUltra high-fidelity DNA polymerase (Stratagene) and then the parental plasmid was digested by DpnI enzyme digestion. Since DpnI only cuts methylated target sequences, the newly synthesized mutated plasmids were not affected by DpnI and were transformed into *Escherichia coli* DH5 α . Plasmid preparations of all constructs were fully confirmed by automated DNA sequencing and then the α -1.S11A mutant was used to make the double mutant α -1.S11A/S18A, which was also fully confirmed by DNA sequencing.

OK cells that stably express the rat AT_{1a} receptor (T35 cells [17]) were transfected with plasmids expressing wild-type or mutant rat α -1 proteins by electroporation, as described previously [7]. Since the rat kidney Na⁺/K⁺-ATPase is resistant to inhibition by ouabain, the cells were selected for expression of the rat α -1 subunit by addition of 10 μ M ouabain to the growth medium from the day after transfection. This concentration of ouabain, which fully inhibits the endogenous OK Na⁺/K⁺-ATPase without significantly affecting the expressed rat Na⁺/K⁺-ATPase [7], was maintained throughout subsequent passaging of the cells. Experiments were performed with either a pooled selected population termed E10A [7] or a clonally selected line termed 10C1.5, with equivalent results. For cells expressing the double mutant α -1.S11A/S18A, experiments were performed with a pooled selected population termed D11,18p1. For cells expressing α -1.S938A, experiments were performed with a clonally selected line termed 938Ac1.2.

Na⁺/K⁺-ATPase activity

Cells were grown in supplemented DMEM (Dulbecco's modified Eagle's medium)-F12 at 37 °C on 12-well plates under an atmosphere of 5 %CO₂ to approximately 75 % confluence on the day of the experiment. At 24 h before an experiment, the medium was replaced with DMEM-F12 without serum or ouabain. At 2 h before the addition of isotope, the medium was replaced with DMEM-F12 containing either 0, 1 μM or 10 mM ouabain. ⁸⁶RbCl was added (at a final concentration of 12.5 μCi/ml) and cells were incubated with periodic shaking for 10 min in the same environment in which they had been grown. Thereafter the plates were put on ice, the extracellular isotope was aspirated, and the cells were washed with ice-cold buffer containing 140 mM choline chloride, 1.2 mM MgCl₂, 3 mM BaCl₂, 10 mM Hepes and 2 %BSA (pH 7.4). The cells were lysed with 2 %SDS, ⁸⁶Rb⁺ was counted and the rate of K⁺ uptake was calculated per milligram of total cellular protein [measured by BCA (bicinchoninic acid)], with the assumption that K⁺ was taken up at the same rate as ⁸⁶Rb⁺.

Since the endogenous Na⁺/K⁺-ATPase in the parental OK cells stably transfected with the rat AT_{1a} receptor (T35 cells) is almost fully inhibited by 1 μM ouabain [7], we estimated the activity of the rat Na⁺/K⁺-ATPase in our cell lines from the rate of ⁸⁶Rb⁺ uptake in the presence of 1 μM ouabain minus the rate in the presence of 10 mM ouabain. The activity of the endogenous Na⁺/K⁺-ATPase in T35 cells was calculated from the difference in ⁸⁶Rb⁺ influx between zero and 10 mM ouabain, and the activity of the endogenous OK Na⁺/K⁺-ATPase in the other cell lines was calculated from the difference in ⁸⁶Rb uptake between zero and 1 μM ouabain.

Testing how Ang II and phosphorylation of Na⁺/K⁺-ATPase regulates Populations #1 and #2 of Na⁺/K⁺-ATPase

To determine whether phosphorylation of Ser¹¹/Ser¹⁸ and/or Ser⁹³⁸ mediates how Ang II differentially regulates Populations #1 and #2, we used a previously developed procedure, which is to compare the amount and rate at which these two populations of rat kidney Na⁺/K⁺-ATPase elute from a digoxin-affinity column [7]. In these experiments, cells were treated with or without Ang II and then disrupted by osmotic lysis using a solution similar to one (Buffer R) previously used to prepare basolateral membranes from epithelial cells in the absence of detergents [18]. These conditions generate two populations of right-side-out vesicles from the plasma membrane containing Na⁺/K⁺-ATPase activity that can be separated from each other on the basis of differences in buoyant densities [18,19]. In our experiments we suggest that these vesicles, which were large enough [20] to contain multiple copies of the Na⁺/K⁺-ATPase, were separated from each other on the basis of average differences in the conformational state of the Na⁺/K⁺-ATPase. Any intracellular vesicles containing Na⁺/K⁺-ATPase present in the original lysate would be expected to pass through the digoxin-affinity column without binding, because the binding site for digoxin would be on the inside of the vesicle.

In each experiment, OK cells co-expressing the rat AT_{1a} receptor and either the wild-type or mutant forms of the rat α-1 subunit of Na⁺/K⁺-ATPase were treated for 5 min with or without 10 nM Ang II. These conditions were chosen because these are the conditions that have previously been shown to alter the elution of wild-type rat kidney Na⁺/K⁺-ATPase from the digoxin-affinity column [7]. Also, 10 nM Ang II stimulates the transport of Na⁺ across T35 cells [17] and is within the range of Ang II concentrations in the lumen of proximal tubules [21,22]. In each experiment, the same number of cells was treated with or without Ang II and each of these two groups of cells was separately osmotically lysed as described previously [7]. Nuclear-free cellular lysates were then prepared in the presence of a binding solution containing 30 mM NaCl, 3 mM ATP, 4 mM Mg²⁺, 25 mM imidazole, 1

mM 4-(2-aminoethyl) benzene sulfonyl fluoride, 0.8 μ M aprotinin, 20 μ M leupeptin, 40 μ M bestatin, 15 μ M pepstatin A, 14 μ M E-64, 1 μ M okadaic acid, 1 μ M microcystin and 5 μ M phenylarsine oxide (pH 7.4) [7]. This solution contains all the necessary ligands to put Na^+/K^+ -ATPase into the E_2P conformation, which binds to cardiac glycosides [9]. In each experiment one column was loaded with protein from control cells and a second column was loaded at the same time with an equal amount of protein from cells treated with Ang II. The pair of columns in each experiment contained the same bed volume of fresh digoxin-affinity medium, and both columns were run side by side under the same conditions. The amount of protein loaded on to the paired columns varied from experiment to experiment, but was consistently between 3 and 4 mg. Unbound protein was removed by washing the columns with the binding solution [7]. Each column was then eluted with Solution #1 [30 mM NaCl, 3 mM ATP, 4 mM EDTA and 25 mM imidazole (pH 7.4)] [7]. After most of Solution #1 had passed through the column, Solution #2 [150 mM NaCl, 3 mM ATP, 4 mM EDTA and 25 mM imidazole (pH 7.4)] was added to the top of the column. After Solution #2 drained to the top of the affinity matrix, a solution containing 2 % SDS was added to the top of the column to denature the protein that still remained bound to the column [7]. In these experiments there was a delay of approximately one fraction before a new solution added to the top of the column matrix could be collected at the bottom. The amount of protein in individual fractions was measured in duplicate using BCA. Six independent experiments were performed for each cell line. We note that we have already demonstrated that cellular protein only binds to the affinity column via a direct interaction between Na^+/K^+ -ATPase and digoxin and that the amount of Na^+/K^+ -ATPase in a given fraction is directly proportional to the amount of eluted protein [7].

Data analysis

The data for each cell line were analysed using a two-way ANOVA with repeated measures with two between subject factors [7]. If there was a significant difference between the 'second between subject factor' ($P < 0.05$), a post-hoc analysis was performed to determine whether there was a significant difference in the amount of protein in individual fractions from control compared with Ang II-treated cells [7]. Fractions that were shown to be significantly different ($P < 0.05$) by means of the post-hoc test are marked in the Figures with an asterisk. Fractions that exhibited a strong trend ($P < 0.1$), but failed to reach statistical significance, are marked in the Figures with a hash. Note that the statistical analysis is designed to detect differences with or without Ang II in the amount of protein in each fraction within each experiment, and to factor out differences in the absolute amount of protein between experiments. Therefore for each fraction in each experiment we also determined the amount of protein eluted with cells treated with Ang II minus the amount eluted from control cells. We then calculated and graphed the mean and S.E.M. of these data for all six experiments for each cell line.

To compare the relative rate at which each solution eluted Na^+/K^+ -ATPase from the digoxin-affinity column, we determined the natural log of the fraction of the protein that remained on the column as each fraction was collected and plotted these data as a function of the fraction number. Since the fractions are collected as a function of time, this approach is formally the equivalent of determining the first-order rate constant for the unidirectional efflux of a solute from a cell [23]. Finally, to determine whether Ang II significantly affected the amount of protein eluted by Solution #1, Solution #2 and SDS we compared the amount of protein eluted from control and Ang II-treated cells in each experiment using a paired Student's *t* test.

Development of polyclonal antibodies against rat kidney Na⁺/K⁺-ATPase α -1 phosphorylated at Ser¹¹ and Ser¹⁸

Antibodies obtained from Cell Signaling Technology were developed in rabbits immunized with phosphopeptides that represent the regions flanking Ser¹¹ [anti-P-Ser¹¹ (phosphorylated Ser¹¹)] or Ser¹⁸ [anti-P-Ser¹⁸ (phosphorylated Ser¹⁸)] of rat Na⁺/K⁺-ATPase α -1. Affinity-purified antibodies were tested for selectivity and specificity by Western blotting of OK cells that had been stably transfected to express either wild-type rat kidney Na⁺/K⁺-ATPase α -1 or α -1.S11A, or α -1.S18A mutant proteins.

Characterization of phosphospecific antibodies

The OK cells were grown on 100-mm plates [7]. The day before the experiment the medium was replaced with DMEM-F12. When the cells were ~ 75 % confluent and the plate contained 1.2–1.4 mg of protein. 10C1.5 cells, α -1.S11A cells or α -1.S18A cells were pretreated for 30 min $\pm$ 10 μ M BIM-1 (bisindolylmaleimide-1). Thereafter, the cells were incubated $\pm$ 0.2 μ M PMA for 5 min. Plates were placed on ice, rinsed with ice-cold Hanks buffer, and cells lysed with 1 ml of Laemmli sample buffer [24] containing the protease inhibitors [7] 1 μ M microcystin, 1 μ M okadaic acid and 1 mM sodium orthovanadate. The cellular lysate was harvested by scraping, further disrupted by repeated passage through a 26-gauge needle and frozen at -80°C until analysed. Samples were heated to 45°C for 20 min and proteins were separated by SDS/PAGE using an 8 % separating gel. Proteins were transferred on to PVDF, which was then probed for either phosphorylation at Ser¹¹ or Ser¹⁸ using site-directed phosphospecific antibodies developed with Cell Signaling Technology. Both of these antibodies were used at a dilution of 1:1000. Similarly, cells expressing either the wild-type rat α -1 subunit or the S938A mutant were pretreated $\pm$ 100 μ M IBMX (isobutylmethylxanthine) and $\pm$ 100 μ M H-89 for 30 min. Cells treated with IBMX were then treated for 5 min $\pm$ 20 μ M FSK (forskolin) and processed as described above for the other phosphospecific antibodies prior to analysing with an antibody specific for the phosphorylated version of Ser⁹³⁸ (Santa Cruz Biotechnology). The antibody against phosphorylated Ser⁹³⁸ was used at a dilution of 1:1500.

Western blotting was performed as described previously [25]. The protein content of cell lysates was standardized prior to loading ~ 30 μ g/lane. Following the detection of phosphorylated forms of Na⁺/K⁺-ATPase α -1, the blots were stripped and reprobed for total Na⁺/K⁺-ATPase using either an antibody raised against the rat α -1 subunit of Na⁺/K⁺-ATPase (Cell Signaling Technology, catalogue number 3010) or to the α -1 subunit of sheep kidney Na⁺/K⁺-ATPase (Sigma, catalogue number M8-P1-A3). These immunoblots are displayed in the lower panels of each relevant Figure. Results were recorded by enhanced chemiluminescent detection on to film. Each of the immunoblots shown is representative of a minimum of two independent experiments.

Testing how Ang II changes phosphorylation of Na⁺/K⁺-ATPase at Ser¹¹, Ser¹⁸ and Ser⁹³⁸

OK cells (E10A) expressing the wild-type α -1 subunit or α -1.S11A/S18A cells were grown on 100-mm culture plates and, prior to experiments, were treated as described above for the characterization of the phosphospecific antibodies. Cells were treated with or without 10 nM Ang II for 5 min and then analysed for the effect of Ang II on the phosphorylation at Ser¹¹, Ser¹⁸ and Ser⁹³⁸. Cells analysed for phosphorylation at Ser¹¹ and Ser¹⁸ were pretreated for 30 min with or without 1 μ M herbimycin A, a selective inhibitor of tyrosine kinases, and 10 μ M BIM-1 and H-89, selective inhibitors of PKC and PKA respectively, in DMEM-F12.

Materials

We purchased $^{86}\text{Rb}^+$ from PerkinElmer. Culture media and SDS/PAGE reagents were from Fisher Scientific. Acrylamide was from Bio-Rad Laboratories. PVDF was from Millipore. The 96-well plates were from Corning Costar. BCA was from Pierce Biotechnology. HRP (horseradish peroxidase)-conjugated secondary antibodies were from Jackson ImmunoResearch. KPL chemiluminescence reagents were from Insight Biotechnology. H-89 and BIM-1 were from LC Laboratories. Anti-P-Ser¹¹ (catalogue number 4020), anti-P-Ser¹⁸ (catalogue number 4006) and anti- Na^+/K^+ -ATPase (catalogue number 3010) antibodies were from Cell Signaling Technology. Anti-P- Na^+/K^+ -ATPase α (Ser⁹³⁸) (SC16710-R) antibody was from Santa Cruz Biotechnology. pRc/CMV was from Invitrogen. PfuUltra high-fidelity DNA polymerase was from Stratagene. Phosphatase inhibitors were from Axxora. Dry epoxy-activated Sepharose was purchased from Amersham Biosciences. All other reagents, including the antibody against the α -subunit of sheep kidney Na^+/K^+ -ATPase (M8-P1-A3) were purchased from Sigma-Aldrich.

RESULTS

Stable expression of wild-type and mutant forms of rat α -1 subunit of Na^+/K^+ -ATPase in OK cells

The rat wild-type α -1 subunit (rat α -1.wild-type) and two mutant forms (α -1.S11A/S18A and α -1.S938A) were functionally expressed in T35 cells (Table 1), a line of OK cells in which the rat AT_{1a} receptor has been stably expressed [17]. In each of these cell lines the activity of the rat Na^+/K^+ -ATPase was not significantly different than the activity of the endogenous OK Na^+/K^+ -ATPase in T35 cells (Table 1). In each cell line, the activity of the endogenous OK Na^+/K^+ -ATPase was reduced to approximately half of its wild-type level, as measured in T35 cells (Table 1). Also, the activity of the respective rat Na^+/K^+ -ATPase accounted for approximately two-thirds of the total Na^+ pump activity in each cell line (Table 1).

Three populations of Na^+/K^+ -ATPase in plasma membranes of α -1.S11A/S18A and α -1.S938A cells

The Na^+/K^+ -ATPase from α -1.S11A/S18A (Figure 1A) and α -1.938 (Figure 1B) cells elute from the digoxin-affinity column in three distinct populations, as previously shown for α -1.wild-type cells [7]. Under control conditions ~ 17 % of the Na^+/K^+ -ATPase from α -1.S11A/S18A cells bound to digoxin-affinity columns was eluted by Solution #1 and therefore is in Population #1 (Figure 1A and Table 2). Another 50 % of bound Na^+/K^+ -ATPase was subsequently eluted by Solution #2 and is in Population #2 (Figure 1A and Table 2). The remaining Na^+/K^+ -ATPase was subsequently eluted with denaturing concentrations of SDS and is in Population #3 (Figure 1A and Table 2). The Na^+/K^+ -ATPase from control α -1.938A cells was distributed between the three populations in a similar manner (Figure 1B and Table 2). Treating α -1.S11A/S18A and α -1.938A cells with Ang II significantly altered how the Na^+/K^+ -ATPase in each of these cell types was eluted from the digoxin-affinity column compared with Na^+/K^+ -ATPase from control cells (Figures 1A and 1B respectively), as previously shown for α -1.wild-type cells [7].

Effect of Ang II on the elution from the digoxin-affinity column

The effect of Ang II in α -1.wild-type cells was to decrease the amount of Na^+/K^+ -ATPase in Population #1 initially eluted by Solution #1 (Figure 2A, fractions 2 and 4). In contrast, Ang II stimulated the amount of Na^+/K^+ -ATPase in Population #2 initially eluted by Solution #2 (Figure 2A, fraction 11) and markedly reduced the amount of Na^+/K^+ -ATPase in Population #2 eluted in later fractions (Figure 2A, fractions 13–18). This pattern of effects

evident in α -1.wild-type cells was not present in either α -1.S11A/S18A cells or α -1.938A cells (Figures 2B and 2C). For instance, Ang II treatment did not reduce the amount eluted by Solution #1 in α -1.S11A/S18A cells (Figure 2B) and it did not reduce the amount of Na^+/K^+ -ATPase eluted by Solution #2 in α -1.938A cells (Figure 2C).

Ang II significantly decreased the total amount of Na^+/K^+ -ATPase in Population #2 in α -1.wild-type cells (Table 2). Adding Ang II to α -1.S11A/S18A cells also significantly reduced the amount of Na^+/K^+ -ATPase in Populations #1 and #2 (Table 2).

To evaluate the rate at which the Na^+/K^+ -ATPase was eluted from the digoxin-affinity columns from the three cell lines with or without Ang II, we plotted the natural log of the fraction of total protein that remained on the column at the time each fraction was collected as a function of the fraction number. In these graphs the slope of the curve is an estimate of the first-order rate constant in all three cell lines (Figure 3). Treatment of α -1.wild-type cells with Ang II (Figure 3A) slowed the initial elution rate of Population #1 by 33 % (Table 3, fractions 2–5), increased the initial rate of elution of Population #2 by 35 % (Table 3, fractions 10–12), and decreased the rate of elution of Population #2 in later fractions by 37 % (Table 3, fractions 14–17). In α -1.S11A/S18A cells (Figure 3B), Ang II had no effect on the rate of elution of Population #1 (Table 3, fractions 2–5), decreased the initial rate of elution of Population #2 by 40 % (Table 3, fractions 10–12), and had no effect on the rate of elution of Population #2 (Table 3, fractions 10–17). In α -1.938A cells (Figure 3C), Ang II slowed the rate of elution of Population #1 by 46 % (Table 3, fractions 2–5), but had no effect on the elution of Population #2 (Table 3, fractions 10–17).

Effect of Ang II on the phosphorylation of the α -subunit of Na^+/K^+ -ATPase

Characterization of the antibody against phosphorylated Ser¹¹—To test whether 10 nM Ang II increased phosphorylation of Ser¹¹ under the experimental conditions used in the present study, we developed and characterized an antibody against phosphorylated Ser¹¹. Incubating α -1.wild-type cells with PMA increased the signal of anti-P-Ser¹¹ antibody at the position of the rat kidney α -subunit (Figure 4A, top panel, lane 5 compared with lane 1 at the position of the arrow). The band at the arrow was identified as the α -subunit of the rat Na^+/K^+ -ATPase by stripping and reprobing the immunoblot in the top panel of Figure 4(A) with an antibody raised against the α -1 subunit of rat Na^+/K^+ -ATPase (Figure 4A, bottom panel). Adding BIM-1 blocked the stimulatory effect of PMA (Figure 4A, top panel, lane 5 compared with lane 6 at the position of the arrow). When α -1.S11A cells were incubated with or without PMA there was no signal at the position of the rat kidney α -subunit either in the absence or presence of PMA (Figure 4A, top panel, lane 7 and lane 11 at the position of the arrow). In addition to responding to changes in the phosphorylation of the α -subunit of rat kidney Na^+/K^+ -ATPase, the antibodies against phosphorylated Ser¹¹ also, on occasion, recognized a band that ran slightly higher than the rat kidney Na^+/K^+ -ATPase in both α -1.wild-type and α -1S11A cells, and responded to stimulation by PMA and Ang II similar to the rat α -1 subunit (Figure 4A, top panel, immediately above the arrow).

Effect of Ang II on phosphorylation of Ser¹¹—Treating α -1.wild-type cells with 10 nM Ang II increased the signal of anti-P-Ser¹¹ antibody at the position of the α -subunit of the rat kidney (Figure 4B, top panel, lane 1 compared with lane 3 at the position of the arrow). The band at the position of the arrow in the top panel of Figure 4(B) was identified as the rat kidney Na^+/K^+ -ATPase, because when this blot was stripped and reprobed with the antibody raised against the rat α -1 subunit there was only one band and it coincided with the lower band in the top panel of Figure 4(B). The stimulatory effect of 10 nM Ang II (Figure 4B, top panel, lane 1 compared with lane 3 at the position of the large arrow) was not present if the cells were pretreated with a kinase inhibitory cocktail containing BIM-1,

H-89 and herbimycin A (Figure 4B, top panel, lane 3 compared with lane 4). In these experiments there was also a second band that ran just above the rat α -1 subunit which was also increased by 10 nM Ang II and inhibited by the kinase inhibitory cocktail (Figure 4B, top panel, immediately above the arrow).

Characterization of the antibody against phosphorylated Ser¹⁸—To test whether Ang II increased phosphorylation of Ser¹⁸, we also developed and characterized an antibody against phosphorylated Ser¹⁸. Incubating α -1.wild-type cells with PMA produced a large response to anti-P-Ser¹⁸ at the position of the α -subunit (Figure 5A, top panel, lane 1 compared with lane 3 at the position of the arrow). PMA did not produce a signal at this position if it was added in the presence of BIM-1 (Figure 5A, top panel, lane 3 compared with lane 4 at the position of the arrow). Anti-P-Ser¹⁸ antibody also did not detect a signal at the position of the arrow in the lysates of α -1.S18A cells with or without PMA (Figure 5A, top panel, lane 5 compared with lane 7 at the position of the arrow). The band at the position of the arrow in the top panel of Figure 5(A) was identified as the α -subunit of rat Na⁺/K⁺ -ATPase, because it was in the same position as the signal obtained when the blot was stripped and reprobed with the antibody raised against the rat kidney α -1 subunit (Figure 5A, bottom panel). Anti-P-Ser¹⁸ antibody also detected another protein that was not the Na⁺/K⁺ -ATPase that ran below the α -subunit (Figure 5A, top panel, lanes 1–8). The intensity of this lower band did not change following treatment with BIM-1 or PMA.

Effect of Ang II on phosphorylation of Ser¹⁸—Treating cells expressing the wild-type rat α -1 subunit with 10 nM Ang II increased the signal from anti-P-Ser¹⁸ antibody at the position of the rat α -1 subunit compared with control values (Figure 5B, top panel, lane 1 compared with lane 3 at the position of the arrow). Ang II did not increase the signal intensity in cells pretreated with the inhibitor cocktail containing BIM-1, H-89 and herbimycin A (Figure 5B, top panel, lane 3 compared with lane 4 at the arrow). The band at the position of the arrow was identified as the α -subunit of rat Na⁺/K⁺ -ATPase after the blot in the top panel of Figure 5(B) was stripped and reprobed with an antibody against the rat α -1 subunit (Figure 5B, bottom panel).

Characterization of the antibody against phosphorylated Ser⁹³⁸—To test whether anti-P-Ser⁹³⁸ antibody was able to detect changes in phosphorylation at Ser⁹³⁸, OK cells expressing wild-type rat α -1 subunit or α -1.S938A cells were treated IBMX/FSK with or without H-89. Adding IBMX/FSK to cells expressing the rat wild-type α -1 subunit increased the signal at the position of the α -1 subunit of Na⁺/K⁺ -ATPase (Figure 6A, top panel, lane 1 compared with lane 5 at the position of the arrow). The band at the position of the arrow was identified as the α -subunit of Na⁺/K⁺ -ATPase by stripping and reprobing the immunoblot in the top panel of Figure 6(A) with an antibody raised against the sheep α -1 subunit. The stimulatory effect of IBMX/FSK was partially inhibited by H-89 (Figure 6A, top panel, lane 5 compared with lane 6 at the position of the arrow). Adding IBMX/FSK to α -1.S938A cells produced almost no increase in the signal at the position of the α -subunit (Figure 6A, top panel, lane 7 compared with lane 11 at the position of the arrow).

Effect of Ang II on phosphorylation of Ser⁹³⁸—Treating cells expressing the wild-type rat α -1 subunit with 10 nM Ang II increased the signal from anti-P-Ser⁹³⁸ antibody at the position of the α -1 subunit compared with control values (Figure 6B, top panel, lane 1 compared with lane 2 at the position of the arrow). The band at the position of the arrow was identified as the α -subunit of Na⁺/K⁺ -ATPase on the basis of aligning the bands with the signals in the bottom panel of Figure 6(B) obtained by stripping the immunoblot in the top panel of Figure 6(A) and reprobing with an antibody against the α -subunit of sheep kidney Na⁺/K⁺ -ATPase. Ang II also increased the signal from anti-P-Ser⁹³⁸ antibody in α -1.S11A/

S18A cells at the position of the α -1 subunit (Figure 6B, top panel, lane 3 compared with lane 4 at the position of the arrow). The increase in response of anti-P-Ser⁹³⁸ antibody to Ang II was not significantly different in the presence of the inhibitor cocktail containing BIM-1, H-89 and herbimycin A (results not shown). In both α -1.wild-type and α -1.S11,18A cells, the antibody against phosphorylated Ser⁹³⁸ also recognized a protein that ran slightly higher than the rat α -1 subunit that was often detected by the antibody against phosphorylated Ser⁹³⁸ (Figure 6B, top panel, above the arrow).

DISCUSSION

The results of the present study clearly show that Ang II-dependent phosphorylation of the rat kidney Na⁺/K⁺ -ATPase at specific sites can regulate how the Na⁺/K⁺ -ATPase releases bound cardiac glycoside, which is one of the basic biochemical properties of the Na⁺/K⁺ -ATPase. The critical evidence for this conclusion, which supports our central hypothesis, is the distinctive phenotype of separate cell lines expressing wild-type or mutant forms of the rat α -1 subunit. Supporting data show that Ang II increases phosphorylation of rat kidney Na⁺/K⁺ -ATPase at all three sites. These experiments also further demonstrate the presence of at least two populations of Na⁺/K⁺ -ATPase molecules in the plasma membrane that are each regulated by Ang II via distinct sets of phosphorylation sites: one via Ser¹¹/Ser¹⁸ and the other primarily via Ser⁹³⁸. Thus it is very likely that Ang II-dependent phosphorylation of rat kidney Na⁺/K⁺ -ATPase can regulate the intrinsic properties of the Na⁺/K⁺ -ATPase in the rat proximal tubule, and that more than one population of Na⁺/K⁺ -ATPase in the plasma membrane is part of this regulatory mechanism. Our ability to detect the multiple populations of Na⁺/K⁺ -ATPase was due to our novel use of a digoxin-affinity column which should also be useful in further purifying these populations and identifying possible interacting proteins that could have been present in our minimally disrupted preparation of plasma membranes.

On the basis of the previous demonstration that two sub-conformations of E₂P have different biochemical properties, as demonstrated by differences in which their immediate products subsequently release bound cardiac glycosides in response to Na⁺ and ATP [9,10], we suggest that Ang II-dependent phosphorylation of rat kidney Na⁺/K⁺ -ATPase at Ser¹¹/Ser¹⁸ shifts the sub-conformation of E₂P in Population #1 before it binds to digoxin. Likewise, phosphorylation of rat kidney Na⁺/K⁺ -ATPase at Ser⁹³⁸ shifts a different sub-conformation of E₂P in Population #2 before it binds to digoxin, changing the rate at which it was subsequently released in response to Na⁺ and ATP. These data implicating an effect of Ang II on E₂P is also of interest, because the formation of E₂P directly precedes one of the rate-limiting steps in the reaction mechanism of the Na⁺/K⁺ -ATPase [26].

In evaluating the biochemical identity of Population #1 and #2 it is important to note that Population #1 is not present in OK cells expressing a truncation mutant missing the first 32 amino acids of the α -1 subunit [7]. Also, in α -1.S938A cells Ang II significantly reduced the size of Population #1 (Figure 1B). Thus these data suggest that there are sites of phosphorylation in the N-terminus of the α -subunit that could play a role in the formation of Population #1. The Na⁺/K⁺ -ATPase in the kidney kinetically functions as a ($\alpha\beta$)₂ heterodimer [27–29]. However, these data do not exclude the possibility that at least some of the Na⁺/K⁺ -ATPase in the kidney plasma membrane could operate as a $\alpha\beta$ protomer. Therefore it is possible that Population #1, which is the smallest of the three populations, consists of protomers, and that Population #2 is made up of diprotomers, with the balance between the two being controlled via unidentified sites of phosphorylation in the N-terminus. Alternatively, both populations could consist of diprotomers, with one set containing a single bound digoxin and the other initially containing two. On the basis of the kinetic characteristics of the diprotomer model [8,27] it has been proposed that the classical

Albers–Post reaction mechanism for the kidney [30,31] be replaced with a two-gear bicycle model in which the Na^+/K^+ -ATPase pumps Na^+ and K^+ at a low rate when ATP is bound to one protomer and at a high rate when ATP is bound to both [28]. A key feature as to how the bicycle shifts gears is the extent to which the respective α -subunits within a diprotomer interact [28]. Therefore one of the mechanisms by which Ang II-dependent phosphorylation could regulate the kinetic properties of the diprotomer would be to modify one or both α -subunits within a diprotomer. Evidence for one of the α -subunits within a diprotomer being post-translationally modified was presented many years ago [32] and the idea that one or both could be modified has been with us for over 20 years [10].

The kidney Na^+/K^+ -ATPase of all mammalian species, including humans, have sites that could be phosphorylated by Ang II binding to AT_1 receptors on the plasma membrane [11,33]. Human kidney Na^+/K^+ -ATPase has both Ser^{11} and Ser^{938} , but not Ser^{18} [33]. Phosphorylation of Ser^{18} is required for Ang II to stimulate the activity of the rat kidney Na^+/K^+ -ATPase when it is expressed in OK cells, but not when expressed in LLCPK1 cells [34]. Ser^{938} has not been previously implicated in a mechanism by which Ang II regulates the kidney Na^+/K^+ -ATPase in any species. Furthermore, there has been a long standing controversy over whether or not Ser^{938} can be phosphorylated at all under physiological conditions [35,36]. Now, however, there is growing evidence implicating this site in the regulation of kidney Na^+/K^+ -ATPase in all mammalian species [33] and one of the predicted effects is regulating the affinity for intracellular Na^+ [37]. Interestingly, in the present study Ang II-induced phosphorylation at Ser^{938} may not have been due to the activation of PKA, because it was not blocked by H-89. Thus the increase in phosphorylation could have been due to the inhibition of a phosphatase, or there is an additional unidentified kinase that is capable of increasing phosphorylation at Ser^{938} .

Conclusion

The present study shows that Ang II-dependent phosphorylation of the rat kidney Na^+/K^+ -ATPase can rapidly alter two sub-conformations of E_2P in the plasma membranes of proximal tubules. This is the first demonstration that Ang II-dependent phosphorylation of any form of Na^+/K^+ -ATPase can regulate any of the basic biochemical properties of the Na^+/K^+ -ATPase. On the basis of these data we suggest that these phosphorylation-dependent changes in E_2P are part of a mechanism by which Ang II rapidly and acutely regulates the functional properties of the rat kidney Na^+/K^+ -ATPase, complimenting phosphorylation-dependent changes in trafficking of Na^+/K^+ -ATPase to the plasma membrane.

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Abbreviations used

Ang II angiotensin II

BCA	bicinchoninic acid
BIM-1	bisindolylmaleimide-1
CMV	cytomegalovirus
DMEM	Dulbecco's modified Eagle's medium
FSK	forskolin
IBMX	isobutylmethylxanthine
OK	opossum kidney
PKA	protein kinase A
PKC	protein kinase C

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Figure 1. Elution from digoxin-affinity columns of plasma membrane protein containing Na^+/K^+ -ATPase Populations #1 (fractions 1–9), #2 (fractions 10–18) and #3 (fractions 19–22) from control and Ang II-treated α -1.S11A/S18A cells (A) and α -1.S938A cells (B)

The data points shown for each fraction are the means of the values from six separate experiments. Fractions that are significantly different on the basis of our post-hoc analysis ($P < 0.05$) are marked with *. Fractions that exhibited a strong trend ($P < 0.1$), but failed to reach statistical significance are marked with #.

Figure 2. Relative effect of Ang II on the elution of plasma membrane protein containing Populations #1, #2 and #3 of Na⁺/K⁺-ATPase from rat α-1.wild-type (A), α-1.S11A/S18A (B) and α-1.S938A (C) cells

Shown is the mean ± S.E.M. of the difference between the amount of protein in each fraction eluted from Ang II-treated cells minus the amount eluted from paired control cells. Fractions that have a significant difference compared with control on the basis of the post-hoc analysis ($P < 0.05$) are marked with *. Fractions that exhibited a strong trend ($P < 0.1$), but failed to reach statistical significance, are marked with #.

Figure 3. Effect of Ang II on the fractional amount of plasma membrane protein that remained on the digoxin-affinity columns as the columns were eluted with Solution #1, #2 and SDS for rat α -1.wild-type (A), α -1.S11A/S18A cells (B) and α -1.S938A cells (C)

The values shown are the natural log of the fraction of total protein that remained on the digoxin-affinity column as each fraction was collected. Shown are the means $\pm$ S.E.M. from the six separate experiments for each cell line.

Figure 4. Phosphorylation of rat kidney Na^+/K^+ -ATPase at Ser¹¹

(A) Top panel: characterization of the anti-P-Ser¹¹ antibody and the response to PMA ± BIM-1, an inhibitor of PKC. Lanes 1–6 contain protein from OK cells (10C1.5) expressing the wild-type (wt) rat α -1 subunit of Na^+/K^+ -ATPase. Lanes 7–12 contain protein from α -1.S11A cells. The samples in lanes 3, 4, 9 and 10 are from experiments in which the cells were exposed to 10 pM Ang II for 10 min prior to lysis. Lanes 13 and 14 contain 2.5 and 5 μg of rat kidney microsomes respectively. The arrow in the top panel is at the position of the rat α -subunit of Na^+/K^+ -ATPase as determined in the bottom panel. Bottom panel: the immunoblot in the top panel after stripping and reprobing with an antibody raised against the rat kidney α -1 subunit of Na^+/K^+ -ATPase showing the location and relative amount of Na^+/K^+ -ATPase. (B) Top panel: the effect of 10 nM Ang II with or without kinase inhibitors on phosphorylation at Ser¹¹ using an anti-P-Ser¹¹ antibody in OK cells (E10A) expressing the wild-type rat α -1 subunit of Na^+/K^+ -ATPase. The arrow in the top panel is at the position of the α -subunit of rat kidney Na^+/K^+ -ATPase as determined in the lower panel. A prolonged exposure is shown to reveal basal and stimulated phosphorylation. The kinase inhibitors include herbimycin A for tyrosine kinases, BIM-1 for PKC and H-89 for PKA (see the Materials and methods section). Bottom panel: the immunoblot in the top panel after stripping and reprobing with an antibody raised against the rat kidney α -1 subunit of Na^+/K^+ -ATPase showing the location and relative amount of Na^+/K^+ -ATPase.

Figure 5. Phosphorylation of rat kidney Na^+/K^+ -ATPase at Ser¹⁸

(A) Top panel: characterization of the anti-P-Ser¹⁸ and the response with or without PMA and with or without BIM-1. Lanes 1–4 contain protein from OK cells (10C1.5) expressing the wild-type (wt) rat α -1 subunit of Na^+/K^+ -ATPase. Lanes 5–8 contain protein from α -1.S18A cells. The arrow in the top panel is at the position of the α -subunit of rat kidney Na^+/K^+ -ATPase determined in the bottom panel. Bottom panel: the immunoblot in the top panel after stripping and reprobing with an antibody raised against the rat kidney α -1 subunit of Na^+/K^+ -ATPase showing the location and relative amount of Na^+/K^+ -ATPase.

(B) Top panel: effect of 10 nM Ang II with or without kinase inhibitors on phosphorylation at Ser¹⁸ assessed using an anti-P-Ser¹⁸ antibody in OK cells (E10A) expressing the wild-type rat α -1 subunit of Na^+/K^+ -ATPase. A prolonged exposure is shown to reveal basal and stimulated phosphorylation. Bottom panel: the immunoblot in the top panel after stripping and reprobing with an antibody raised against the rat kidney α -1 subunit of Na^+/K^+ -ATPase showing the location and relative amount of Na^+/K^+ -ATPase.

Figure 6. Phosphorylation of rat kidney Na⁺/K⁺-ATPase at Ser⁹³⁸

(A) Upper panel: characterization of the anti-P-Ser⁹³⁸ antibody and the response with or without IBMX/FSK and with or without H-89. Lanes 1–6 contain protein from OK cells (10C1.5) expressing the wild-type (wt) rat α -1 subunit of Na⁺/K⁺-ATPase. Lanes 7–12 contain protein from α -1.S938A cells. The samples in lanes 3, 4, 9 and 10 are from experiments in which the cells were exposed to 10 pM Ang II for 10 min. Lanes 13 and 14 contain 2.5 and 5 μ g of rat kidney microsomes respectively. The arrow in the top panel is at the position of the α -subunit of Na⁺/K⁺-ATPase as determined from the bottom panel. Bottom panel: the immunoblot in the top panel after stripping and reprobing with an antibody raised against the α -1 subunit from sheep kidney showing the location and relative amount of Na⁺/K⁺-ATPase. (B) Bottom panel: effect of 10 nM Ang II on phosphorylation at Ser⁹³⁸ using an anti-P-Ser⁹³⁸ antibody in OK cells (E10A) expressing the wild-type rat α -1 subunit and in α -1.S11A/S18A cells. Lane 5 contains 4 μ g of rat kidney microsomes. The arrow in the top panel is at the position of the α -subunit of Na⁺/K⁺-ATPase as determined from the bottom panel. As in Figures 4(B) and 5(B) a prolonged exposure is shown to reveal basal and stimulated phosphorylation. Bottom panel: the immunoblot in the top panel after stripping and reprobing with an antibody raised against the α -1 subunit from sheep kidney showing the location and relative amount of Na⁺/K⁺-ATPase.

Table 1

Endogenous, rat and total Na⁺/K⁺-ATPase activity in OK cells (T35 cells) expressing the rat AT_{1a} receptor and the indicated type of rat α -1 subunit of Na⁺/K⁺-ATPase

Type of rat α -1 subunit expressed	Rat Na ⁺ /K ⁺ -ATPase	Endogenous OK Na ⁺ /K ⁺ -ATPase	Total Na ⁺ /K ⁺ -ATPase	Rat Na ⁺ /K ⁺ -ATPase as a percentage of the total Na ⁺ /K ⁺ -ATPase
None (<i>n</i> = 3)	0	9.5 ± 2.1	9.5 ± 2.1	0
Wild-type α -1 (<i>n</i> = 7)	7.8 ± 1.2	4.1 ± 1.1	12.0 ± 2.0	65
α -1.S11A/S18A (<i>n</i> = 6)	13.1 ± 2.8	4.9 ± 2.9	18.0 ± 5.1	73
α -1.S938A (<i>n</i> = 8)	7.8 ± 1.2	4.4 ± 1.0	12.2 ± 1.9	64

Na⁺/K⁺-ATPase activity is expressed as ouabain-sensitive K⁺ uptake (nmol of K⁺/mg per min), under the experimental conditions where endogenous OK and rat Na⁺/K⁺-ATPase activity can be distinguished on the basis of differences in their sensitivity to ouabain. The values shown are the means ± S.E.M. of the indicated number of experiments.

Amount of protein (μ g) eluted from digoxin-affinity columns by Solutions #1, #2 and SDS and the sum total for control and Ang II-treated cells expressing the indicated type of rat α -1 subunit

Table 2

Type of rat α -1 subunit expressed	Protein eluted by Solution #1 (fractions 1-9 containing Population #1 of Na ⁺ /K ⁺ -ATPase)		Protein eluted by Solution #2 (fractions 10-18 containing Population #2 of Na ⁺ /K ⁺ -ATPase)		Protein eluted by SDS (fractions 19-22 containing Population #3 of Na ⁺ /K ⁺ -ATPase)		Total protein eluted (fractions 1-22)	
	Control (n = 6)	Ang II (n = 6)	Control (n = 6)	Ang II (n = 6)	Control (n = 6)	Ang II (n = 6)	Control (n = 6)	Ang II (n = 6)
Wild type rat α -1	379 $\pm$ 90	385 $\pm$ 78	1002 $\pm$ 198	771 $\pm$ 103*	591 $\pm$ 51	636 $\pm$ 93	1972 $\pm$ 222	1792 $\pm$ 238
α -1. S11A/18A	283 $\pm$ 75	238 $\pm$ 61*	860 $\pm$ 147	608 $\pm$ 164*	536 $\pm$ 79	591 $\pm$ 55	1679 $\pm$ 172	1436 $\pm$ 170
α -1. S938A	272 $\pm$ 52	199 $\pm$ 29*	895 $\pm$ 108	988 $\pm$ 116	741 $\pm$ 133	831 $\pm$ 123	1908 $\pm$ 108	2018 $\pm$ 36

Shown are the means $\pm$ S.E.M. for the six experiments performed with each type of cell. Those that are statistically different ($P < 0.05$) are indicated with *.

Table 3

Calculated rate constants for the elution of rat kidney Na^+/K^+ -ATPase from digoxin-affinity columns for control and Ang II-treated cells expressing different forms of the rat α -1 subunit

Type of rat α -1 subunit expressed	Solution #1 (fractions 2-5)			Solution #2 (fractions 10-12)			Solution #2 (fractions 14-17)			SDS (fractions 19-22)		
	Control	Ang II	Percentage change for Ang II compared with the control	Control	Ang II	Percentage change for Ang II compared with the control	Control	Ang II	Percentage change for Ang II compared with the control	Control	Ang II	Percentage change for Ang II compared with the control
α -1.wild-type	-0.038	-0.026	-33%	-0.141	-0.189	+ 35%	-0.0935	-0.059	-37%	-10.9	-11.76	+ 8%
α 1.S11A/S18A	-0.029	-0.027	-8%	-0.199	-0.120	-40%	-0.0476	-0.0450	-5%	-11.6	-11.17	-4%
α -1.S938A	-0.022	-0.012	-46%	-0.189	-0.160	-15%	-0.0533	-0.051	-5%	-11.5	-11.76	+ 2%

Rates are for the intervals indicated by the specified fractions. The values are taken from Figure 3.