Phosphorylation of Rat Kidney Na, K-pump at Ser-938 Is Required for Rapid Angiotensin II-dependent Stimulation of Activity and Trafficking in Proximal Tubule Cells

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Running head: Ser-938 mediates Ang II-dependent stimulation of Na, K-pump
Abstract

How angiotensin II (Ang II) acutely stimulates the Na, K-pump in proximal tubules is only partially understood, limiting insight into how Ang II increases blood pressure. Here we tested whether Ang II increases the number of pumps in plasma membranes of native rat proximal tubules under conditions of rapid activation. We found Ang II increased the amount of Na, K-pump in plasma membranes of native tubules by 33% under conditions (100 pM Ang II for 2 min) previously shown to increase the affinity of the Na, K-pump for Na and stimulate activity 3 fold. Second, we tested if previously observed increases in phosphorylation of the Na, K-pump at S938 were part of the stimulatory mechanism. These experiments were carried out in opossum kidney (OK) cells, cultured proximal tubules, stably co-expressing the AT\textsubscript{1} receptor and either wild-type or a S938A mutant of rat kidney Na, K-pump under conditions found by others to stimulate activity. We found that a 10 min incubation in 10 pM Ang II stimulated the activity of wild-type pumps from 2.3 to 3.5 nmoles K/mg protein/min and increased the amount of pump in the plasma membrane by 80%, but had no effect on cells expressing the S938A mutant. We conclude that acute stimulation of Na, K-pump activity in native rat proximal tubules includes increased trafficking to the plasma membrane and that phosphorylation at S938 is part of the mechanism by which Ang II directly stimulates the activity and trafficking of the rat kidney Na, K-pump in OK cells.

58  Protein kinase C, protein kinase A, Na, K-ATPase

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Abbreviations

α-1.wild-type, OK cell line developed from T35 cells that stably co-express the rat AT₁α receptor and the rat wild-type α-subunit; α-1.S938A, OK cell line developed from T35 cells that stably co-express the rat AT₁α receptor and a form of the rat wild-type α-subunit in which Ser-938 was mutated to an alanine; α-1.Δ32 cells, OK cell line developed from T35 cells that stably co-express the rat AT₁α receptor and a truncation mutant of the rat kidney Na, K-pump missing the first 32 amino acids of the N-terminus; ACE, angiotensin converting enzyme; Ang II, angiotensin II; anti-P-Ser938, phosphospecific antibody to the α-1 isoform of the Na, K-pump that is phosphorylated on Ser-938; BCA, bicincheninic acid; BIM 1, bisindolylmaleimide; FSK, forskolin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; H-89, N-[2-(p-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride; IBMX, isobutyl-1-mthylxanthine; NaKα, α-subunit of Na, K-pump; NBC, sodium bicarbonate co-transporter; NHE3, sodium hydrogen exchanger type 3; OK, opossum kidney; PCT, proximal convoluted tubule cells; PKA, protein kinase A; PKC, protein kinase C; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; T35 cells, OK cells that stably express the rat AT₁α receptor.
**Introduction**

The cellular and molecular mechanisms by which the hormone angiotensin II (Ang II) acutely stimulates the Na, K-pump and in proximal tubules are only partially understood, limiting insight into how Ang II dependent stimulation of sodium reabsorption in the proximal tubule increases blood pressure (13, 18). The ATP driven Na, K-pump provides the driving force for all sodium reabsorption in the kidney and Ang II plays a major role in controlling blood pressure via both its pressor effects and its stimulation of sodium reabsorption in the kidney.

It was first reported that Ang II directly stimulated Na, K-ATPase activity in native rat proximal tubules by increasing the affinity of the Na, K-ATPase for intracellular sodium (1), where Na, K-ATPase activity is a measure of the Na, K-pump’s ability to hydrolyze ATP and intracellular sodium is the pump’s rate-limiting substrate. We then showed in isolated rat proximal tubules that a 2 min exposure to 100 pM Ang II directly stimulated Na, K-pump activity 3 fold at rate-limiting concentrations of intracellular sodium and that stimulation was associated with a shift in the sodium activation curve to the left, clearly demonstrating an increase in the affinity for sodium (30). Nevertheless, in opossum kidney (OK) cells expressing wild-type and mutant forms of the rat α-1 subunit of the Na, K-pump others concluded that Ang II only directly stimulates Na, K-pump activity in proximal tubules by increasing the trafficking of the Na, K-pump to the plasma membrane (7). The evidence for this conclusion was that mutating either Ser-11 or Ser-18 to an alanine blocks both Ang II-dependent trafficking and Ang II-dependent stimulation of Na, K-pump activity (7). However, in native rat
proximal tubules immunocytochemical studies have shown that all of the Na, K-pump in
native rat proximal tubules appears to be closely associated with the plasma membrane
(14). Thus, for over 10 years the evidence has suggested that the activation mechanism
in native proximal tubules from rats was different than when the rat kidney Na, K-
ATPase was expressed in OK cells: a shift in kinetic properties in rats and increased
trafficking in OK cells expressing the rat kidney Na, K-ATPase.

We, however, then demonstrated that incubating OK cells stably co-expressing
the AT1 receptor and the rat kidney α-1 subunit of the Na, K-ATPase in the presence of
Ang II shifted the conformation of the rat kidney Na, K-pump (20). This result is
consistent with an Ang II-dependent shift in the kinetic properties of the rat kidney Na,
K-pump when it is expressed in OK cells, as previously observed in native rat proximal
tubules. In fact, shifts in conformation were observed under two sets of conditions (20).
One of these depends on increased phosphorylation of the α-subunit of the Na, K-pump at
S11/S18, which we tested together, and the other to Ang II-dependent phosphorylation
of the α-1 subunit at S938 (20). Those results were the first evidence that Ang II-
dependent phosphorylation of Ser-938 may be involved in the mechanism by which Ang
II activates the Na, K-pump in the proximal tubule. The potential role of S938 is of
special interest, because in contrast to S18, S938 is a conserved site of phosphorylation in
all mammalian forms of the Na, K-pump (24), which makes it potentially important for
human physiology. In contrast, despite being a major site of PKC phosphorylation in rat
kidney (11), Ser-18 is absent from the α-1 subunit of human kidney Na, K-pump (24).

Therefore, in the present study we have examined two fundamental issues
relevant to how Ang II can rapidly stimulate the activity of the Na, K-pump in the
proximal tubule. The first is to test the hypothesis that Ang II can rapidly increase the amount of Na, K-pump in the plasma membranes of native proximal tubules. The second hypothesis is that phosphorylation of Ser-938 in the α-subunit of the Na, K-pump is part of the mechanism by which Ang II acutely and directly stimulates the activity and trafficking of rat kidney Na, K-pump expressed in OK cells.

**Materials and Methods**

*Ethical Approval:* Experiments were performed in male Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) housed under controlled conditions (21–23°C; lights on, 0700-1900). They were permitted free access to water and standard rat chow. The rats were cared for in accordance with the principles of the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. All protocols were approved by the Wayne State University Institutional Animal Care and Use Committee.

*Isolation of proximal tubules:* Proximal convoluted tubule cells (PCT) from male Sprague-Dawley rats (200-225 g) were prepared by collagenase dispersion and isolated on Percoll gradients as previously described (30). Harvested PCT were suspended in an ice-cold Buffer A containing 75 mM choline chloride, 4 mM NaHCO₃, 5 mM KCl, 0.74 mM NaH₂PO₄, 5 mM glucose, 20 mM HEPES, 1.2 mM MgSO₄, 0.56 mM Na₂HPO₄, 4 mM lactate, 1 mM Na pyruvate, 0.1% BSA, 0.5 mM CaCl₂·2H₂O, 1 mM glutamine, L-alanine, and 1 mM butyrate, pH 7.4 and washed twice at 36 x g for 10 min.

*Effect of Ang II on amount of Na, K-pump in the plasma membranes of rat proximal tubules:* We tested if Ang II increases the amount of Na, K-pump in the plasma membranes of rat proximal tubules under the same conditions that we previously used to demonstrate that 100 pM Ang II directly stimulates rat Na, K-pump activity ~ 3-
fold at a normal intracellular sodium concentration of ~10 mM (30). Accordingly, after the tubules were isolated they were divided into two groups and suspended in buffer A at 37° C. Ang II was added to one group at a final concentration of 100 pM, an equal volume of vehicle was added to the other, and the tubules were incubated for 2 min. Thereafter, small volumes of three concentrated stocks were quickly added to both groups: Na acetate to increase the sodium concentration to 28 mM; choline chloride to increase its concentration to 97 mM, and monensin to achieve a final concentration of 15 µM. The tubules were then incubated for two more minutes at 37° C and then placed on ice.

After an aliquot was removed to determine the protein concentration the remaining tubules were labeled with biotin for isolation on immobilized streptavidin. The procedure we used was a modification of the procedure developed by Ortiz (23). Accordingly, the tubules were washed 3 times with ice-cold Buffer B containing 10 mM boric acid, 140 mM NaCl, 4 mM KCl and 1.8 mM CaCl2, pH 9.0 and centrifugation at 36 x g. The tubules were suspended in oxygenated ice-cold Buffer B containing 1.5 mg/ml biotin and incubated for 90 min at 4°C. The tubules were then collected by centrifugation and washed 3 times with ice-cold PBS supplemented with 100 mM lysine. The tubules were then lysed in ice-cold RIPA buffer (50 mM TRIS-HCl, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, pH 7.4) supplemented with protease [1.04 mM 4-(2-aminoethyl) benzene sulfonyl fluoride, 15 µM pepstatin A, 14 µM E-64, 36 µM bestatin, 21 µM leupeptin, and 0.8 µM aprotinin] and phosphatase inhibitors [1 µM microcystin, 1µM okadaic acid, and 1 mM sodium orthovanadate]. After 30 min the lysates were centrifuged and the total protein in the supernatant was
measured as described above. A sample containing 0.15 mg of total protein was mixed with 500 µl of immobilized streptavidin and incubated overnight at 4°C. Thereafter, the immobilized streptavidin and its bound proteins were washed with RIPA buffer, then with Buffer C (500 mM NaCl, 0.1% Triton X-100, 50 mM HEPES, pH 7.5, 0.1% SDS), followed by a solution containing 50 mM Tris-HCl, pH 7.4. The proteins were removed from the streptavidin using Laemmli sample buffer (17) at 45°C supplemented with 50 mM dithiothreitol and the protease and phosphatase inhibitors used above. The eluted proteins were separated by 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF by electrophoresis. The amount of Na, K-pump in each sample was quantified by immunoblotting as previously described using known amounts of rat kidney microsomes on each immunoblot as standards (32).

Development and Characterization of OK cell lines: OK cell lines stably co-expressing the rat AT_{1A} receptor and either the wild-type (α-1.wild-type) or S938A mutant (α-1.S938A cells) form of the rat α-1 isoform were developed, characterized and grown as previously described (20). The activity of the rat Na, K-pump in α-1.wild-type cells is 7.8 ± 1.2 nmoles K/mg/min and the activity of the remaining endogenous OK Na, K-pump is 4.1 ± 1.1 nmoles K/mg/min (20). In α-1.S938A cells the activity of the rat kidney Na, K-pump is also 7.8 ± 1.2 nmoles K/mg/min and the activity of the endogenous OK Na, K-pump is 4.4 ± 1.0 nmoles K/mg/min (20).

Effect of Ang II on Na, K-pump activity: In OK cells the activity of the endogenous Na, K-pump is fully inhibited by 1 µM ouabain (31). Therefore, rat Na, K-pump activity was measured as the difference in the rate of ^{86}Rb uptake in media containing 1 µM ouabain minus uptake in the presence of the same media containing 10
mM ouabain as previously described (20). Cells were treated $\pm$ 10 pM Ang II at $37^\circ$ C for 15 min, $^{86}$RbCl was added (12.5 $\mu$Ci/ml final conc.) and the uptake of isotope was measured over the subsequent 10 min period at $37^\circ$ C. In the calculation of Na, K-pump activity we assumed that $^{86}$Rb was being pumped at the same rate as the potassium ion.

**Effect of Ang II on amount of Na, K-pump in the plasma membranes of OK cells:** Cells were put in serum-free media twenty-four hours before an experiment. Each of the cell lines were treated $\pm$ 10 pM Ang II at $37^\circ$ for 10 min., which are conditions predicted to activate the rat kidney Na, K-pump expressed in OK cells (7). The cells were then labeled with biotin for isolation on immobilized streptavidin and the amounts of biotinylated Na, K-pump in control and Ang II-treated samples was determined using the protocol outlined above for rat proximal tubules.

**Development of a polyclonal antibody against rat kidney Na,K-ATPase $\alpha1$ phosphorylated at Ser-938:** Antibodies were developed by Cell Signaling Technology in rabbits immunized with phosphopeptides that represent the regions flanking Ser-938 [anti-P-Ser938 (phosphorylated Ser-938)] of rat Na, K-ATPase $\alpha1$. Affinity-purified antibodies were tested for selectivity and specificity by immunoblotting of four OK cell lines: $\alpha1$.wild-type cells, $\alpha1$.S938A cells, T-35 cells and $\alpha1$.Δ32 cells, which are T-35 cells stably expressing the rat AT1a receptor and a truncation mutant of the rat Na, K-pump missing the first 32 amino acids of its N-terminus (31).

**Characterization of anti-P-Ser938:** The procedures used were similar to those previously described (20) except where noted below. T-35 cells, $\alpha1$.wild-type cells, $\alpha1$.S938A cells, and $\alpha1$.Δ32 cells cells were grown on-100 mm plates until they were ~70% confluent. The day before the experiment the medium was replaced with DMEM-
F12 (20). Cells were pretreated at 37°C for 10 min with ± 10 µM H-89. IBMX was then added to a final concentration of 100 µM and the cells incubated for an additional 25 min at which time 20 µM FSK was added for 5 minutes. The incubation media was removed by aspiration and 250 µl of Laemmli sample buffer (17) pre-warmed to 60°C and containing 1 µM microcystin, 1 mM orthovanadate and protease inhibitors (31) was added to each plate. The cells were then dislodged from the surface of the plate by scraping and the resulting cellular lysate collected in a microcentrifuge tube and incubated on the plates for an additional 20 min at 60°C. The lysate was then further disrupted by repeated passage through a 26-gauge needle and frozen at -80°C. Samples were warmed to 45°C for 20 min and the proteins were subjected to SDS/PAGE using a 7.5% separating gel. Proteins were transferred to nitrocellulose. For antibody detection assays, membranes were incubated for 1 hour at room temperature in blocking buffer (Tris-buffered saline [TBS], pH 7.4, with 0.1% Tween-20 [TBST] containing 5% skim milk powder), washed four times with TBST, and incubated overnight at 4°C with primary antibody in TBST with 2% BSA (anti-phospho Ser938 1:1000). After washing three times with TBST, membranes were incubated for 1 hour with horseradish peroxidase–conjugated secondary antibody (anti-rabbit IgG, 1:5,000 dilution) in blocking buffer. Membranes were again washed six times with TBST. Bound antibodies were detected by chemiluminescence on film. Responses were quantified by densitometry using a Fugi LAS-4000 and ImageReader software.

Effect of Ang II on the phosphorylation status of Na, K-pump at Ser-938: Both α-1.wild-type and α-1.S938A cells were grown and treated as previously described (20).
Cells from each group were incubated with and without Ang II for 10 min at 37° C and then whole-cell lysates were prepared as described above.

**Statistics:** Differences between control and Ang II –treated samples were analyzed using a paired t-test. Values marked with “*” represent a significant difference ($P \leq 0.05$).

**Materials:** Materials were purchased from the following vendors as indicated: $^{86}$RbCl was from PerkinElmer (Waltham, MA); culture media and SDS-PAGE reagents from Fisher Scientific (Hanover Park, IL); acrylamide from BioRad Laboratories (Hercules, CA); PVDF from Millipore (Billerica, MA); 96-well plates from Corning Costar (Lowell, MA); BCA, NHS-SS-biotin and immobilized streptavidin from Pierce Biotechnology (Rockford, IL); HRP-conjugated secondary antibodies from Jackson ImmunoResearch (Westgrove, PA); KPL chemiluminescence reagents from Insight Biotechnology (Wembley, U.K.); BIM 1 from LC Laboratories (Woburn, MA); (Danvers, MA); DNA polymerase from Stratagene (La Jolla, CA); phosphatase inhibitors (microcystin and okadaic acid) from Axxora, LLC. (San Diego, CA); and forskolin, 7-Deacetyl-7-[O-(N-methylpiperazino)-γ-butyryl]-, dihydrochloride from Calbiochem (San Diego, CA). The molecular weight markers were MagicMark from Invitrogen (Carlsbad, CA). The antibody to the α-1 subunit of Na, K-ATPase (M8-P1-A3) and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

**Results**

**Effect of Ang II on the amount of Na, K-pump in the plasma membranes of isolated rat proximal tubules:** Ang II acutely increased the amount of biotinylated Na, K-pump in the plasma membranes of rat proximal tubules compared to vehicle-treated
tubules (Fig. 1A). The percent increase in biotinylated Na, K-pump was 33% (Fig. 1A) and occurred under the same conditions that Ang II stimulates Na, K-pump activity ~3 fold (30). Under the experimental conditions shown in Fig. 1 biotin, only labeled proteins that were in the plasma membrane; not intracellular proteins (Fig. 1B). For instance, the intracellular protein GAPDH, which was present in the whole cell lysate prepared after the cells were biotinylated, was among the proteins that passed through the streptavidin column without binding and was not present in the samples that were specifically eluted from the streptavidin columns with reducing agent (Fig. 1B).

**Effect of Ang II on rat Na, K-pump activity in OK cells:** Ang II stimulated the activity of the rat kidney Na, K-pump stably expressed in OK cells compared to the same cells under control conditions (Fig. 2). Ang II, however, had no effect on the activity of rat kidney Na, K-pump activity in α-1.S938A cells (Fig. 2). In these experiments all of the measured Na, K-pump activity is coming from the expressed rat kidney Na, K-pump without any contribution from the remaining endogenous OK Na, K-pump, because all solutions contained at least 1 µM ouabain, which does not inhibit the rat kidney Na, K-pump, but fully inhibits the endogenous Na, K-pump (31). Furthermore, the concentration of Ang II and the length of exposure were chosen to mimic the conditions used by others to show that Ang II directly activates the rat kidney Na, K-pump expressed in OK cells (7).

**Effect of Ang II on the amount of Na, K-pump in the plasma of α-1 wild-type and α-1.S938A cells:** Under the same conditions that it stimulated rat kidney Na, K-pump activity (Fig. 2) Ang II significantly increased the amount of biotinylated Na, K-pump in the plasma membranes of α-1.wild-type cells (Fig. 3). In contrast, Ang II had
no effect on the amount of biotinylated Na, K-pump in the plasma membranes of α-1.S938A cells (Fig. 3). These results support the conclusion that Ser-938 is part of the mechanism by which Ang II increases the amount of Na, K-pump in the plasma membranes of OK cells.

**Effect of Ang II on the phosphorylation of Na, K-pump at Ser-938:** We developed and characterized an antibody to detect changes in the phosphorylation of Na, K-pump at Ser-938, which is known to be a site of PKA phosphorylation (2, 12). The addition of IBMX/FSK to α-1.wild-type cells significantly increased the response of the antibody (Fig. 4A). The prior addition of H-89, an inhibitor of PKA, significantly decreased the response to IBMX/FSK (Fig. 4A). In α-1.S938A cells, in which ~2/3 of the total Na, K-pump is the S938A mutant (20), the addition of IBMX/FSK produced a more modest, albeit significant, increase in the signal compared to control, which was completely blocked by H-89 (Fig. 4A). In T35 cells, which are OK cells stably expressing the AT₁α receptor and only the endogenous Na, K-pump (20), the addition of IBMX/FSK also produced an increase in the signal compared to control conditions, which was blocked with the PKA inhibitor H-89 (Fig. 4B). Finally, the addition of IBMX/FSK to α-1.Δ32 cells, which do not contain either Ser-11 or Ser-18, significantly increased the response (Fig. 4B), which was also significantly reduced by the prior addition of H-89 (Fig. 4B). Thus, this phospho-specific antibody is detecting increases in the phosphorylation of Ser-938.

Ang II at a concentration of 10 pM increased the phosphorylation of Na, K-pump at Ser-938 in α-1.wild-type cells relative to control (Fig. 5A and Fig. 5B). These increases occurred under the same conditions that Ang II stimulated the activity of the rat
kidney Na, K-pump (Fig. 2) and increased the amount of Na, K-pump in the plasma membrane (Fig. 3). Ang II at 10 nM also increased the phosphorylation at Ser-938 relative to control (Fig. 5B, lane 1 versus lane 4).

**Discussion**

We have shown that a brief exposure (≤ 2 min) to Ang II increased the amount of Na, K-pump in the plasma membranes of native rat proximal tubules by 33%. This result supports the hypothesis that rapid stimulation of Na, K-pump activity in native rat proximal tubules includes increased trafficking of the Na, K-pump to the plasma membrane. The increase occurred under conditions previously shown to increase Na, K-pump activity in the rat proximal tubule over 3 fold at resting levels of intracellular sodium (30). Thus, the observed increase in the amount of Na, K-pump in the plasma membrane can only partially account for the observed stimulation of Na, K-pump activity. Intracellular sodium is the rate-limiting substrate for the Na, K-pump under physiological conditions and in our experiments treatment of proximal tubules with Ang II shifted the sodium activation curve to the left (30). Therefore, we conclude that rapid Ang II-dependent stimulation of the Na, K-pump activity in rat proximal tubules is due to a combination of two mechanisms: increased trafficking of the Na, K-pump to the plasma membrane and a change in the kinetic properties of the Na, K-pump, which includes an increased affinity for sodium. Whether or not increased trafficking to the plasma membrane and changes in the kinetic properties of the Na, K-pump always occur together in response to Ang II is another issue. It is possible that their relationship to each other could depend on other factors, such as the concentration of intracellular...
sodium (7), calcium (6, 29) or the extent to which specific cell signaling pathways are activated and which sites on the Na, K-pump have been phosphorylated (15).

Previous evidence in native rat proximal tubules for a population of Na, K-ATPase that could be trafficked to the plasma membrane includes the demonstration that rat proximal tubules contain Na, K-pump in clathrin-coated vesicles and early endosomes (4) and that cAMP acutely increases the amount of Na, K-pump in plasma membranes of rat proximal tubules (5). As to the location of these vesicles in the proximal tubules, work in OK cells expressing the rat kidney Na, K-pump suggest they reside in a pool of intracellular vesicles closely associated with the plasma membrane (9). This location would be consistent with all of the Na, K-pump in the rat proximal tubule being at or near the plasma membrane (14).

Our new data also directly support the hypothesis that phosphorylation of Ser-938 is part of the mechanism by which Ang II directly stimulates the activity and trafficking of rat kidney Na, K-pump stably expressed in OK cells. Specifically, we have shown that if Ser-938 is mutated to an alanine the ability of Ang II to stimulate rat kidney Na, K-pump activity (Fig. 2) and trafficking to the plasma membrane (Fig. 3) of OK cells is lost. We also showed that Ang II increases the net phosphorylation of Na, K-pump at Ser-938 in whole-cell lysates of α-1.wild-type cells (Figs. 5 and 6). Thus, we have identified a conserved site of phosphorylation potentially important for understanding how Ang II stimulates the Na, K-pump in both rat and human proximal tubules.

Ser-938 was identified as a site of PKA phosphorylation over twenty years ago (2, 12). Ever since there has been significant controversy over its physiological role and the extent to which it could be phosphorylated by PKA under physiological conditions (3, 19,
In our studies, Ang II-dependent increases in the phosphorylation of Ser-938 are not blocked by H-89 (20), a well-established inhibitor of PKA. H-89 did, however, effectively inhibit IBMX/FSK stimulated increases in Ser-938 phosphorylation in all of our cell lines (Fig. 4). Furthermore, in OK cells that stably express the rat AT1a receptor, the addition of Ang II to the outside of the cell subsequently decreases intracellular cAMP (27). Thus, it is unlikely that activation of the AT1 receptor in the proximal tubule increases phosphorylation at Ser-938 via PKA.

Ser-938 is in the cytoplasmic loop between helices 8 and 9 (22). Simulations based on the crystal structure predict that increased phosphorylation of Ser-938 would decrease the affinity for sodium (25), which is the opposite of what we have observed in rat proximal tubules (30). Thus, it is possible that the increased phosphorylation of Ser-938 that we observed in whole cell lysates represents Na, K-pump in intracellular vesicles. If this is the case, then increased phosphorylation at Ser-938 along with sites in the N-terminus of the Na, K-pump (7) could be part of the trigger to increase the interaction of the Na, K-pump with proteins (8, 10, 16, 33) that mediate trafficking of Na, K-pump to the plasma membrane.

In conclusion, we have shown that Ang II rapidly (≤ 2 min) increases the membrane abundance of the Na, K-pump in native rat kidney proximal tubules. We have also demonstrated that Ser-938, a conserved site of phosphorylation in both rats and humans, is part of the mechanism by which Ang II acutely stimulates rat kidney Na, K-pump activity and trafficking in OK cells. Based on these new observations and our previous work detailed above, we conclude that Ang II rapidly stimulates Na, K-pump activity in native rat kidney proximal tubules by both increasing the trafficking of Na, K-
pump to the plasma membrane and by changing the kinetic properties of the Na, K-pump, including an increase in the affinity of the Na, K-pump for intracellular sodium. In OK cells stably expressing the rat kidney Na, K-pump we conclude that Ser-11, Ser-18, and Ser-938 are all part of the mechanism by which Ang II directly stimulates the activity and trafficking of the rat kidney Na, K-pump.

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Author Contributions

Katherine Massey did most of the experimental work, including the biotinylation studies, measurements of ouabain-sensitive ^86^Rb uptake, and characterization of antibodies. Susan Keezer developed the phospho-specific antibody to phosphorylated Ser-938. Quanwen Li mutated the phosphorylation sites on the Na, K-pump. Noreen Rossi provided the methodology to isolate rat proximal tubules, 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 phospho-specific antibodies. Douglas Yingst supervised the overall project and wrote the manuscript. All authors contributed to the experimental design, the analysis of data, and editing of the manuscript.
References


Fig. 1A: The amount of biotinylated α-subunit of Na, K-pump in the plasma membranes of native rat proximal tubules acutely exposed ± 100 pM Ang II at 37°C. The values are means ± SE, where N = 5 separate rat preparations each measured once. The “*” denotes a significant difference ($P \leq 0.05$).
Fig. 1 B: A representative immunoblot run in parallel from one of the 5 experiments shown in Fig. 1A. Lanes 1 shows the relative amounts of the α-subunit of Na, K-pump and GAPDH in the lysates of rat proximal tubules not treated with Ang II at the time they were mixed with streptavidin. Lane 2 shows the relative amounts of both proteins from these control cells at the time they were specifically eluted from the streptavidin column with reducing agent. Lane 3 shows the relative amount of both proteins from Ang II-treated cells eluted from the streptavidin column with reducing agent. Lane 4 shows the relative amount of both proteins in the lysates of proximal tubules previously exposed to Ang II at the time they were mixed with streptavidin. Lanes 5, 6, 7, and 8 contain 0.6, 1.2, 2.5, and 5 μg of rat kidney microsomes (30), respectively.
**Fig 2:** Rat kidney Na, K-pump activity ± 10 pM Ang II in α-1.wild-type and α-1.S938A cells.

The values are means ± SE, where N = 5 and 6, respectively, where N is the number of independent experiments each measured once.
**Fig. 3:** The relative amount of biotinylated Na, K-pump in the plasma membranes of α-1.wild-type and α-1.S938A cells ± 10 pM Ang II compared to untreated control cells in the same experiment. The values are means ± SE, where N = 5 for α-1.wild-type cells and 6 for α-1.S938A cells, where N is the number of independent experiments each measured once. The “*” denotes that the ratio is significantly different from 1.0 ($P \leq 0.05$).
Fig 4A: Response of the antibody designed to detect phosphorylation of the Na, K-pump at S938, which is phosphorylated by PKA, in \( \alpha \)-1.wild-type and \( \alpha \)-1.S938A cells.

IBMX/FSK was added to increase cAMP and H-89 to inhibit PKA. The values are means ± SE, where N = 5 for \( \alpha \)-1.wild-type cells, 4 for \( \alpha \)-1.S938A cells, where N is the number of independent experiments each measured once. The experimental details are outlined in the Materials and Methods section under the subheading “Characterization of anti-P-Ser938.”

![Phosphorylation at Ser938](image.png)
Fig. 4B: A representative immunoblot for T-35 and α-1.Δ32 cells carried out under the same conditions as the experiments in Fig. 4A.
**Fig. 5A:** The relative effect of 10 pM Ang II on the phosphorylation of the Na, K-pump at Ser-938 in α-1.wild-type and α-1.S938A cells compared to untreated control cells in the same experiment. The values are means ± SE, where N = 3 for α-1.wild-type cells and 3 for α-1.S938A cells, where N is the number of independent experiments each measured once. The “*” denotes that the ratio is significantly different from 1.0 (P ≤ 0.05).
Fig. 5B: Representative immunoblot showing the relative effect of 10 pM and 10 nM Ang II in α-1 wild-type cells in increasing the phosphorylation of Ser-938 compared to IBMX/FSK to stimulate PKA.