Intracellular Na⁺ Regulates Dopamine and Angiotensin II Receptors
Availability at the Plasma Membrane and Their Cellular
Responses in Renal Epithelia*

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The balance and cross-talk between natriuretic and antinatriuretic hormone receptors plays a critical role in
the regulation of renal Na⁺ homeostasis, which is a ma-

or determinant of blood pressure. Dopamine and angio-
tensin II have antagonistic effects on renal Na⁺ and
water excretion, which involves regulation of the
Na⁺,K⁺-ATPase activity. Herein we demonstrate that
angiotensin II (Ang II) stimulation of AT1 receptors in
proximal tubule cells induces the recruitment of
Na⁺,K⁺-ATPase molecules to the plasmalemma, in a
process mediated by protein kinase Cβ and interaction
of the Na⁺,K⁺-ATPase with adaptor protein 1. Ang II
stimulation led to phosphorylation of the α subunit
Ser-11 and Ser-18 residues, and substitution of these
amino acids with alanine residues completely abolished
the Ang II-induced stimulation of Na⁺,K⁺-ATPase medi-
ated Rb⁺ transport. Thus, for Ang II-dependent stimula-
tion of Na⁺,K⁺-ATPase activity, phosphorylation of these
serine residues is essential and may constitute a
triggering signal for recruitment of Na⁺,K⁺-ATPase mol-
ecules to the plasma membrane. When cells were treated
simultaneously with saturating concentrations of do-
pamine and Ang II, either activation or inhibition of the
Na⁺,K⁺-ATPase activity was produced dependent on the
intracellular Na⁺ concentration, which varied in a
very narrow physiological range (9–19 mM). A small in-
crease in intracellular Na⁺ concentrations induces the
recruitment of D1 receptors to the plasma membrane
and a reduction in plasma membrane AT1 receptors.
Thus, one or more proteins may act as an intracellular
Na⁺ concentration sensor and play a major regulatory
role on the effect of hormones that regulate proximal
tubule Na⁺ reabsorption.

Sodium excretion by the kidney is a tightly regulated proc-
ess, and several cardiovascular diseases are usually associated
with its abnormal regulation. Variations in salt intake may
affect total body Na⁺ balance and induce a rise in blood pres-
ure unless homeostasis is maintained (1). The balance be-
tween natriuretic and antinatriuretic hormones plays a critical
role in the regulation of renal Na⁺ transport and excretion (1, 2). In proximal tubules, where more than 70% of filtered Na⁺
reabsorption occurs, the interplay between the antagonistic
actions of the natriuretic dopamine (DA)1 and the anti-natu-
retic angiotensin II (Ang II) represents an important mecha-
nism to regulate renal Na⁺ and water excretion (3). During salt
derprivation, enhanced Ang II production and AT1 receptor
expression in proximal tubules increase Na⁺ and water reab-
sorption (4–7). Conversely, Na⁺ load causes increased produc-
tion of DA and DA receptor expression in proximal tubule
epithelial cells promoting renal Na⁺ and water excretion (8–
11). Ang II antagonizes the natriuretic response elicited by DA
stimulation of D1 receptors (3, 12), and DA opposes the
Ang II-induced stimulation of Na⁺ uptake in proximal tubules (13, 14). Therefore, the balanced action of DA and Ang II has a
primary impact on proximal tubule Na⁺ reabsorption. The
process that determines the balance of hormonal action, and
how DA can reduce proximal tubule Na⁺ reabsorption in the
presence of saturating concentrations of the antagonist Ang
II, are some of the mechanistic aspects that remain to be
elucidated. Although some insights regarding the regulation by
DA have been reported by us and other researchers, the mo-
lecular mechanism by which Ang II regulates the activity of the
proteins involved in proximal tubule Na⁺ reabsorption is still
unknown (10, 11, 15–18). In this study, we have investigated
the cellular mechanisms beyond the antagonistic actions of Ang
II and DA and how these mechanisms are dependent on
changes in intracellular Na⁺ concentration.

EXPERIMENTAL PROCEDURES

Materials—Cells culture supplies were purchased from Invitrogen
and HyClone Laboratories (Logan, UT). Molecular biology reagents
were obtained from New England Biolabs (Beverly, MA), Promega
(Madison, WI), Stratagene (La Jolla, CA), and Sigma. Angiotensin II,
dopamine, PMA, and the anti-phosphoserine antibody were purchased
from Sigma. Sulfo-NHS-biotin was obtained from Pierce (Rockford, IL).
Antibodies against AP1 and AP2 were purchased from Upstate Bio-
tech (Lake Placid, NY). Antibody against D1 was obtained from
Alpha Diagnostics (San Antonio, TX). Antibody against AT1 was pur-
chased from Santa Cruz Biotechnology (Santa Cruz, CA). [32P]RbCl
was obtained from PerkinElmer Life Sciences. LY333531 was a kind
gift from Eli Lilly (Indianapolis, IN). Protein kinase C (PKC) isozyme
inhibitor peptides were a generous gift from Dr. Moely-Rosen (Stan-

1 The abbreviations used are: DA, dopamine; Ang II, angiotensin II;
NKA, Na⁺,K⁺-ATPase; DMEM, Dulbecco’s modified Eagle’s medium;
[Na⁺]i, intracellular Na⁺ concentration; OK, opossum kidney; PRC,
protein kinase C; PMA, phorbol 12-myristate 13-acetate; AP, adaptor
protein; sulfo-NHS, N-hydroxysulfosuccinimide.
ford University, Stanford, CA). All other reagents were of the highest grade available.

**Cell Culture and Transfection**—Studies were performed with the established opossum kidney (OK) epithelial cell line, which is often used as a physiological model system of renal proximal tubule function (19–21). OK cells were maintained at 37 °C (10% CO2) in Dulbecco’s modified Eagle’s medium with 10% calf serum and antibiotics (DMEM-10). Mutants of renal Na+–K+–ATPase (NKA) α1 subunit cDNA were prepared as described previously (22–25) from a plasmid containing the wild type α1 subunit sequence and complementary oligonucleotides containing the desired change. Briefly, annealed plasmid and oligonucleotides were subjected to PCR amplification with Pfu polymerase, followed by restriction of the original wild type template with DpnI. After transformation of bacteria, the recovered mutant plasmids were evaluated by restriction analysis and direct sequencing of the altered region. Plasmids containing the wild type and mutated α1 subunit cDNAs were transfected into OK cells using LipofectAMINE 2000 liposomes (Invitrogen) as described previously (22–25). Selection for cells expressing the highest level of rodent α subunit was achieved by exposing the cells to a medium containing 3 μM ouabain. Because the endogenous Na+ pump of OK cells is completely inhibited by this concentration of ouabain (22–25), only successful recipients of transfected rodent α subunit would be able to survive. Resistant colonies were expanded and maintained in DMEM containing 3 μM ouabain. Experiments were performed with a mix of at least 20 independent clones for each cell line, and NKA of mock-transfected cells (vector alone, vector plus liposomes, or liposomes alone) had the same activity and sensitivity to ouabain as non-transfected host cells.

**Determination of Rb Transport**—Measurements of NKA-mediated transport by Rb+ uptake were performed with attached cells as described previously (22–24). Briefly, cells were transferred to serum-free DMEM containing 50 mM HEPES, pH 7.4 (DMEM-HEPES), and either 1.5 mg/ml sulfo-NHS-biotin. After incubation for 1 h at 37 °C, the cells were washed three times with ice-cold saline and dissolved with SDS, and accumulated radioactivity was determined. NKA-mediated Rb+ transport was calculated from the difference between tracer uptake between same samples incubated in 3 μM and 5 mM ouabain. The ouabain-insensitive Rb+ transport (measured in the presence of 5 mM ouabain) was 25–30% of the total Rb+ transport measured. In some experiments, cells were treated with hormones, activators, and inhibitors before the Rb+ transport determination. The concentrations used and the time of treatment are described in the respective figures.

**Protein Biotin Labeling to Separate the Plasma Membrane Pool of NKA**—The experiments were performed with OK cells expressing the rodent wild type α1 subunit and grown to 80–90% confluency in six-well plates. After treatment of the cells with Ang II, the medium was changed to ice-cold 50 mM Tris-HCl, pH 7.5, 2 mM CaCl2, 150 mM NaCl, 1.5 mM MgSO4, 0.1% sulfo-NHS-biotin. After incubation for 1 h at 4 °C, the cells were scraped in immunoprecipitation buffer (20 mM Tris, 2 mM EDTA, 2 mM EGTA, 30 mM sodium pyrophosphate, pH 7.3) containing a protease inhibitor mixture, frozen in liquid nitrogen, thawed rapidly, probe-sonicated twice on ice-water bath, and frozen-thawed again. The cell suspension was centrifuged at 14,000 g for 5 min. The supernatant was separated, and protein concentration was determined. Aliquots containing equal amounts of protein were transferred to clean tubes, and 1% Triton X-100 and 0.2% SDS were added. Anti-α1 antibody was added, and the suspension was incubated for 1 h at 4 °C with end-over-end shaking and overnight with protein A/G-agarose, which had been pre-washed three times with phosphate-buffered saline and one wash with immunoprecipitation buffer containing 1% Triton X-100. After separation, the agarose beads were washed four times with immunoprecipitation buffer containing 1% Triton X-100 and 0.1% SDS and once with 50 mM Tris-HCl, pH 7.4, and finally resuspended in Laemmli sample buffer. Electrophoresis, Western blot analysis with anti-AP2 and anti-α1 antibodies, and autoradiography were performed as described previously (24).

**Determinations of DA and Ang II Receptors at the Plasma Membrane**—The experiments were the same as described for the determination of plasma membrane NKA α1, except that antibodies against D1 and AT1 were used. Other details are described in the respective figures.

**Monitoring Ionic Changes in OK Cells**—Optical determinations of the intracellular Na+ concentration ([Na+]i) with the Na+–binding benzo[26]furazan-isophthalate were performed as described previously (22, 23, 26). Based on the changes in intracellular Na+ produced by different concentrations of monensin, the following equation was deduced:

\[ [\text{Na}^+]_i = (2.2 \pm 0.1 \times 10^{-3} \text{[monensin]} + (8.9 \pm 0.7 \text{mm}) (26) \]

This equation was used to calculate the [Na+]i, which corresponds to the concentration of monensin in the cell medium.

**Determination of AP1 and AP2 Co-precipitation with NKA α1**—After treatment with Ang II, OK cells were dissolved, and the NKA α1 was immunoprecipitated with anti-α1 antibody. The precipitated material was separated by SDS-PAGE, and the proteins were transferred to a piece of polyvinylidene difluoride membrane. This was assayed by Western blot analysis with anti-AP1 antibody. After development and scanning, the membrane was stripped and tested successively with anti-AP2 and anti-α1 antibodies. The protein bands were developed and scanned. Each experiment was repeated three times.

**Other Determinations and Data Analysis**—Unless indicated otherwise, all treatments were performed at 23 °C. Reagents were dissolved in water, except for DA, which was dissolved in 0.5% sodium metabisulfite, FMA in 100% dimethyl sulfoxide, and monensin in 95% ethanol. For each assay, equal amounts of solvents were added to control and test samples. However, these amounts were minimal, and they did not appreciably change the ouabain-sensitive NKA-mediated Rb+ transport. Determinations of protein concentration and immunoprecipitation of NKA were performed as described previously (24). Comparisons between groups were performed by either Student’s t test for unpaired data or analysis of variance, as indicated in the figure legends.

**RESULTS**

**Ang II Has a Biphasic Action on Proximal Tubule NKA**—Picomolar concentrations of the hormone stimulate, whereas micromolar concentrations inhibit proximal tubule Na+ reabsorption (7, 18, 27). A similar effect was described for the Ang II-induced activation of proximal tubule NKA (3, 28). Importantly, under physiological conditions, only stimulating picomolar concentrations of Ang II are produced (18). We observed that treatment of OK cells with Ang II induced a concentration-dependent increase in Rb+ transport, and a maximal activation was achieved with 1 pM Ang II (Fig. 1A). The basal Rb+ transport was 7.6 ± 0.9 nmol/mg/min, and it was stimulated to a maximum of 10.6 ± 0.5 nmol/mg/min by 1 pM Ang II. Higher picomolar concentrations of Ang II resulted in lower stimulation of Rb+ transport (Fig. 1A). As reported by other researchers (7, 18, 27), we also observed that micromolar concentrations of Ang II inhibited Rb+ transport (data not shown). At all tested picomolar concentrations of Ang II, activation of NKA was prevented by addition to the cell medium of 10 nM of the PKCβ inhibitor LY333531 (Fig. 1A). This inhibitor had no effect on the basal NKA-mediated Rb+ transport by itself. At the concentration used (10 nM), LY333531 is a very specific inhibitor of the β-isofoms of PKC (29–31). Therefore, Ang II induces the stimulation of NKA activity via activation of PKCβ.

On the basis of pharmacological studies, the effects of Ang II on Na+ and fluid transport were attributed to AT1 receptors (32, 33). Consistent with this observation, Fig. 1B shows that the AT1 receptor antagonist candesartan (34) antagonized, in a concentration-dependent manner, the NKA stimulatory effect of a maximally effective dose (1 pM) of Ang II. In the absence of Ang II, candesartan had no effect on the basal Rb+ transport. Therefore, Ang II acting through AT1 receptors induced the stimulation of OK cell NKA-mediated Rb+ transport.

Treatment of the cells with either 1 pM Ang II or 1 μM PMA produced about the same level of stimulation of Rb+ transport (Fig. 2A). This level of NKA activation was not further increased when cells were treated simultaneously with PMA and Ang II. The fact that the stimulatory effects of PMA and Ang II on Rb+ transport were not additive (PMA, 34.6 ± 7.5%; Ang II, 32.2 ± 4.5%; PMA plus Ang II, 36.8 ± 7.5%) suggests that PMA and Ang II share a common signaling pathway to activate the NKA. Consistent with this conclusion, we have demonstrated previously that PMA-induced activation of NKA is also mediated by the β-isofoms of PKC (35). Fig. 2A also shows that 0.1 μM staurosporine prevented the stimulation of Rb+ transport.
inhibits all of the classic and novel PKC isoforms of change for each experimental condition was calculated with respect to a control in the absence of PMA and/or Ang II. *, $p < 0.01$ with respect to basal value, not treated with Ang II; $B$, the AT1 receptor antagonist candesartan blocks the Ang II-induced activation of NKA. Cells were treated with different concentrations of candesartan for 30 min before Ang II treatment. The percentage of change for each experimental condition was calculated with respect to a control in the absence of Ang II and candesartan. *, $p < 0.05$ with respect to control.

by either PMA or Ang II. At the concentration of 0.1 $\mu$M, staurosporine inhibits all of the classic and novel PKC isoforms but not the atypical PKC$\xi$ (29, 31).

**Ang II Induces Phosphorylation of the NKA $\alpha$ Subunit**—Ang II induces a significant increase in the phosphorylation level of NKA $\alpha$ subunit (Fig. 2B). As $\alpha_1$ Ser-11 and Ser-18 are targets for phosphorylation by PKC (15, 36, 37), the level of $\alpha_1$ phosphorylation induced by Ang II was determined in cells expressing $\alpha_1$ mutants in which either Ser-11 (S11A cells) or Ser-18 (S18A cells) residues were substituted by alanine residues. The basic level (without Ang II) of $\alpha_1$ phosphorylation was not significantly different between the mutants and wild type $\alpha_1$ subunits (Fig. 2B). Ang II induced an increased phosphorylation of S11A and S18A $\alpha_1$ mutants, but the final level of phosphorylation was lower in either mutant than in wild type $\alpha_1$ (Fig. 2B). Taking into consideration the variations inherent to these measurements, the sum of the levels of phosphorylation in Ser-11 and Ser-18 approximately corresponds to the level of phosphorylation in wild type $\alpha_1$. Therefore, Ang II treatment induced the phosphorylation of both Ser-11 and Ser-18 in NKA $\alpha_1$.

$\alpha_1$ Ser-11 and Ser-18 Are Essential for Ang II-induced Stimulation of NKA-mediated $Rb^+$ Transport—Activation of NKA by Ang II was not observed in cells expressing a truncated $\alpha_1$ in which the first 26 $\text{NH}_2$-terminal amino acids of $\alpha_1$ were eliminated ($\Delta 1$–26; see Fig. 3A). To further characterize $\alpha_1$ amino acids involved in Ang II activation of NKA, experiments were performed in cells expressing the $\alpha_1$ S11A and S18A mutants. The basal ouabain-sensitive $Rb^+$ transport was the same in cells transfected with wild type and mutant rodent $\alpha_1$ cDNAs (Fig. 3A). In cells expressing the wild type $\alpha_1$, treatment with Ang II resulted in increased levels of ouabain-sensitive $Rb^+$ transport. However, substitution of either Ser-11 or Ser-18 with alanine residues (S11A and S18A) greatly im-

Fig. 1. A, effect of the PKC$\beta$ inhibitor LY335531 on Ang II-induced activation of NKA-mediated $Rb^+$ transport. Cells were treated with 10 nM LY335531 for 30 min before treatment with various concentrations of Ang II for 10 min. The percentage of change for each experimental condition was calculated with respect to a control measured in the absence of Ang II and LY335531. Data were analyzed using analysis of variance ($p < 0.01$) and $t$ tests ($*, p < 0.01$ with respect to control values; $#$, $p < 0.01$ with respect to basal value, not treated with Ang II). B, the AT1 receptor antagonist candesartan blocks the Ang II-induced activation of NKA. Cells were treated with different concentrations of candesartan for 30 min before Ang II treatment. The percentage of change for each experimental condition was calculated with respect to a control in the absence of Ang II and candesartan. *, $p < 0.05$ with respect to control.

Fig. 2. A, the stimulatory effects of Ang II and PMA on NKA-mediated $Rb^+$ transport are not additive. Cells were treated with 1 $\mu$M PMA and/or 1 $\mu$M Ang II for 10 min before the $Rb^+$ transport assay. When indicated, cells were treated with 0.1 $\mu$M staurosporine for 30 min. The percentage of change for each experimental condition was calculated with respect to a control in the absence of PMA and/or Ang II. *, $p < 0.05$ with respect to control. B, ANG II induces phosphorylation of NKA $\alpha_1$ Ser-11 and Ser-18 residues. OK cells stably transfected with the rodent $\alpha_1$ subunit wild type and the S11A and S18A mutants were treated with 1 $\mu$M Ang II for 10 min. NKA $\alpha_1$ was immunoprecipitated, and the phosphorylation level of phosphorylation induced by Ang II was determined in cells expressing the rodent $\alpha_1$ subunit wild type and the S11A and S18A mutants treated with 1 $\mu$M Ang II for 10 min. NKA $\alpha_1$ was immunoprecipitated, and the phosphorylation level was determined using an anti-phosphoserine antibody. Representative Western blots with anti-phosphoserine (Ph-Ser) and anti-$\alpha_1$ (NKA$\alpha$) antibodies are shown in the upper panel. The ratio of phosphorylation level (as determined in Ph-Ser) to protein (as determined in NKA$\alpha$) was calculated for each experimental condition and presented in the lower panel as a percentage change of Ang II-induced phosphorylation with respect to a non-treated wild type control (WT). *, $p < 0.05$ with respect to the increase of phosphorylation produced by Ang II in the wild type $\alpha_1$. 

 Intracellular $Na^+$ Affects D1 and AT1 Receptors Trafficking

28721
paired the stimulation of Rb⁺ transport (Fig. 3A). The same results were observed with PMA (Fig. 3A). Because none of the mutations altered the basal Rb⁺ transport measured in the absence of either Ang II or PMA, the results illustrated in Fig. 3A suggest that either S11A or S18A mutations have affected specifically the mechanism of NKA activation and not the intrinsic mechanism of NKA activity. We have demonstrated previously that the effect of PMA was specific and mediated by PKC and that 4α-phorbol 12,13-didecanoate, a phorbol ester that does not stimulate PKC, had no effect on the level of ouabain-sensitive Rb⁺ transport (22, 23).

Ang II-induced NKA Stimulation Is the Result of Recruitment of NKA Molecules to the Plasma Membrane—The Ang II-induced increase in NKA-mediated Rb⁺ transport may result from a more rapid ATPase rate of enzyme molecules already present at the plasma membrane or from the translocation of NKA molecules from intracellular compartments to the plasma membrane. Thus, we studied the effect of Ang II on the size of the plasmalemmal pool of NKA molecules. After treatment with Ang II, the temperature of the cell medium was reduced to 4 °C to label plasma membrane proteins with sulfo-NHS-biotin. The low temperature impeded the trafficking of NKA molecules between the plasma membrane and intracellular compartments locking the recruited molecules at the cell plasma membrane during treatment with sulfo-NHS-biotin. This reagent reacts with primary amino groups and does not permeate across biological membranes; thus, protein side chains containing primary amines that are exposed to the extracellular medium were biotinylated. Then, cells were lysed, and NKA-α1 was immunoprecipitated, and Western blot analysis was performed with AP1, AP2, and NKA-α1 antibodies. As described under “Experimental Procedures,” each blotted membrane was tested with the three antibodies. A representative Western blot for each antibody is illustrated in left panel. Quantitation data of precipitated AP1, AP2, and NKA-α1 are presented in the bar graph as a percentage change of Ang II-induced co-precipitation with respect to a non-treated control.

Fig. 3. A, both Ser-11 and Ser-18 residues of α1 are essential for the stimulation of NKA-mediated Rb⁺ transport induced by either Ang II or PMA. Rb⁺ transport mediated by NKA was determined in OK cells expressing the wild type and S11A, S18A, and Δ1–26 mutants of α1. S11A and S18A indicate substitutions by alanine residues of α1 Ser-11 and Ser-18. Δ1–26 represents a mutant in which amino acids 1–26 of the mature α1 subunit were deleted. The percentage of change in Rb⁺ transport for each condition was calculated with respect to a control in the absence of either Ang II or PMA. *, p < 0.05 with respect to control. B, Ang II induces the recruitment of NKA molecules to the plasma membrane. Cells were treated with 1 pM Ang II for 10 min and then the abundance of NKA molecules at the plasma membrane was determined by biotinylation as described under “Experimental Procedures.” A representative Western blot is shown in the upper panel. Quantitation data of biotinylated NKA-α1 are presented in the lower panel as the percentage change of Ang II-induced biotinylation with respect to a non-treated control. C, AP1, but not AP2, is involved in the Ang II-induced recruitment of NKA molecules to the plasma membrane. Cells were treated with 1 pM Ang II for 10 min and then NKA-α1 was immunoprecipitated, and Western blot analysis was performed with AP1, AP2, and NKA-α1 antibodies. As described under “Experimental Procedures,” each blotted membrane was tested with the three antibodies. A representative Western blot for each antibody is illustrated in left panel. Quantitation data of precipitated AP1, AP2, and NKA-α1 are presented in the bar graph as a percentage change of Ang II-induced co-precipitation with respect to a non-treated control.
plasma membrane pool of NKA molecules elicited by Ang II treatment (Fig. 3B) is consistent with the hormone-induced activation of Rb<sup>+</sup> transport illustrated in Fig. 3A. Therefore, Ang II-induced stimulation of NKA activity is produced by recruitment of NKA molecules to the plasma membrane.

**Ang II Induces the Interaction between Adaptor Protein 1 and NKA Molecules**—Recruitment of plasma membrane proteins occurs by selective recognition of the target protein, located in intracellular compartments, by interaction with AP1 followed by the protein translocation into the plasma membrane via clathrin-coated vesicles (38). To determine whether this mechanism is involved in the recruitment of NKA molecules elicited by Ang II, the level of co-precipitation of AP1 with NKA molecules was determined. As shown in Fig. 3C, there was no significant difference in the amount of NKA immunoprecipitated from samples treated or not with Ang II. However, the co-precipitation of AP1 and NKA molecules is increased by Ang II treatment. On the contrary, Ang II has no effect on the level of co-precipitation of NKA molecules and AP2 (Fig. 3C), which is responsible for clathrin vesicle formation during plasma membrane endocytosis. Indeed, we have demonstrated previously (39–42) that DA induces the interaction of AP2 with NKA molecules that are retrieved from the plasma membrane by clathrin-vesicle-mediated endocytosis.

**Small Changes of Intracellular Na<sup>+</sup> Concentration Modulate the Ang II-induced Stimulation of NKA Activity**—The Na<sup>+</sup> ionophore monensin was used to produce stable incremental concentrations of [Na<sup>+</sup>]<sub>i</sub>. Monensin has been used extensively as an Na<sup>+</sup> ionophore, and stable incremental increases in [Na<sup>+</sup>]<sub>i</sub>, by graded concentrations of monensin have been described in several tissues and cell lines (43–45). Monensin works as an Na<sup>+</sup> transporter when it binds to the cell membrane. Then, maintaining extracellular Na<sup>+</sup> at a physiological concentration (155 mM), Na<sup>+</sup> enters the cell in a saturable fashion that depends on the concentration of monensin added to the cell medium (26). In contrast, other Na<sup>+</sup> ionophores (e.g., gramicidin D) equilibrate Na<sup>+</sup> across the membrane and dissipate the Na<sup>+</sup> gradient. Because we are studying a process that depends on the maintenance (and modulation) of the Na<sup>+</sup> gradient across the cell membrane, it was important to perform the experiments under conditions in which the Na<sup>+</sup> gradient was maintained. Changes in [Na<sup>+</sup>]<sub>i</sub> were monitored by digital fluorescence microscopy of cells loaded with the Na<sup>+</sup> indicator Na<sup>+</sup>-binding benzofuran-isophthalate (26). Then, keeping extracellular Na<sup>+</sup> at 155 mM, cells were treated with 1, 2, 3, and 5 μM monensin to increase the [Na<sup>+</sup>]<sub>i</sub> from 9 mM (basal) to 11, 13, 15, and 19 mM, respectively. Therefore, the maximal change of [Na<sup>+</sup>]<sub>i</sub> was 10 mM. As the NKA activity is limited by the availability of intracellular Na<sup>+</sup>, the elevated [Na<sup>+</sup>]<sub>i</sub> elicited by monensin produced stimulation of basal NKA-mediated Rb<sup>+</sup> transport (26). Because of this, data presented in Fig. 4 represent the change in Rb<sup>+</sup> transport produced by DA and/or Ang II in the presence of different monensin concentrations, expressed as a percent of the Rb<sup>+</sup> transport measured in the presence of the corresponding concentration of monensin alone (no DA or Ang II). Although NKA inhibition induced by DA was higher at increasing [Na<sup>+</sup>]<sub>i</sub>, NKA activation induced by Ang II was reduced as the [Na<sup>+</sup>]<sub>i</sub> was raised (Fig. 4). When the cells were treated simultaneously with DA and Ang II, NKA stimulation was observed at basal [Na<sup>+</sup>]<sub>i</sub> (9 mM). However, this stimulation was stepwise reduced to become an inhibition at increasing [Na<sup>+</sup>]<sub>i</sub>. It can be argued that in the presence of both DA and Ang II, the NKA inhibition observed at 19 mM [Na<sup>+</sup>]<sub>i</sub>, is because of a shift of the action of Ang II to an inhibitory effect. This is clearly not the case, because at 19 mM [Na<sup>+</sup>]<sub>i</sub>, Ang II alone has no significant effect on NKA-mediated Rb<sup>+</sup> transport (Fig. 4).

Furthermore, in the presence of both Ang II and DA, the level of NKA inhibition at 19 mM [Na<sup>+</sup>]<sub>i</sub>, is the same as that produced by DA alone. Between 11 and 13 mM [Na<sup>+</sup>]<sub>i</sub>, treatment of the cells with both DA and Ang II would not translate into a significant modification of Rb<sup>+</sup> transport. It is likely that at these [Na<sup>+</sup>]<sub>i</sub>, the stimulatory effect of Ang II is compensated by the inhibitory effect of DA. Although we determined that the increased [Na<sup>+</sup>]<sub>i</sub>, was accompanied by a transient elevation in intracellular free calcium (Ca<sup>2+</sup>) concentration, we have also demonstrated that the intracellular Ca<sup>2+</sup> concentration was at or below the basal level when determinations of Rb<sup>+</sup> transport were performed, and the same results were obtained when a Ca<sup>2+</sup> chelator was introduced into the cells (26). Therefore, modulation of the actions of DA and Ang II as illustrated in Fig. 4 is not produced by changes in intracellular Ca<sup>2+</sup> but the result of different [Na<sup>+</sup>]<sub>i</sub>.

The intracellular signaling pathways associated with D1 or AT1 receptors can be stimulated directly by treatment of the cells with PMA (see Fig. 2A and Fig. 3A and Ref. 24). As illustrated in Fig. 4, whereas 1 μM PMA activates NKA at basal [Na<sup>+</sup>]<sub>i</sub>, the phorbol ester inhibits the NKA at 19 mM [Na<sup>+</sup>]<sub>i</sub>. Therefore, [Na<sup>+</sup>]<sub>i</sub>, modulates the stimulatory and inhibitory hormonal actions on NKA even when the hormonal receptors are by-passed by direct stimulation of the signaling pathways. We have demonstrated previously (35) that PKC<sub>i</sub> mediates the NKA inhibition induced by DA. However, PMA cannot activate...
PKCζ (31). To determine the PKC isozyme involved in this inhibition, peptides that inhibit the interaction of the PKC isozymes and their anchoring proteins (RACKs) were used (46). As illustrated in Table I, although PKCζ is the PKC isozyme involved in DA-induced inhibition of NKA, both PKCζ and PKCε participate in the PMA-elicited inhibition of NKA at 19 mM [Na⁺]. So far, we do not know the molecular mechanism involved in PMA stimulation of the DA pathway, but the above results suggest that PMA activates PKCε, which may then activate PKCζ molecules that are components of the DA intracellular pathway.

The Level of [Na⁺], Modulates the Plasma Membrane Pool of Ang II and DA Receptors—It has been described (47) that DA induced the recruitment of D1 receptors to the plasma membrane. Thus, we studied the effect of increased [Na⁺], on the level of plasma membrane D1 receptors. After the cells were treated with 5 μM monensin to increase [Na⁺], from 9 to 19 mM, the abundance of plasma membrane D1 receptors was determined at different times (Fig. 5A). This produced a steady increase of D1 receptors, and after 30 min of monensin treatment (the maximum time measured), a four to five times increase in plasma membrane D1 receptors was determined. Addition of 1 μM DA for 5 min produced a constant increase in the number of plasma membrane D1 receptors, and the level of increase (on top of those increased by monensin) was the same at the different times (Fig. 5A). Therefore, treatment for 5 min with 1 μM DA increased a fixed amount of D1 receptors independently of how many receptors were already at the plasma membrane.

In the absence of monensin treatment, DA produced a very small change in the plasma membrane abundance of D1 receptors (Fig. 5B). When added after an [Na⁺], increase from 9 to 19 mM, DA steadily increases the plasma membrane abundance of D1 receptors at the plasma membrane that reaches maximum at 20 min of DA treatment (Fig. 5B). At this point, D1 receptors at the plasma membrane have increased two and a half times. The DA-induced plasma membrane increase of D1 receptors is in addition to those receptors already recruited by monensin treatment.

Contrary to the effect on D1 receptors, an increase of [Na⁺], induced a reduction in AT1 receptors, and after 30 min about 50% of the AT1 receptors have been internalized (Fig. 5C). The opposite effects of [Na⁺], on D1 and AT1 receptors (Fig. 5) is consistent with the effect of [Na⁺], on the action of DA and Ang II on NKA activity (Fig. 4).

**TABLE I**

| Peptide inhibitors | RB⁺ transport | PMA | Monensin/PMA | Monensin/DA |
|--------------------|---------------|-----|--------------|-------------|
| Scrambled peptide  | 57 ± 11       | -44 ± 6 | -49 ± 8     |             |
| PKCζ peptide      | 58 ± 7        | -26 ± 5 | -43 ± 10    |             |
| PKCε peptide      | 54 ± 8        | -28 ± 6 | -28 ± 7     |             |

*p < 0.05 with respect to the scrambled peptide control.

**DISCUSSION**

The present study demonstrates that changes in [Na⁺], modulate the number of DA and Ang II receptors present at the plasma membrane. These changes lead to significant differences in cellular responses. Increases in [Na⁺], lead to a higher number of D1 receptors in the plasma membrane that is paralleled by a reduced abundance of AT1 receptors. Whereas at basal [Na⁺], Ang II stimulates NKA, this effect is blunted by rising the [Na⁺]. Conversely, at higher [Na⁺], there is an increased abundance of D1 receptors at the plasma membrane associated with a reduction in NKA-mediated RB⁻ transport.

We demonstrate that stimulation of OK cell AT1 receptors by Ang II leads to recruitment of NKA molecules to the plasma membrane in a process mediated by PKCζ and an increased interaction between NKA and AP1 molecules. The recruitment of NKA molecules to the plasma membrane is responsible for the increased NKA activity. This conclusion is supported by the observation that Ang II stimulation of AT1 receptors produced comparable increases in both the plasma membrane pool of NKA molecules and the ouabain-sensitive RB⁻ transport. That the stimulation of NKA activity by Ang II is because of a direct effect on NKA and not a consequence of increased Na⁺ permeability is shown by the fact that the stimulation is prevented by amino acid substitutions (S11A or S18A) or the deletion (Δ1–26) of the NKA α1 NH₂ terminus. LY333531 prevented the Ang II-dependent activation of NKA. It has been demonstrated previously (29–31) that 10 nM LY333531 inhibits (in vivo and in vitro) the activity of the PKCζ without any effect on other PKC isoforms, protein kinase A, Ca²⁺-calmodulin kinase, casein kinase, and Src tyrosine kinase. Therefore, the Ang II-induced activation of NKA is mediated by PKCζ. We have demonstrated previously that the β- and ε-isoforms of PKC are present in OK cells and that PKCζ is involved in the DA-dependent inhibition of NKA activity (35).

Determinations of the levels of RB⁻ transport and phosphorylation with S11A and S18A mutants suggest that Ang II-dependent stimulation of RB⁻ transport is exclusively dependent on PKC-mediated phosphorylation of Ser-11 and Ser-18. The fact that the presence of both serine residues is essential and that they are phosphorylated by stimulation of AT1 receptors suggests that phosphorylation is indeed involved in the mechanism of Ang II activation of NKA. Because activation of NKA produced by stimulation of AT1 receptors results from recruitment of NKA molecules to the plasma membrane, phosphorylation of Ser-11 and Ser-18 may be the signal that triggers this process, and thereby, only phosphorylated NKA molecules may be translocated from intracellular compartments to the plasma membrane. The fact that Ang II increases the co-precipitation of AP1 and NKA molecules suggests that translocation of NKA from intracellular compartments to the plasma membrane is a clathrin vesicle-mediated process.

DA treatment of proximal tubule cells results in inhibition of NKA activity (22, 23, 25, 48). DA acts through PKCζ and endocytosis of NKA molecules, and only phosphorylation of α1 Ser-18 is essential (24). Although DA inhibition of NKA is increased, stimulation of this activity by Ang II is reduced at higher [Na⁺]. That inhibition or activation of NKA may be observed when both DA and Ang II are added to the cell medium at maximal activating concentrations is dependent on the level of [Na⁺]. Therefore, the effect of hormones that regulate the rate of Na⁺ translocation across the proximal tubule epithelial cells, and thereby Na⁺ excretion, may be modulated by the level of [Na⁺]. The regulation of hormonal action by [Na⁺], may explain the observation that DA is able to reduce proximal tubule Na⁺ reabsorption in the presence of concentrations of Ang II that should totally override the effect of DA (18). Although luminal proximal tubule Ang II concentrations are in the 0.1–1 nM range, plasma concentrations are in the picomolar range (18, 49, 50). How can low concentrations of DA inhibit Na⁺ reabsorption in the presence of saturating concentrations of the antagonistic Ang II? Our results suggest that, even in the presence of maximal activating concentrations of...
the hormones, it is the level of $[\text{Na}^+]_i$ that may determine whether the DA or Ang II signaling pathway is activated. One possible mechanism by which $[\text{Na}^+]_i$ modulates the effects of these antagonistic hormones is the abundance of their receptors present at the plasma membrane. Consistent with this, we have shown that increasing $[\text{Na}^+]_i$ from 9 to 19 mM leads to an increased abundance of plasma membrane D1 receptors with a parallel decrease in AT1 receptors.

We have observed that the DA-induced recruitment of D1 receptors is very small in the absence of an increased $[\text{Na}^+]_i$, but also a pre-existent increased $[\text{Na}^+]_i$ is required for DA to induce the recruitment of D1 receptors to plasma membrane. These results represent the first report that $[\text{Na}^+]_i$ induces endocytosis of AT1 receptors. Cells were treated with 5 mM monensin for the indicated times. Then, the abundance of AT1 receptors at the plasma membrane was determined by cell surface biotinylation and Western blot analysis with an anti-AT1 antibody as described under "Experimental Procedures." A representative Western blot is shown in the upper panel. Quantitation data are presented in the lower panel as linear regressions of plasma membrane AT1 receptors in the presence or absence of monensin with respect to controls that were not treated with DA. The dashed line represents the plasma membrane abundance of D1 receptors in cells treated with DA in the absence of monensin. C. increased $[\text{Na}^+]_i$, induces endocytosis of AT1 receptors. Cells were treated with 5 mM monensin for the indicated times. Then, the abundance of AT1 receptors at the plasma membrane was determined by cell surface biotinylation and Western blot analysis with an anti-AT1 antibody as described under "Experimental Procedures." A representative Western blot is shown in the upper panel. Quantitation data are presented in the lower panel as linear regression of plasma membrane AT1 receptors with respect to a control non-treated with monensin.

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Intracellular Na\(^+\) Affects D1 and AT1 Receptors Trafficking

28726

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Intracellular Na\(^+\) Regulates Dopamine and Angiotensin II Receptors Availability at the Plasma Membrane and Their Cellular Responses in Renal Epithelia

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