We tested the hypothesis that the level of intracellular sodium modulates the hormonal regulation of the Na\(^{+},\text{K}^{+}\)-ATPase activity in proximal tubule cells. By using digital imaging fluorescence microscopy of a sodium-sensitive dye, we determined that the sodium ionophore monensin induced a dose-specific increase of intracellular sodium. A correspondence between the elevation of intracellular sodium and the level of dopamine-induced inhibition of Na\(^{+},\text{K}^{+}\)-ATPase activity was determined. At basal intracellular sodium concentration, stimulation of cellular protein kinase C by phorbol 12-myristate 13-acetate (PMA) promoted a significant increase in Na\(^{+},\text{K}^{+}\)-ATPase activity; however, this activation was gradually reduced as the concentration of intracellular sodium was increased to become a significant inhibition at concentrations of intracellular sodium higher than 16 mM. Under these conditions, PMA and dopamine share the same signaling pathway to inhibit the Na\(^{+},\text{K}^{+}\)-ATPase. The effects of PMA and dopamine on the Na\(^{+},\text{K}^{+}\)-ATPase activity and the modulation of these effects by different intracellular sodium concentrations were not modified when extracellular and intracellular calcium were almost eliminated. These results suggest that the level of intracellular sodium modulates whether hormones stimulate, inhibit, or have no effect on the Na\(^{+},\text{K}^{+}\)-ATPase activity leading to a tight control of sodium reabsorption.

The molecular mechanism by which hormone receptors coupled to stimulation of protein kinase C (PKC)\(^{2}\) regulate sodium reabsorption in renal proximal convoluted tubules is not well understood. The Na\(^{+},\text{K}^{+}\)-ATPase, located within the basolateral membrane of tubule epithelial cells, maintains a transmembrane concentration gradient for sodium, ensuring the net reabsorption of this cation. Hormonal short term regulation of Na\(^{+},\text{K}^{+}\)-ATPase activity may contribute to the ability of the kidney to adjust sodium reabsorption. In recent years, an increasing number of publications (1–4) have reported the short term regulation of kidney Na\(^{+},\text{K}^{+}\)-ATPase by hormones and intracellular second messengers that modulate proximal tubule sodium reabsorption. Renal Na\(^{+},\text{K}^{+}\)-ATPase activity is regulated by phosphorylation/dephosphorylation processes, and both cAMP-dependent protein kinase and protein kinase C (PKC) phosphorylate the Na\(^{+},\text{K}^{+}\)-ATPase (1–10). We have demonstrated that Ser-18 of the α-subunit is essential for the inhibition of the Na\(^{+}\)-pump activity by dopamine and that both Ser-18 and Ser-11 are essential for the stimulation of this activity by PMA (10–15). These amino acids are phosphorylated by different PKC isoforms during these processes (10–15). We also described that although dopamine inhibition of Na\(^{+},\text{K}^{+}\)-ATPase is mediated by endocytosis of plasma membrane Na\(^{+},\text{K}^{+}\)-ATPase molecules, PMA stimulation is due to recruitment of Na\(^{+},\text{K}^{+}\)-ATPase molecules from intracellular compartments to the plasma membrane (10, 15).

Decreased Na\(^{+},\text{K}^{+}\)-ATPase activity induced by dopamine is partly responsible for reduced sodium reabsorption during a high salt diet, and impaired regulation of the Na\(^{+},\text{K}^{+}\)-ATPase activity in renal tubules has been linked to the development of high blood pressure (1–4). In vivo, increases in dietary sodium intake or acute sodium loading lead to natriuresis accompanied by elevated urinary dopamine excretion, which suggested that dopamine produced endogenously by the epithelial proximal tubule cells might contribute to the natriuretic response (2–4). In this model, endogenously produced dopamine would be transported outside the proximal tubule cells where it binds to specific cell membrane receptors. The question arising is how an external effect, the acute sodium load, is translated into activation of the intracellular dopaminergic system. Our hypothesis is that an increased filtered load of sodium may lead to a transient elevation in intracellular sodium concentration that triggers the dopaminergic response. The present study was performed to test the hypothesis that the level of intracellular sodium modulates the tight control of Na\(^{+},\text{K}^{+}\)-ATPase activity by different agonists. We present evidence that the intracellular sodium concentration of kidney cells determines the level of inhibition of the Na\(^{+},\text{K}^{+}\)-ATPase activity by dopamine. We also demonstrate that direct stimulation of cellular protein kinase C by the phorbol ester PMA leads to either activation or inhibition of Na\(^{+},\text{K}^{+}\)-ATPase activity depending on the intracellular sodium concentration.

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** To whom correspondence should be addressed: College of Pharmacy, University of Houston, Houston, Texas 77204, the Department of Molecular Medicine, Karolinska Institutet, S-17176 Stockholm, Sweden, the Centro de Investigacion en Ciencias Agropecuarias, INTA, CC25, 1712 Castelar, Buenos Aires, Argentina, and the Department of Anatomy and Cell Biology, State University of New York, Brooklyn, New York 11203.

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EXPERIMENTAL PROCEDURES

Materials—Cell culture supplies were purchased from Invitrogen and HyClone Laboratories (Logan, UT). Molecular biology reagents were from New England Biolabs (Beverly, MA), Promega (Madison, WI), Stratagene (La Jolla, CA), and Sigma. Oubain was purchased from Calbiochem, PMA, ethyloxyresorufin, and dopamine were obtained from Sigma. [125I]RbCl was obtained from PerkinElmer Life Sciences. Other chemicals were of the highest quality available.

Cell Culture and Transfer—Ospossum kidney (OK) cells were maintained at 37 °C (10% CO2) in Dulbecco’s modified Eagle’s medium with 10% calf serum and antibiotics (DMEM-10). The expression vector pcMV containing the rodent Na+/pump α- subunit cDNA was obtained from PharMingen. Mutants of α1 were prepared, as previously described (12–15), from a plasmid containing the wild type α-subunit sequence and cDNA encoding 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 plasmid 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 α-subunit cDNAs were transfected into OK cells using liposomes, as described previously (12–15). Selection for cells expressing the highest level of rodent α-subunit was achieved by exposing them to a medium containing 3 μM ouabain. Because the endogenous Na+/pump of OK cells is completely inhibited by this concentration of ouabain, only successful recipients of transfected rodent α-subunit would be able to survive. Resistant colonies were expanded and maintained in DMEM-10 containing 3 μM ouabain. Experiments were generally carried out with at least two independent cell lines. The Na+/K+·ATPase 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 Protein Concentration—Cells were solubilized with SDS, and aliquots were used for protein determination. Protein concentration was determined by the bicinchoninic acid method (Pierce) using bovine serum albumin as a standard.

Determination of Rb+ Transport—Measurements of Na+/K+·ATPase-mediated transport by Rb+ uptake were performed with attached cells as described previously (12, 13, 15). Transfected cells grown in DMEM-10 were exposed for 2 h to 2 mM EGTA to facilitate access of ligands to Na+/K+·ATPase. To measure Rb+ transport, cells were transferred to serum-free DMEM containing 50 mM HEPES, pH 7.4 (DMEM/HEPES), and either 3 mM or 5 mM ouabain (incubation medium). Cells were incubated with these amounts of ouabain for first 20 min at 37 °C in an air atmosphere and then for 10 min at 25 °C. All treatments and determinations were performed at 25 °C. After treatment, a trace amount of [125I]RbCl was added to the cell medium. After 20 min, cells were washed four times with ice-cold saline and dissolved with SDS, and accumulated radioactivity was determined. Na+/K+·ATPase-mediated transport was calculated as the difference in Rb+ uptake between samples incubated in 3 mM and 5 mM ouabain. The ouabain-insensitive Rb+ transport was 25–30% of the total Rb+ transport measured. As PMA was dissolved in MeSO, the same volume of solvent was added to control samples. For the experiments with ethyloxyresorufin, the drug was dissolved in ethanol. The volume of solvent used did not alter the Rb+ transport of control samples. Each experiment was made in triplicate, and results are the mean ± S.D. of at least three independent experiments. Data are expressed as nanomoles of Rb+ transported per mg of protein per min, or as the percentage of Rb+ transported with respect to a control sample. When monensin was used, control sample was the ouabain-sensitive Rb+ transport of cells treated with monensin.

Experiments in a Low Calcium Medium—Several experiments were performed with cells in a low calcium medium. DMEM has a concentration of calcium of 1.8 mM. To reduce calcium concentration, 4 mM EGTA was added to the medium. Assuming that the total calcium in DMEM is free calcium, 4 mM EGTA would reduce the free calcium concentration to 0.12 μM. Several hours of incubation in this low calcium medium does not affect the attachment of the cells or their morphology as observed by microscopy. To further check this, experiments with 1,2-bis(o-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid (BAPTA) were used. The membrane-permeant acetoxymethyl form of BAPTA (BAPTA-AM) was added to the cell incubation solution at the concentration of 100 μM and incubated for 1 h at 25 °C, as indicated by the manufacturer (Molecular Probes, Eugene, OR).

Monitoring Ionic Changes in OK Cells—Optical determinations of intracellular sodium with a sodium-sensitive dye were performed as described previously (12, 13). Fluorescence measurements of [Na+]i were performed using the membrane-permeant tetra(ace toxyethyl) ester of the sodium-binding benzofuran-isophthalate (SBFI-AM, Molecular Probes, Eugene, OR) following standard protocols (16, 17). Cells were loaded for 3 h with the dye at room temperature in DMEM/HEPES 15% and then washing 2–5 times with SBFI-AM and 0.1% w/v Pluronic F-127 (Molecular Probes, Eugene, OR). After loading, the cells were washed several times with DMEM/HEPES and incubated for 30 min in the same medium to allow de-esterification of SBFI-AM. The complete hydrolysis of SBFI-AM to SBFI was judged by changes in the excitation and emission spectra (16). Optical measurements were performed in temperature-controlled room temperature medium. Since fluorescence measurements of (Ca2+), were performed using 1,6-aminocarboxy-2(5-carboxy-2-ox azolyl)-5-benzofuranxylxyloxy)-2(2-amino-5-methylphenoxycethane-N,N,N’,N’-tetraacetic acid (Fura-2). OK cells were loaded with the membrane-permeant tetra(ace toxyethyl) ester of this dye (Fura-2 AM, Molecular Probes, Eugene, OR) following similar protocols as with the SBFI-AM dye. Cells were loaded for 1–2 h with the dye at room temperature in DMEM/HEPES medium containing 2 μM Fura-2 AM and 0.1% w/v Pluronic F-127. Cells were then washed several times with DMEM/HEPES and incubated for 30 min in the same medium to allow de-esterrification of the dye. Optical signals were acquired in DMEM/HEPES medium at room temperature.

Optical Setup—The general plan of the optical setup used to monitor Na+ and Ca2+ levels in OK cells was similar to other standard methods using a dual-excitation fluorescence imaging system. Essentially the optical system consists of an inverted fluorescence microscope (Olympus, Melville, NY) with a video camera (Pentamax, Princeton Instruments, Trenton, NJ) attached to its video port. Light from a 75-watt xenon lamp (model 1600, Optic Quip, Highland Mills, NY) is collimated and rendered quasi-monochromatic by one of several interference filters, focused by means of a quartz UV-grade condenser and reflected to the preparation by a dichroic mirror. Excitation wavelengths were selected through a computer-controlled filter changer using excitation filters having wavelengths of 340 and 380 nm (5 nm bandwidth, Omega Optical, Brattleboro, VT). These excitation filters were selected because they are adequate for ratio measurements using either SBFI or Fura-2 indicators. By using these indicators, the fluorescence emission was detected above 420 nm after passing through a dichroic mirror (400 nm, Omega Optical, Brattleboro, VT) and a 420 nm highpass filter (Omega Optical, Brattleboro, VT). To ensure optical stability in the recordings and avoid possible photobleaching effects, the excitation light levels were reduced by neutral density filters until the emitted fluorescence intensity remained constant for 200 s of illumination. Significant levels of photobleaching were observed, and drugs at the concentrations used did not affect or quench fluorescence levels. No detectable change in the SBFI or Fura-2 ratios was observed in the pH range (7.4–7.1) tested in the present study. To improve efficiency, fluorescent light from the cells was collected by high numerical aperture (×20 or 40, Fluor; Nikon), which formed a real image on the CCD sensor of the video camera located in the image plane of the microscope. Optical sensitivity was optimized by controlling the number of times the CCD sensor recorded the fluorescence according to background fluorescence levels of the cells and the size of the fluorescence changes to be detected. By using the protocol previously described, fluorescence measurements of Na+ levels in cells loaded with SBFI usually required image exposures of 250–300 ms. For free Ca2+ determinations with Fura-2, exposures in the order of 100– 120 ms were needed. The chambers containing loaded cells were alternately excited at 340 and 380 nm by rapidly switching optical filters, and ratiometric determinations usually corresponded to image pairs taken within 800 ms. Sequential image pairs were usually collected every 6 s, although in some experiments faster time resolution was used.

Ionic Determination—Fluorescence measurements of [Na+]i and [Ca2+]i were performed using traditional ratiometric determination protocols (12, 13, 18, 19). Terms of the equation were assessed by in situ calibration at the end of each experiment with solutions of known ionic concentrations. Cytosolic Na+ levels were calculated according to the original equation described previously (18) with a K1 value for SBFI- Na+ of 18 mM. Calibrations of the excitation ratio were performed with membrane-permeabilized with gramicidin D (1 μM) and superfused with different standard Na+-containing solutions (12, 19). The free Ca2+ concentration was measured by determining the ratio of Fura-2 fluorescence at 340 nm (F340) and 380 nm (F380) excitations. There was no detectable photobleaching during measurements as determined by the isobestic wavelength for both dyes. The fluorescence ratio F340/ F380 of Fura-2 was calibrated in situ according to standard protocols using the same equation (18). In this case, calibrations were performed...
in OK cells permeabilized with the Ca\(^{2+}\) ionophore ionomycin. These calibrations were confirmed with cells permeabilized with either digitonin or saponin. \(F_{\text{max}}\) and \(F_{\text{min}}\) were determined in Ringer’s solution (1 mM Ca\(^{2+}\)) to saturate the Ca\(^{2+}\) indicator and then bathing the cell in low Ca\(^{2+}\) Ringer’s solution supplemented with 5 mM EGTA.

Image Processing and Statistical Analysis of Optical Data—Standard computer-based image analysis software was used for the analysis of Na\(^+\) and Ca\(^{2+}\) images. Video images were acquired at 8 bits resolution and stored in real time in a Pentium IBM-compatible computer system. Final ionic determinations were obtained applying standard ratiometric processing algorithms. In the figures, ionic changes in single cells are illustrated by pseudocolors reflecting ionic concentration ranges as determined according to ratiometric determinations (see above). Temporal plots of Na\(^+\) and Ca\(^{2+}\) transients were obtained from averaged values over 6 × 6 pixel kernels. To improve signal-noise ratio of SBFI fluorescence measurements, \([\text{Na}^+]_i\), determinations at each time results from the averaging of multiple samples acquired at faster time resolutions. Usually, individual points represent the average of 6 ratio determinations taken every 10 s (6 paired-frame/min), although in some experiments faster time resolution was used.

Statistical Analysis—Comparisons between groups were performed by Student’s \(t\) test for unpaired data.

RESULTS

Monensin Induces a Dose-dependent Increase of Intracellular Sodium in OK Cells—The sodium ionophore monensin was used to produce stable incremental elevations of intracellular sodium in OK cells. To determine the intracellular concentration of free sodium, OK cells were loaded with a sodium-sensitive dye, and the level of emitted fluorescence was monitored using a video imaging system, as described previously (12, 13). Fluorescent images of OK cells loaded with SBFI and excited at 380 nm are shown at the left of Fig. 1, A–C. Upon excitation at 340 and 380 nm, the level of intracellular sodium was calculated from the ratio of emitted fluorescence that was calibrated by loading the cells with standard sodium concentrations. Ratiometric images of SBFI-loaded cells, displayed in pseudocolor, were obtained at different times of treatment and concentrations of monensin. Images in the center of Fig. 1, A–C (XI), illustrate the basal sodium concentration (no monensin treatment). Basal levels of intracellular sodium ranged from 5.3 to 13.1 mM with an average of 7.8 ± 3.3 mM (n = 24). Images on the right of Fig. 1, A–C (XII) illustrate the intracellular sodium concentration 5 min after the addition of 6, 9, or 12 \(\mu\)M monensin, respectively, to the cell medium. Fig. 1, D and E, illustrate intracellular sodium levels at various times after addition of different monensin concentrations to the cell medium. In situ calibration of the excitation ratio of SBFI at various intracel-
Because the activity of the Na⁺,K⁺-ATPase has no effect on the Na⁺,K⁺-ATPase activity, but a steady increase in the inhibition of the Na⁺,K⁺-ATPase activity by dopamine was observed at increasing concentrations of intracellular sodium (Fig. 2B, left panel). The inhibitory effect illustrated in Fig. 2B is due to dopamine and not to monensin because in the range of concentrations this reagent was used it only produced “activation” of the Na⁺,K⁺-ATPase activity (Fig. 2A). Data presented in Fig. 2B, (left panel) represent the change in Na⁺,K⁺-ATPase activity produced by dopamine in the presence of different monensin concentrations, expressed as a percent of the Na⁺,K⁺-ATPase activity measured in the presence of that concentration of monensin alone.

We have demonstrated previously (11–13, 15) that treatment of OK cells with phorbol esters promoted a significant stimulation of Na⁺,K⁺-ATPase activity. This stimulatory effect, which occurs with cells containing basal concentrations of sodium, is illustrated in Fig. 2B. However, when increasing the intracellular sodium concentration, the activation of Na⁺,K⁺-ATPase induced by treatment with 1 μM PMA was gradually reduced to become a significant inhibition at concentrations of monensin higher than 3 μM (16 mM intracellular sodium) (Fig. 2B, right panel). Interestingly, as shown in Fig. 2B (right panel), there is a range of monensin concentration (1–2 μM) corresponding to 11–13 mM intracellular sodium concentration in which the treatment of the cells with PMA did not translate into any significant modification of the Na⁺,K⁺-ATPase activity. It is likely that at these concentrations of intracellular sodium the stimulatory and inhibitory effects of PMA are compensated. Data presented in Fig. 2B (left panel) represent the change in Na⁺,K⁺-ATPase activity produced by PMA in the presence of different monensin concentrations, expressed as a percent of the Na⁺,K⁺-ATPase activity measured in the presence of that concentration of monensin alone.

All of the following experiments to determine Rb⁺ transport were performed with cells treated with 5 μM monensin, which increased the intracellular sodium concentration from ~9 to ~20 mM. This concentration of monensin (5 μM) was chosen because the increase of intracellular sodium (~11 mM) produced is believed to be in the physiological range. The inhibition of Na⁺,K⁺-ATPase elicited by PMA resembles that produced by dopamine (Fig. 2B). Therefore, we performed several experiments to determine whether both reagents stimulate the same signaling pathway. We determined whether the effects of both agonists are additive. Also we determined whether phosphorylation of Na⁺,K⁺-ATPase α-subunit Ser-18 and production of 20-HETE are essential for the inhibition of Na⁺,K⁺-ATPase activity by both PMA and dopamine. Determination of Rb⁺ transport in cells treated simultaneously with both PMA and dopamine in the presence of 5 μM monensin (~20 mM intracellular sodium) showed that the inhibitory effects of PMA and dopamine on the Na⁺,K⁺-ATPase activity were not additive (PMA, ~45 ± 7%; dopamine, ~52 ± 9%; PMA and dopamine, ~56 ± 7%) (Fig. 3). Note that data in Fig. 3 represent the change of activity produced by PMA and/or dopamine, expressed as a percentage of the activity measured in cells treated with monensin alone.

We have demonstrated previously (10, 14) that whereas phosphorylation of the α-subunit Ser-18 is essential for dopamine inhibition of the Na⁺,K⁺-ATPase, activation of the Na⁺,K⁺-ATPase in response to PMA requires the phosphorylation of both Ser-11 and Ser-18 (15). Fig. 4 illustrates that the inhibition of Na⁺,K⁺-ATPase by PMA plus monensin requires

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**Fig. 2. A,** monensin increases Na⁺,K⁺-ATPase activity. Cells were incubated with the indicated concentrations of monensin for 30 min before Rb⁺ transport assay. *, p < 0.02 with respect to non-treated cells. B, intracellular sodium level modulates the effects of dopamine and PMA on the Na⁺,K⁺-ATPase activity. Cells were incubated with the indicated concentrations of monensin for 30 min before treatment with 1 μM dopamine for 5 min or 1 μM PMA for 10 min. The percentage of change for each concentration of monensin was calculated with respect to a control in the absence of either dopamine or PMA. *, p < 0.02 with respect to control. Assays were performed as indicated under “Experimental Procedures” and in the text.
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Fig. 3. The inhibitory effects of PMA and dopamine on the Na\textsuperscript{+},K\textsuperscript{-}-ATPase activity of cells treated with monensin are not additive. Cells were incubated with 5 \mu M monensin for 30 min before treatment with 1 \mu M PMA for 10 min and/or 1 \mu M dopamine for 5 min. In the experiment with both PMA and dopamine, dopamine was added 5 min after the beginning of PMA treatment. The percentage of change for each experimental condition was calculated with respect to a control in the absence of dopamine or/and PMA. * \( p < 0.05 \) with respect to control. Assays were performed as indicated under “Experimental Procedures” and in the text.

Fig. 4. Ser-18 is essential for both PMA- and dopamine-elicited inhibition of Na\textsuperscript{+},K\textsuperscript{-}-ATPase in cells treated with monensin. Rb\textsuperscript{+} transport mediated by the Na\textsuperscript{+},K\textsuperscript{-}-ATPase of cells expressing the wild type rodent \( \alpha \)-subunit and three \( \alpha \)-mutants was determined. \( \Delta (1-26) \) is the mutant in which amino acids 1–26 of the mature \( \alpha \)-subunit were deleted. The percentage of change for each condition was calculated with respect to a control in the absence of either dopamine or PMA. * \( p < 0.05 \) with respect to control. Assays were performed as indicated under “Experimental Procedures” and in the text.

the integrity of Ser-18 and that substitution of Ser-11 by an alanine residue prevented the stimulation of Na\textsuperscript{+},K\textsuperscript{-}-ATPase by PMA, but it did not affect the inhibition of this activity elicited by PMA plus monensin. Elimination of the first 26 amino acids of the \( \alpha \)-subunit totally blunted the inhibitory effects of both PMA and dopamine. None of these mutations affected the basal Na\textsuperscript{+},K\textsuperscript{-}-ATPase activity. Thus, the inhibition of Na\textsuperscript{+},K\textsuperscript{-}-ATPase by either PMA or dopamine requires the integrity of Ser-18.

The arachidonic acid metabolite 20-hydroxyeicosatetraenoic acid (20-HETE) is an important component of the signaling pathway stimulated by dopamine for inhibition of the Na\textsuperscript{+},K\textsuperscript{-}-ATPase activity (20, 21). Preincubation of the cells with ethoxyresorufin, an inhibitor of the cytochrome P450-dependent monoxygenase which produces 20-HETE (20), totally prevented the inhibition of Na\textsuperscript{+},K\textsuperscript{-}-ATPase activity induced by either dopamine plus monensin (control, \(-49 \pm 7\% \); +ethoxyresorufin, \(4 \pm 9\%\) ) or PMA plus monensin (control, \( -40 \pm 5\% \); +ethoxyresorufin, \( -6 \pm 9\%\) ) (Fig. 5). The effect of ethoxyresorufin appears to be specific for the inhibitory pathway because it has no effect on the basal Na\textsuperscript{+},K\textsuperscript{-}-ATPase or on the stimulation of this activity produced by PMA in the absence of monensin. Therefore, results illustrated in Figs. 2–5 support the hypothesis that PMA and dopamine stimulate the same intracellular messenger pathway to inhibit the Na\textsuperscript{+},K\textsuperscript{-}-ATPase.

Calcium Does Not Affect the Inhibition of Na\textsuperscript{+},K\textsuperscript{-}-ATPase by Dopamine and PMA—So far our results indicate that an increased intracellular sodium concentration is essential for the inhibition of the Na\textsuperscript{+},K\textsuperscript{-}-ATPase. Because changes in intracellular sodium produced by the monensin treatment may be associated with changes in intracellular calcium, we determined whether the effects described above were due to alterations in the intracellular calcium concentration. To reduce the entry of calcium into the cells, in some experiments, 4 mM EGTA was added to the cell medium. Because the incubation medium has a calcium concentration of 1.8 mM, a concentration of 0.12 \mu M free calcium was calculated assuming that the initial calcium concentration corresponded to free calcium. However, if some of the calcium in the cell medium was forming complexes with other molecules, the free calcium concentration in the presence of 4 mM EGTA should then be lower than 0.12 \mu M.

The intracellular free calcium concentration was monitored in cells loaded with the specific free calcium indicator Fura-2-AM, the membrane-permeant acetoxymethyl form of Fura-2. Fluorescent images of OK cells loaded with Fura-2-AM are shown on the left of Fig. 6, A and B. Determinations were performed with cells in a medium containing either 1.8 mM (Fig. 6A) or 0.12 \mu M (Fig. 6B) calcium. The intracellular level of free calcium (Fig. 6) and the effect of external calcium on the intracellular sodium concentration (Fig. 7) were determined in cells treated with 12 \mu M monensin to have the “worst case” scenario. To determine the intracellular free calcium concentration, the level of emitted fluorescence upon excitation at 340 and 380 nm was monitored. Images of Fura-2-AM loaded cells (displayed in pseudocolor) obtained at different times and concentrations of monensin illustrate the change in intracellular free calcium levels. Images labeled XI (Fig. 6, A and B) illustrate the basal free calcium concentration (no monensin treat-
Monensin produces a transient increase in cytosolic Ca\(^{2+}\). Images in A and B illustrate cytosolic Ca\(^{2+}\) levels in individual OK cells in a medium containing either 1.8 mM or 0.12 \(\mu\)M free calcium, respectively. The left image in each panel shows fluorescence video images of Fura-2-AM-loaded OK cells excited at 380 nm. Subsequent pseudocolored images correspond to ratiometric \([Ca^{2+}]\) determinations performed immediately before and 1.5 and 6 min after the addition of 12 \(\mu\)M monensin to the cell medium. Pseudocolored images in A and B represent ratio fluorescent intensity values proportional to cytosolic Ca\(^{2+}\) concentrations as determined by in situ calibration at the end of the experiments. Calibration bar corresponds to 15 \(\mu\)M. Plots on C illustrate the time course of \([Ca^{2+}]\), changes elicited by 12 \(\mu\)M monensin in OK cells in a medium containing either 1.8 mM (curve A) or 0.12 \(\mu\)M (curve B) free calcium. Arrows (X1, X2 and X3) indicate the time points when the individual images shown in A and B were taken. Measurements correspond to the mean \pm S.D. of 6 ratio determinations taken at 1-min intervals and performed in a 6 \times 6 pixel region of each cell. Assays were performed as indicated under “Experimental Procedures” and in the text.

The rise in intracellular free calcium elicited by monensin was totally dependent on the presence of extracellular calcium. When the determination of intracellular free calcium was repeated with cells in a medium with very low calcium concentration (0.12 \(\mu\)M), the rise in intracellular free calcium elicited by monensin was blunted (Fig. 6C, curve B). Although a small “bump” is apparent in curve B, the change in intracellular free calcium was not significant with respect to the basal concentration measured in the absence of monensin. After the bump, there was a steady reduction in intracellular free calcium concentration, and 13 min after the addition of monensin, the intracellular free calcium concentration was about 1/4 of that measured in the absence of monensin (Fig. 6C, curve B). Therefore, independent of the concentration of extracellular calcium (0.12 \(\mu\)M or 1.8 mM), intracellular calcium was at or below the basal level when the determinations of Rb\(^{+}\) transport were performed.

The change in intracellular sodium produced by monensin was dependent on the level of extracellular calcium (Fig. 7). The new steady state level of intracellular sodium elicited by 12 \(\mu\)M monensin was reached faster when the concentration of free-calcium in the cell medium was reduced from 1.8 mM to 0.12 \(\mu\)M (Fig. 7C). Four minutes after the addition of monensin, cells in 0.12 \(\mu\)M free calcium medium had about twice the concentration of intracellular sodium as compared with cells in medium containing 1.8 mM calcium. Although the level of ex-
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...was calculated with respect to a control in the absence of dopamine. *p < 0.02 with respect to control. Assays were performed as indicated under “Experimental Procedures” and in the text.

tracellular calcium affects the rate of intracellular sodium increase, the final steady state levels of intracellular sodium elicited by monensin appears not to be significantly different at the two extracellular calcium concentrations.

To determine whether the transient increase in intracellular free Ca\(^{2+}\) level produced by monensin is involved in the inhibition of Na\(^+\),K\(^-\)-ATPase by dopamine, the assay of ouabain-sensitive Rb\(^+\) transport was performed with cells in the presence of either 1.8 mM or 0.12 \(\mu\)M extracellular calcium (\(+\)EGTA). The right bar illustrates results obtained with cells incubated in a 0.12 \(\mu\)M extracellular calcium medium and loaded with 100 \(\mu\)M BAPTA-AM. Cells were incubated with 5 \(\mu\)M monensin for 30 min before treatment with 1 \(\mu\)M dopamine for 5 min. The percentage of change for each experimental condition was calculated with respect to a control in the absence of dopamine. *p < 0.02 with respect to control. Assays were performed as indicated under “Experimental Procedures” and in the text.

**FIG. 8.** Inhibition of Na\(^+\),K\(^-\)-ATPase activity by dopamine is not affected by the level of extra- and intracellular calcium. For the determinations, cells were incubated in a medium containing either 1.8 mM or 0.12 \(\mu\)M extracellular calcium (+EGTA). The right bar illustrates results obtained with cells incubated in a 0.12 \(\mu\)M extracellular calcium medium and loaded with 100 \(\mu\)M BAPTA-AM. Cells were incubated with 5 \(\mu\)M monensin for 30 min before treatment with 1 \(\mu\)M dopamine for 5 min. The percentage of change for each experimental condition was calculated with respect to a control in the absence of dopamine. *p < 0.02 with respect to control. Assays were performed as indicated under “Experimental Procedures” and in the text.

This is the first time that changes in intracellular sodium were measured to correlate accurately changes in this ion concentration with the regulation of the Na\(^+\),K\(^-\)-ATPase activity by dopamine and PMA. The present results demonstrate that regulation of the Na\(^+\),K\(^-\)-ATPase activity by dopamine and PMA is modulated by changes of the cell intracellular sodium concentration. Several lines of evidence indicate the modulation of the regulation of the Na\(^+\),K\(^-\)-ATPase activity by the level of intracellular sodium. This conclusion is supported by several lines of evidence: 1) Dopamine does not inhibit the Na\(^+\),K\(^-\)-ATPase activity unless intracellular sodium is increased (Fig. 2). 2) Basal intracellular sodium stimulation of PKC with PMA activates the Na\(^+\),K\(^-\)-ATPase, and the phorbol ester inhibits the Na\(^+\),K\(^-\)-ATPase activity at elevated intracellular sodium concentration (Fig. 2). 3) The level of the inhibition of Na\(^+\),K\(^-\)-ATPase activity by either PMA or dopamine is increased at increasing intracellular sodium concentrations (Fig. 2). Moreover, the inhibitory action by either PMA or dopamine occurs by triggering the same intracellular signaling pathway (Figs. 3–5). Thus, we observed a direct correlation between the level of intracellular sodium and the regulation of Na\(^+\),K\(^-\)-ATPase activity by PMA and dopamine.

The change in intracellular sodium concentration is likely to be exerting a permissive role on a particular signaling pathway involved in cellular homeostasis regulation. Effects of the level of intracellular sodium concentration on cell homeostasis have been suggested previously. A stimulation of the synthesis de novo of Na\(^+\),K\(^-\)-ATPase molecules by elevated intracellular sodium has been described in several tissues and cells (27–29), and a specific mechanism that involves a transcriptional regulation stimulated by elevated sodium has been reported (24).

These effects, however, are long term; the ones we describe in this report are short term regulations that do not involve the synthesis of new Na\(^+\),K\(^-\)-ATPase molecules (9–15). More related to short term regulation is the report that elevated intracellular sodium stimulates the production of dopamine in proximal tubule cells (30–32). The experiments described in this report relied on exogenously added dopamine and PMA. Hence, it is likely that inhibition of Na\(^+\),K\(^-\)-ATPase by dopamine or PMA in the presence of monensin is the result of a permissive effect of elevated intracellular sodium on signaling molecule(s) that are downstream of the dopamine receptor. Finally, reports from other researchers (30–32) and this work suggest that intracellular sodium acts in several different signaling pathways and at different levels to regulate cellular homeostasis.

The free intracellular sodium concentration of OK cells was determined in situ by digital imaging fluorescence microscopy from the changes in fluorescence produced by the sodium indicator SBFI (22). On the basis of these determinations, the effect of the various drugs on the Na\(^+\),K\(^-\)-ATPase activity was tested with cells treated with 5 \(\mu\)M monensin which produced an increase in intracellular sodium from \(-9\) to \(-20\) mM. Because proximal tubule epithelial cells should normally support changes in this range of intracellular sodium concentration, we assumed that 5 \(\mu\)M monensin produced an elevation of intracellular sodium concentration within the physiological range. This is very important because it is likely that many changes in protein function are produced when the intracellular ionic concentration is changed to limits in which the integrity and survival of the cell are in question. The increased intracellular sodium level produced by monensin was accompanied by a transient elevation in intracellular free calcium concentration, and the rate of increase of intracellular sodium concentration promoted by monensin was greater in cells assayed in the absence of extracellular calcium. Although interesting, the study of the mechanism responsible for increased calcium entry into the cell was not the object of this project and was not pursued further. Nevertheless, independent of the mechanism involved, at the time the Rb\(^+\) transport assay was performed (30 min after monensin addition), the intracellular calcium concentration was at or lower than the basal level, and intracellular sodium had reached its new steady state concentration. Therefore, no effect due to calcium should be expected under these conditions. That calcium is not involved in the modulation of the hormonal regulation of Na\(^+\),K\(^-\)-ATPase was also supported by the observations that the inhibition of Na\(^+\),K\(^-\)-ATPase by either PMA or dopamine was not affected by removal of external calcium, and by loading the cells with the calcium chelator BAPTA. Therefore, modulation of the PMA and dopamine effects on Na\(^+\),K\(^-\)-ATPase appears to be dependent exclusively on the level of intracellular sodium.

We and other investigators have described previously (4,
11–13, 15) that treatment of OK cells and rat renal proximal tubules with PMA results in stimulation of the Na⁺,K⁺-ATPase activity. The present results demonstrate that the level of activation of Na⁺,K⁺-ATPase by PMA is reduced and even reversed to an inhibition by an increase of the OK cell intracellular sodium concentration. On the basis of these results, we hypothesized that PMA can activate two different pathways for either inhibition or stimulation of Na⁺,K⁺-ATPase activity. Which one is activated would depend on the intracellular sodium concentration. While at basal intracellular sodium concentration PMA stimulated the signaling pathway that leads to Na⁺,K⁺-ATPase activation; at higher intracellular sodium concentrations PMA stimulated a different pathway that leads to inhibition of Na⁺,K⁺-ATPase. Under the latter conditions, PMA appears to stimulate the signaling pathway that is normally activated by dopamine to inhibit the Na⁺,K⁺-ATPase activity. Several lines of evidence support this conclusion. 1) The higher the intracellular sodium concentration, the greater the inhibition of Na⁺,K⁺-ATPase activity by both PMA and dopamine. 2) The inhibition produced by PMA and dopamine is not additive when both reagents were added simultaneously to the cell medium. 3) The integrity of the intracellular sodium concentration PMA stimulated the signaling pathway produced only a transient increase of intracellular calcium (24, 25). However, this effect has not been observed in experiments where the sodium gradient was present. It has been described that high monensin works as a sodium transporter when it binds to the tubule cells and the translocation of dopamine receptors from cytosolic storage compartments to the plasma membrane (37), there must be an intracellular mechanism that senses the presence of the sodium load. Because sodium enters the kidney proximal tubule cells through the apical domain by a gradient-dependent mechanism, an elevation of luminal sodium should translate in increased intracellular sodium. This, alone or in combination with other cellular factors, may determine and modulate the hormonal regulation of proteins involved in sodium reabsorption. Understanding the mechanisms of such intracellular networks would provide alternative strategies to treat disorders associated with altered sodium homeostasis such as salt-sensitive hypertension.

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