

# Stimulation of Protein Kinase C Rapidly Reduces Intracellular $\text{Na}^+$ Concentration via Activation of the $\text{Na}^+$ Pump in OK Cells

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## SUMMARY

$\text{Na}^+$  reabsorption is regulated in proximal tubules by hormones that stimulate protein kinase C (PKC). To determine whether stimulation of PKC causes a reduction in intracellular  $\text{Na}^+$  concentration ( $[\text{Na}^+]_i$ ) that might link  $\text{Na}^+$  pump activation to increased  $\text{Na}^+$  reabsorption,  $[\text{Na}^+]_i$  was measured in kidney cells loaded with the  $\text{Na}^+$ -sensitive fluorescent indicator SBFI. Rapid digital imaging fluorescence microscopy determinations were performed in epithelial kidney cells transfected with the rodent  $\text{Na}^+$  pump  $\alpha 1$  cDNA. In 42 determinations, the basal  $[\text{Na}^+]_i$  was  $19.7 \pm 2.4$  mM. Stimulation of PKC reduced the  $[\text{Na}^+]_i$  to  $5.6 \pm 0.6$  mM in  $\sim 10$  sec. This drastic change in  $[\text{Na}^+]_i$  requires a transient 74–120-fold increase in  $\text{Na}^+$  pump activity.

After the new steady state  $[\text{Na}^+]_i$  is reached, the  $\text{Na}^+$  pump is 58% activated. The entry of  $\text{Na}^+$  into the cells is not affected by stimulation of PKC; therefore, the reduction in  $[\text{Na}^+]_i$  is exclusively dependent on activation of the  $\text{Na}^+$  pump. Accordingly, PKC stimulation does not affect the  $[\text{Na}^+]_i$  of cells expressing a mutant  $\text{Na}^+$  pump that is not stimulated by PKC. The decrease in  $[\text{Na}^+]_i$  observed in cells transfected with the rodent  $\text{Na}^+$  pump  $\alpha 1$  cDNA is large and sufficiently fast that it is expected to stimulate rapidly passive  $\text{Na}^+$ -influx into the cells, thereby accounting for the observed PKC-induced stimulation of  $\text{Na}^+$  reabsorption.

The molecular mechanism by which certain hormones with receptors coupled to stimulation of PKC increase  $\text{Na}^+$  reabsorption in proximal convoluted tubules is not well understood (1–5). The  $\text{Na}^+$  pump, which is located in the basolateral membrane of the tubular epithelial cells, maintains a transmembrane concentration gradient for  $\text{Na}^+$ , ensuring the net reabsorption of  $\text{Na}^+$  (6, 7). Some researchers have proposed that short term hormonal regulation of the  $\text{Na}^+$  pump may contribute to the ability of the kidney to adjust  $\text{Na}^+$  reabsorption (8, 9). Although a compelling proposal, it is not obvious by what mechanism changes in pump activity may regulate  $\text{Na}^+$  transport across epithelia.

In proximal tubules, luminal  $\text{Na}^+$  enters the cell through  $\text{Na}^+$ -coupled systems for amino acids, hexoses, inorganic phosphate, and a  $\text{Na}^+/\text{H}^+$  exchanger (10, 11). These transporters are located in the apical membrane and constitute the limiting step in  $\text{Na}^+$  reabsorption. It follows that any regulatory mechanism seeking to alter urinary  $\text{Na}^+$  reab-

sorption must modulate the passive  $\text{Na}^+$  influx across the apical membrane of epithelial cells. An attractive possibility is that hormones adjust the free intracellular  $\text{Na}^+$  concentration ( $[\text{Na}^+]_i$ ) via modulation of the  $\text{Na}^+$  pump. A decrease in free  $[\text{Na}^+]_i$  would stimulate turnover of the  $\text{Na}^+/\text{H}^+$  exchanger, thereby increasing passive  $\text{Na}^+$  influx. This possibility has not yet been systematically explored.

Without a reliable measurement of free  $[\text{Na}^+]_i$ , it is not possible to predict whether the reported hormonal modulation of the  $\text{Na}^+$  pump activity (8, 9, 12, 13) leads to changes in free  $[\text{Na}^+]_i$ . If this change occurs, it is important to determine whether its time course is consistent with the expected short term regulation of  $\text{Na}^+$  reabsorption. In this report, we describe rapid determinations of free  $[\text{Na}^+]_i$  by digital imaging fluorescence microscopy of kidney cells loaded with the fluorescent  $\text{Na}^+$  indicator SBFI (14).

Stable transfected mammalian cells have been shown to be a useful system in which to study  $\text{Na}^+$  pump structure-function relationships (15–17). In this system, transfected cells expressing wild-type or active mutants of the rodent  $\alpha 1$  subunit are readily identified and selected by their ability to survive in a cell medium containing ouabain. Price and Lin-

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**ABBREVIATIONS:** PKC, protein kinase C; DMSO, dimethylsulfoxide;  $[\text{Na}^+]_i$ , intracellular sodium concentration; PMA, phorbol-12-myristate 13-acetate; PDD, phorbol-12,13-didecanoate; MIA, 5-(*N*-methyl-*N*-isobutyl)-amiloride; DMEM-10, Dulbecco's modified Eagle's medium with 10% calf serum and antibiotics; PVDF, polyvinylidene difluoride; EGTA, ethylene glycol bis( $\beta$ -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate.

grel (15) and Price *et al.* (16) have shown that transfected cells grown in micromolar ouabain survive by virtue of their successful expression of the introduced rodent  $\alpha$  subunit. Maintaining transfected cells in ouabain permits the selection of cells with essentially no endogenous Na<sup>+</sup> pump activity because ouabain remains bound to the high affinity binding site of the endogenous Na<sup>+</sup> pump molecules (17). We have used this expression system to study the effect of PKC stimulation on the activity of the Na<sup>+</sup> pump and demonstrated that stimulation of PKC produces a very rapid decrease of [Na<sup>+</sup>]<sub>i</sub> via activation of the Na<sup>+</sup> pump.

## Experimental Procedures

**Materials.** Cell culture supplies were purchased from GIBCO (Grand Island, NY) and Hyclone Laboratories (Logan, UT). Molecular biology reagents were from New England Biolabs (Beverly, MA), DuPont (Wilmington, DE), Promega (Madison, WI), and United States Biochemical (Cleveland, OH). Ouabain was purchased from Calbiochem (San Diego, CA). Phorbol esters were obtained from Sigma Chemical (St. Louis, MO). SBFI and Pluronic F-127 were obtained from Molecular Probes (Eugene, OR). Other reagents were of the highest quality available.

**Cell culture and transfection.** The expression vector pCMV containing the rodent Na<sup>+</sup> pump  $\alpha$ 1 subunit cDNA was obtained from Pharmingen (San Diego, CA). The preparation of the expression vector (myc/1.32) that encodes a mutant of the  $\alpha$ 1 subunit was previously described (18). This vector expresses a rodent  $\alpha$  subunit in which the first 31 amino acids of the nascent polypeptide are replaced by an initiation methionine and a sequence of 10 amino acids (EQKLISEEDL) from the human *c-myc* oncogene product.

OK cells were maintained at 37° (10% CO<sub>2</sub>) in DMEM-10. Plasmids containing the wild-type and mutant  $\alpha$  subunit cDNAs were transfected into OK cells using liposomes. Cationic liposomes were prepared by sonication with 1 mg of dioleoyl-L- $\alpha$ -phosphatidylethanol-amine and 0.4 mg of dimethyl-dioctadecyl-ammonium-bromide as indicated by Rose *et al.* (19). The day before transfection, OK cells were seeded onto the wells of a 96-well plate (3500 cells/well). The following day, the cells were transfected in 50  $\mu$ l of Opti-MEM I containing 3  $\mu$ g/ml total DNA and 15  $\mu$ l/ml liposomes. Five hours after transfection, 200  $\mu$ l/well DMEM-10 was added. Two days later, cells were transferred to a medium containing 1  $\mu$ M ouabain. Because the endogenous Na<sup>+</sup> pump of OK cells is sensitive to this level of ouabain, only OK cells that express the Na<sup>+</sup> pump containing the rodent  $\alpha$  subunit would be able to survive. After 10 days, cells from the wells that had single colonies were transferred to a medium containing 10  $\mu$ M ouabain to select for cells expressing the highest level of rodent  $\alpha$  subunit. Resistant colonies were expanded and maintained in DMEM-10 containing 10  $\mu$ M ouabain. The Na<sup>+</sup>/K<sup>+</sup>-ATPase (EC 3.6.1.37) of mock-transfected cells (vector alone, vector plus liposomes, or liposomes alone) had the same activity and sensitivity to ouabain as nontreated host cells.

**Preparation of crude plasma membranes to measure Na<sup>+</sup>/K<sup>+</sup>-ATPase.** OK cells were harvested by mild trypsinization and suspended in lysis buffer (10 mM imidazole, 1 mM EDTA, pH 7.5). The cells were probe-sonicated twice for 15 sec with a 15-sec interval in an Ultrasonic homogenizer 4710 (Cole-Parmer Instrument (Chicago, IL)) at 25 W and 80% power output. Samples were maintained in an ice-water bath during sonication. The suspension was centrifuged for 4 min at 1,500  $\times$  g. The resulting supernatant was collected and centrifuged at 513,000  $\times$  g for 15 min at 2° (Optima TLX ultracentrifuge; Beckman Instruments (Columbia, MD)). The pellet was resuspended with a small volume of lysis buffer and used to determine protein and Na<sup>+</sup>/K<sup>+</sup>-ATPase activity.

**Protein determination.** Protein was determined by the bicinchoninic acid method (Pierce Chemical (Rockford, IL)) using BSA as

standard. Cells or cell membranes were homogenized with SDS and aliquots were used for protein determination.

**Determination of Na<sup>+</sup>/K<sup>+</sup>-ATPase.** Protein aliquots (2 mg/ml) were treated with 0.7 mg/ml SDS in the presence of 3 mM ATP, 10 mM imidazole, and 0.4 mM EDTA, pH 7.5, for 10 min at room temperature. Protein samples were then put into an ice-water bath, and BSA was added to a final concentration of 0.4 mg/ml. The SDS treatment was determined to be optimal for exposing latent Na<sup>+</sup>/K<sup>+</sup>-ATPase activity (20). The Na<sup>+</sup>/K<sup>+</sup>-ATPase assay medium contained 0.05 mg/ml membrane protein, 0.3 mg/ml BSA, 0.5 mM EGTA, 130 mM NaCl, 20 mM KCl, 4 mM MgCl<sub>2</sub>, 3 mM ATP, and 50 mM imidazole, pH 7.3. Enzymatic activity was determined as previously described (21) at 37° for 30 min based on the difference between the ATP hydrolysis measured in the absence and presence of ouabain. Experiments were carried out in duplicate and repeated at least five times. Results are the average of at least five experiments and are expressed as average  $\pm$  standard error.

**Determination of Rb<sup>+</sup> transport.** The experiments were performed with cells seeded at ~60% confluence in 24-well plates. To facilitate access of introduced ligands to the Na<sup>+</sup>/K<sup>+</sup>-ATPase, cells grown on plastic were exposed to culture medium containing EGTA before the measurement of Rb<sup>+</sup> uptake (22). No cell detachment from the plastic was observed during incubation. EGTA was not present during treatment with phorbol esters and Rb<sup>+</sup> transport assay. However, we determined that the stimulation of Rb<sup>+</sup> transport by PMA was not affected by the presence of EGTA.

To measure Rb<sup>+</sup> transport, transfected cells (1  $\times$  10<sup>5</sup> cells/well of a 24-well plate) were transferred to serum-free DMEM containing 50 mM HEPES, pH 7.4 (DMEM-HEPES), 2 mM EGTA, and 10  $\mu$ M or 10 mM ouabain (incubation medium). Cells were incubated for 20 min at 37° in an air atmosphere and 10 min at room temperature before addition of 1  $\mu$ M phorbol ester. Five minutes later, a trace amount of [<sup>86</sup>Rb<sup>+</sup>]RbCl was added. Rb<sup>+</sup> uptake was terminated after 20 min by washing the cells four times with ice-cold saline. Cells were dissolved with SDS, and accumulated radioactivity was determined. Na<sup>+</sup>/K<sup>+</sup>-ATPase-mediated Rb<sup>+</sup> transport was estimated based on the difference in tracer uptake between samples incubated in 10  $\mu$ M and 10 mM ouabain. For nontransfected OK cells, Rb<sup>+</sup> transport was measured in the absence and presence of 10 mM ouabain. The nonspecific ouabain-insensitive Rb<sup>+</sup> transport was 15–20% of the total Rb<sup>+</sup> transport measured.

Because phorbol esters were dissolved in DMSO, the same amount of solvent was added to control samples. The amount of solvent used did not alter the Rb<sup>+</sup> transport of control samples. Each experiment was repeated at least four times.

**Determination of Na<sup>+</sup> influx.** Total Na<sup>+</sup> uptake was determined under the same conditions used to measure Rb<sup>+</sup> transport. Cells seeded at ~60% confluence in 24-well plates were transferred to DMEM-HEPES and 2 mM EGTA. Cells were incubated for 20 min at 37° and 10 min at room temperature. Then, the cell medium was exchanged with DMEM-HEPES containing 5 mM ouabain to block Na<sup>+</sup> exit (23). Some samples also received 1  $\mu$ M PMA. Two minutes later, a trace amount of [<sup>22</sup>Na<sup>+</sup>]NaCl was added. Na<sup>+</sup> uptake was terminated after 10 min by washing the cells four times with ice-cold saline. Cells were dissolved with SDS, and accumulated radioactivity was determined. The experiment was repeated four times. Preliminary experiments have shown that Na<sup>+</sup> uptake was linear during the assay.

**Determination of [Na<sup>+</sup>]<sub>i</sub>.** Fluorescence measurements of [Na<sup>+</sup>]<sub>i</sub> were performed using the membrane-permeant tetra-acetoxymethyl ester of the Na<sup>+</sup>-binding dye benzofuran-isophthalate-acetoxymethyl ester (SBFI-AM; Molecular Probes) following standard protocols (24). Cells were loaded by incubating with the dye at room temperature in DMEM-HEPES containing 2–5  $\mu$ M SBFI-AM and 0.1% (w/v) of the nonionic detergent Pluronic F-127. The dye was dissolved from a 1 mM stock solution in DMSO. The dye-incubating medium was sonicated for 5–10 min to facilitate dye dispersion and avoid the adherence of clumps of unsolubilized dye to cells. Cells

were loaded with the dye for 120 min through gentle agitation. Loading in serum-free medium and at room temperature was used to avoid compartmentalization of the dye. After loading, the cells were washed several times in DMEM/50 mM HEPES and incubated 30 min in the same medium to allow de-esterification of SBFI-a.m. The complete hydrolysis of SBFI-AM to SBFI was judged on the basis of changes in the excitation and emission spectra (24). Optical measurement were performed in serum-free HEPES-buffered medium.

The design of the optical setup is based on standard methods (25–27). The system consists of an upright epi-illumination microscope (Nikon Epiphot) with a video camera (MV-1070; Marshal Electronics, Culver City, CA) in the photographic port. Light from a 150-W xenon lamp (Model 1600; Optic Quip, Highland Mills, NY) was collimated and rendered quasimonochromatic by interference filters. The light was focused by a quartz UV-grade condenser (Nikon) and reflected to the preparation by a dichroic mirror. The wavelengths for the excitation and emission filters and the dichroic mirror were selected according to the excitation and emission spectra of SBFI (14). Fluorescence light emitted from the cells was collected by a high numerical aperture water immersion objective ( $\times 20$  or  $\times 40$ ; Fluo; Nikon), which formed a real image on the CCD sensor of the video camera located in the image plane of the microscope. To further improve the sensitivity of the analog camera, image exposures were extended to increase light integration in the CCD sensor. RS-170 (the broadcast video standard) video images were transferred to a frame grabber board (FG100-AT-1024, Imaging Technology; Woburn, MA) plugged directly into the computer bus. This board carried out eight-bit digitization (0.4%, 256 gray levels) and storage of the video images. To take full advantage of the analog/digital conversion range, video signals were manually adjusted for gain and black level (pedestal) so the background was in the middle of the camera output range.

Absolute  $[Na^+]_i$  determinations were performed as described by Harootunian *et al.* (24). SBFI fluorescence was excited by 340- and 385-nm illumination. The ratio of fluorescence intensities excited at 340 and 385 nm (340/385) is proportional to  $[Na^+]_i$ .  $[Na^+]_i$  was calculated according to the equation described by Grynkiewicz *et al.* (28) with a  $K_d$  value of SBFI for  $Na^+$  of 18 mM (14). Other terms of the equation were assessed by *in situ* calibration. Alternate excitation wavelengths of 340 and 385 nm (bandwidth,  $\pm 5$  nm; Omega Optical, Brattleboro, VT) were sampled at 250-msec intervals, and the emission light above 480 nm was collected through the dichroic mirror and a barrier filter (Omega Optical). Changes in excitation wavelengths were obtained with an specially design filter slide changer that could switch filters during a single video frame (33-msec) period. To ensure stability on the recordings at each wavelength and avoid photobleaching effects, the excitation light levels were reduced by neutral density filters until the fluorescence intensity remained constant within 100 sec of illumination. No significant level of autofluorescence was observed in the cells, and the concentration of reagents added to the cell medium did not affect the fluorescence levels as judged by determinations performed at an excitation wavelength corresponding to the isosbestic point of SBFI ( $370 \pm 2.5$  nm; Omega Optical). Temporal plots of  $[Na^+]_i$  were obtained from averaged ratios over  $8 \times 8$  pixels in a region of the cell cytosol. In the pictures,  $[Na^+]_i$  changes are illustrated by pseudocolors resulting from subtraction of the basal level of  $[Na^+]_i$  from those obtained after experimental manipulation. The basal  $[Na^+]_i$  was the same in cells transfected with the wild-type and mutant  $\alpha 1$  cDNA.

At the end of each experiment, *in situ* calibration of the excitation ratio of SBFI was performed to accurately assess  $[Na^+]_i$ . After permeabilization with 10  $\mu M$  gramicidin D (24, 29), cells were superfused with different  $Na^+$  concentrations. The 340/385 intensity ratio decreased stepwise when the extracellular  $Na^+$  concentration was changed from 140 to 90, 60, 30, 12, 6, 3, and 0 mM and raised again after an extracellular  $Na^+$  increase. Calibration curves of  $[Na^+]_i$  were the same for cells transfected with both plasmids. An increase

in the 340/385 intensity ratio was observed when  $K^+$  was removed from the cell medium and after the application of ouabain (100  $\mu M$ ).

**Western blot analysis.** Samples from transfected and nontransfected OK cells were resuspended in sample buffer and warmed to 80° for 15 min. Proteins (100  $\mu g$ /sample) were first separated in a 10% Laemmli gel (30) and then electrotransferred to a piece of PVDF membrane. The PVDF membrane was blocked for 1 hr in 5% w/v nonfat dry milk, 0.1%  $NaNO_3$ , 150 mM NaCl, and 25 mM HEPES, pH 7.4, at room temperature. The membrane was treated with anti- $\alpha$  subunit antibody (1:50 dilution) in blocking solution at room temperature for 1 hr and then washed three times for 5 min with Tris-buffer saline containing 0.1% Tween 20 and three times for 5 min with Tris-buffer saline alone. The membrane was incubated for 1 hr at room temperature with anti-rabbit secondary antibody (dilution 1:1000) conjugated to horseradish peroxidase. The membrane was washed as before. Finally, the immunoreactivity was detected by enhanced chemiluminescence (ECL, Amersham, Arlington Heights, IL). The immunodetected bands were quantified by integration of the density of the total area of each band by use of a Sharp JX-325 scanner interfaced to a Sun Spark Classic computer. The equipment and Quantity One software were obtained from pdi Company (Huntington Station, NY). Determinations were performed at nonsaturating levels of exposure. The primary antibody (NASE) we used was previously described (18).

**Statistical analysis.** Comparisons between groups were performed by Student's *t* test for unpaired data.

## Results

**$Na^+$  pumps containing the rodent  $\alpha$  subunit are expressed at comparable levels in the two OK cell lines.** The experiments were performed in OK cells transfected with either wild-type rodent  $Na^+$  pump  $\alpha$  subunit cDNA or a mutant cDNA that encodes an  $\alpha$  subunit missing the first 31 amino acids of the  $NH_2$ -terminal end (31). There was no difference in the growth rate between nontransfected OK cells and cells transfected with either the wild-type rodent  $\alpha 1$  cDNA or the  $\alpha 1$ -mutant cDNA. Expression of both  $\alpha 1$  cDNAs in OK cells conferred resistance to 10  $\mu M$  ouabain. The endogenous  $Na^+$  pump activity was inhibited by growing the cells and performing the experiments in the presence of 10  $\mu M$  ouabain. Accordingly, any pump-mediated transport that we observed must originate with the  $Na^+$  pumps containing the introduced rodent  $\alpha$  subunit. There was no difference in the ouabain sensitivity between cells transfected with the rodent wild-type and the mutant  $\alpha 1$  cDNAs (31).

The maximal  $Na^+/K^+$ -ATPase activity in nontransfected OK cells was  $2.44 \pm 0.05$   $\mu mol$  of  $P_i$ /mg/hr. Similar levels of activity were observed in membranes of cells transfected with the wild-type ( $2.34 \pm 0.25$   $\mu mol$   $P_i$ /mg/hr) and  $NH_2$  deletion mutant ( $2.15 \pm 0.15$   $\mu mol$   $P_i$ /mg/hr)  $\alpha 1$  cDNAs. Moreover, the same level of ouabain-sensitive  $Rb^+$  transport was determined in transfected ( $\alpha 1$ -wt and  $\alpha 1$ -mut) and nontransfected (OK-wt) cells (Table 1). The  $Na^+/K^+$ -ATPase activity was determined in cell membranes at saturating concentrations of all of the enzyme ligands ( $V_{max}$  condition) while  $Rb^+$  transport was measured in intact cells, in which the  $[Na^+]_i$  is not saturating. Similarities in  $\alpha$  subunit abundance have also been demonstrated in host and transfected COS-1 cells (18). These observations suggest that  $Na^+/K^+$ -ATPases containing the endogenous  $\alpha$  subunits have been replaced by  $Na^+/K^+$ -ATPases containing the ouabain-resistant wild-type or mutant rodent  $\alpha 1$ . For our purposes, an additional important piece of information derived from the determinations described above is that elimination of the  $\alpha$

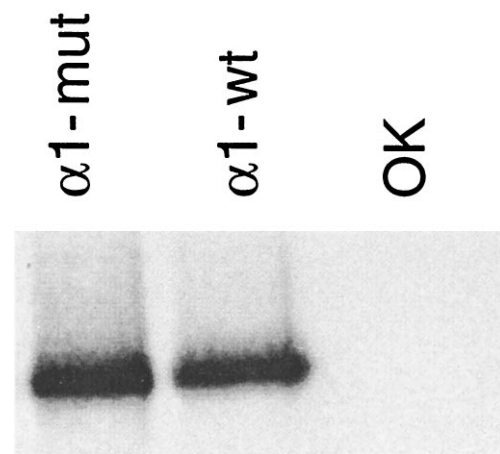


subunit NH<sub>2</sub> terminus did not affect the maximal activity of the Na<sup>+</sup>/K<sup>+</sup>-ATPase and the ouabain-sensitive Rb<sup>+</sup> transport measured in intact cells.

The fact that the maximal Na<sup>+</sup>/K<sup>+</sup>-ATPase activity is the same in cells expressing the rodent wild-type and mutant α1 indicates that these cells have equal amounts of active Na<sup>+</sup> pump molecules. To determine whether these cells express equivalent amounts of rodent α subunit, membrane proteins from cells expressing the rodent wild-type and mutant α1 were separated by SDS-polyacrylamide gel electrophoresis. After blotting the proteins to a piece of PVDF membrane, proteins were reacted with an antibody that recognizes the α subunit (Fig. 1) The anti-α subunit antibody (18) did not bind to the endogenous OK α subunit and allowed us to compare the levels of expression of the wild-type and mutant α1. In three determinations, the intensity (absorbance × mm) of the bands determined by densitometry were 1.06 ± 0.26 and 0.86 ± 0.34 for cells expressing the mutant and wild-type rodent α subunit, respectively. Thus, OK cells expressed the wild-type and mutant α1 at comparable levels.

**Determination of [Na<sup>+</sup>]<sub>i</sub>.** Free [Na<sup>+</sup>]<sub>i</sub> was determined *in situ* by digital imaging fluorescence microscopy in cells transfected with the rodent α1 cDNA. Cells were loaded with the membrane permeant derivative of SBFI (SBFI/AM), and the level of emitted fluorescence on excitation at 340 and 385 nm was monitored using a video imaging system (14, 25). Fig. 2 (top, left) shows a pseudocolored image of SBFI-loaded cells and the *in situ* calibration that was acquired from the same cells. In a monolayer of OK cells, it is almost impossible to distinguish the boundaries between cells by observation at the microscope. In this sense, the image shown in Fig. 2 corresponds to many cells. On the basis of the *in situ* calibration, the steady state basal [Na<sup>+</sup>]<sub>i</sub> was estimated to be 19.7 ± 2.4 mM in 42 determinations. The addition of 5 mM ouabain to the cell medium increased the [Na<sup>+</sup>]<sub>i</sub> consistent with the expected inhibition of the Na<sup>+</sup> pump (Fig. 2). [Na<sup>+</sup>]<sub>i</sub> rose an average of 7.5 mM in 10 min because the passive Na<sup>+</sup> influx was not counteracted by the inhibited Na<sup>+</sup> pump.

**Stimulation of PKC reduced [Na<sup>+</sup>]<sub>i</sub>.** Fig. 3 shows a typical response of cells expressing wild-type rodent α1 to treatment with PMA. Three different determinations are shown. There was a rapid and highly significant decrease in [Na<sup>+</sup>]<sub>i</sub>. Changes in fluorescence were detected almost immediately after the addition of PMA. Each point of the curves corresponds to data accumulated for 250 msec at each excitation wavelength (i.e., each determination required 500 msec). In these measurements, the basal steady state [Na<sup>+</sup>]<sub>i</sub> was ~20 mM, and a new steady state [Na<sup>+</sup>]<sub>i</sub> of ~5 mM was achieved within 10 sec after addition of PMA. In 18 determinations, the steady state [Na<sup>+</sup>]<sub>i</sub> of PMA-treated cells was



**Fig. 1.** Cellular expression of exogenous rodent wild-type and mutant α subunit in OK cells. Cell membranes of nontransfected cells (OK-wt) or cells transfected with the wild-type α1 cDNA (α1-wt) and the NH<sub>2</sub> deletion mutant α1 cDNA (α1-mut) were prepared as indicated in the text. Identical amounts of protein (100 μg) were separated by gel electrophoresis, and the proteins were electrotransferred to a PVDF membrane. This membrane was treated with an antibody that recognizes the rodent α subunit.

5.6 ± 0.6 mM. A similar reduction of [Na<sup>+</sup>]<sub>i</sub> was observed with PDD. Washing out the phorbol esters from the cell medium restored the initial steady state [Na<sup>+</sup>]<sub>i</sub>. PMA and PDD are believed to act by stimulation of PKC (32). Consistent with this idea, phorbol esters that do not stimulate PKC, like 4α-PDD or 4α-PMA, did not affect the [Na<sup>+</sup>]<sub>i</sub> (Fig. 4). Additional support for the involvement of PKC comes from the observation that staurosporine, an inhibitor of protein kinases, blocked the PMA-induced reduction in [Na<sup>+</sup>]<sub>i</sub>. In the absence of PMA, staurosporine did not affect the [Na<sup>+</sup>]<sub>i</sub>. From these data, we conclude that PMA-induced reduction in [Na<sup>+</sup>]<sub>i</sub> was mediated through stimulation of PKC and was not the result of a nonspecific effect of PMA or SBFI. A similar reduction in [Na<sup>+</sup>]<sub>i</sub> was observed in nontransfected OK cells treated with PMA (data not shown).

**The Na<sup>+</sup> pump is responsible for the reduced [Na<sup>+</sup>]<sub>i</sub>.** In OK cells, Na<sup>+</sup> enters the cell through the apical Na<sup>+</sup>/H<sup>+</sup> exchanger (33). As expected, Fig. 5 shows that inhibition of the Na<sup>+</sup>/H<sup>+</sup> exchanger with 8 μM MIA produced a reduction in steady state [Na<sup>+</sup>]<sub>i</sub> from 20 to 5 mM. Thus, one could argue that a PMA-induced inhibition of Na<sup>+</sup> influx may be responsible for the reduced [Na<sup>+</sup>]<sub>i</sub> observed in OK cells expressing the wild-type rodent α1. Even though the Na<sup>+</sup>/H<sup>+</sup> exchanger is the main mechanism of Na<sup>+</sup> entry, the cation is also cotransported into the cell with glucose, amino acids, and phosphate (33). Because we were interested in the effect of PMA on Na<sup>+</sup> entry, independent of the mechanism, we measured total Na<sup>+</sup> influx. This was determined in cells transfected with the wild-type rodent α1 in the presence and absence of PMA. Fig. 6 shows that PMA treatment has no effect at all on the cation uptake. Determination of Na<sup>+</sup>-influx was performed in the presence of ouabain to block Na<sup>+</sup> efflux (23). In this way, any stimulation of Na<sup>+</sup> entry by PMA activation of the Na<sup>+</sup> pump was eliminated.

Because the Na<sup>+</sup> pump is the other major determinant of the steady state [Na<sup>+</sup>]<sub>i</sub>, the PMA-induced reduction in [Na<sup>+</sup>]<sub>i</sub> must have been produced by stimulation of the Na<sup>+</sup> pump

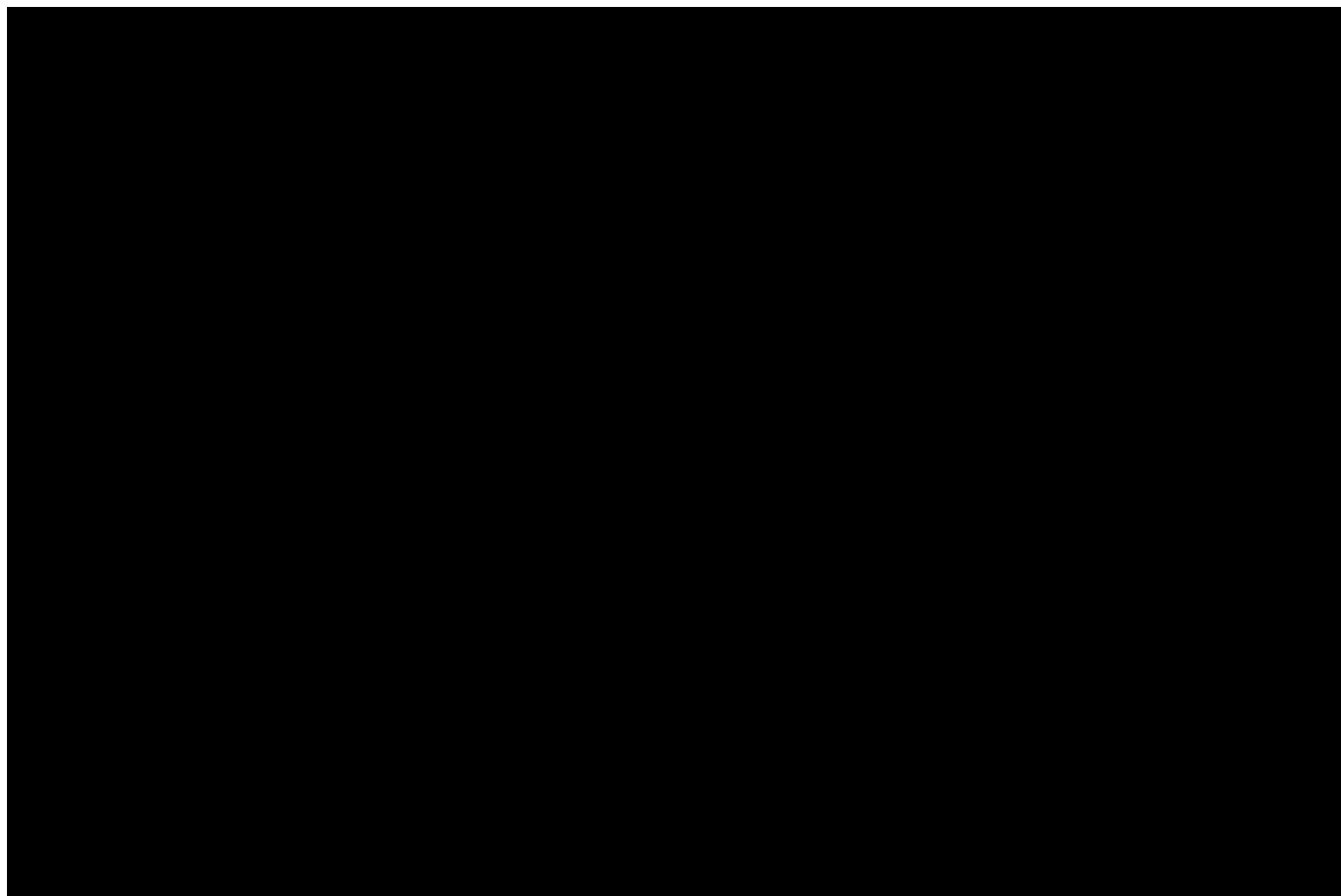
TABLE 1

**Rb<sup>+</sup> transport in transfected and nontransfected OK cells**

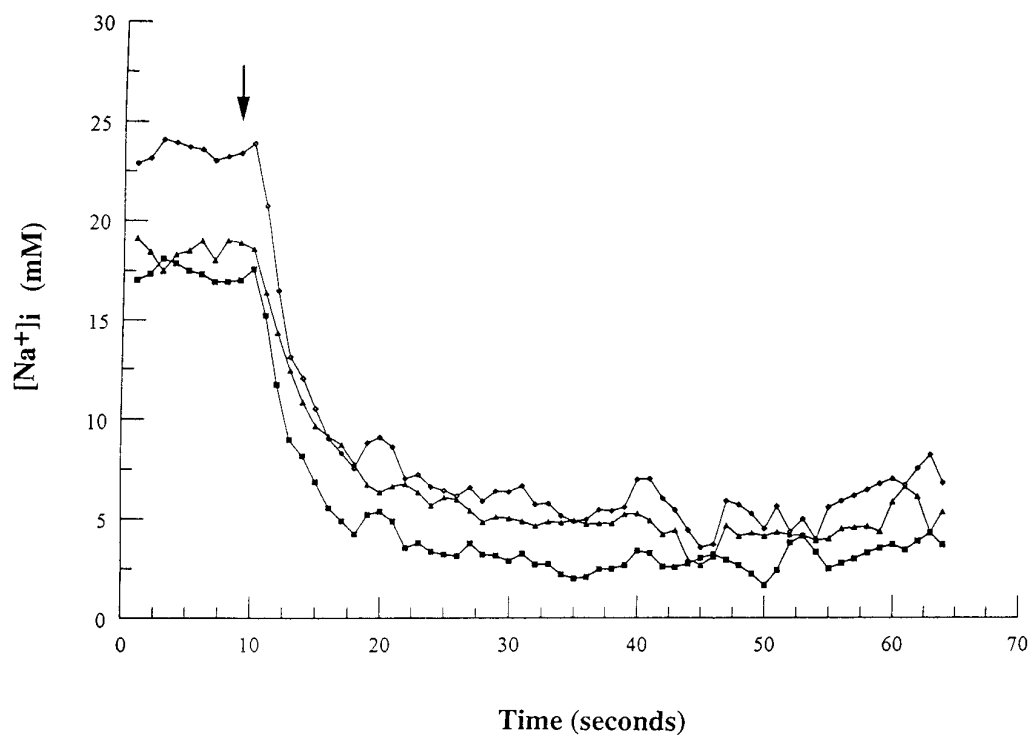
Effect of phorbol ester treatment on Rb<sup>+</sup> transport mediated by the Na<sup>+</sup> pump of nontransfected cells (OK-wt) or cells transfected with the wild-type α1 cDNA (α1-wt) and the NH<sub>2</sub> deletion mutant α1 cDNA (α1-mut).

	Basal	+PMA	+4α-PDD
	nmol/mg/min		
OK-wt	11.6 ± 0.7	14.4 ± 0.7 <sup>a</sup>	11.9 ± 0.3
α1-wt	9.5 ± 1.4	15.0 ± 1.5 <sup>a</sup>	10.3 ± 0.3
α1-mut	11.2 ± 1.9	12.2 ± 0.5	12.1 ± 0.4

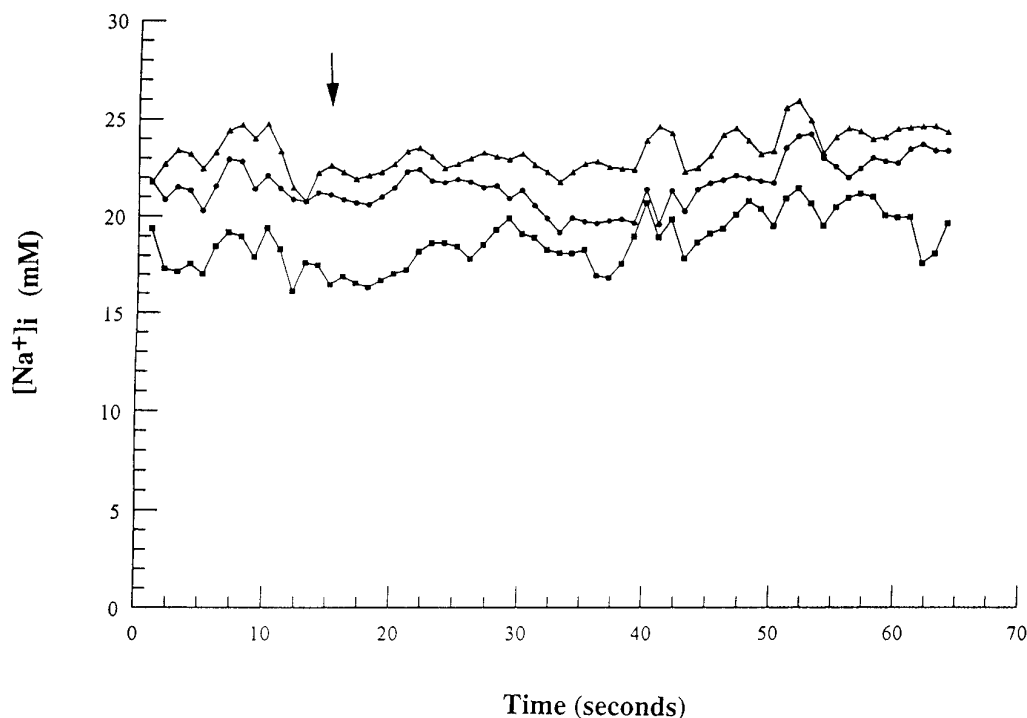
<sup>a</sup> *p* < 0.01 with respect to control without PMA.



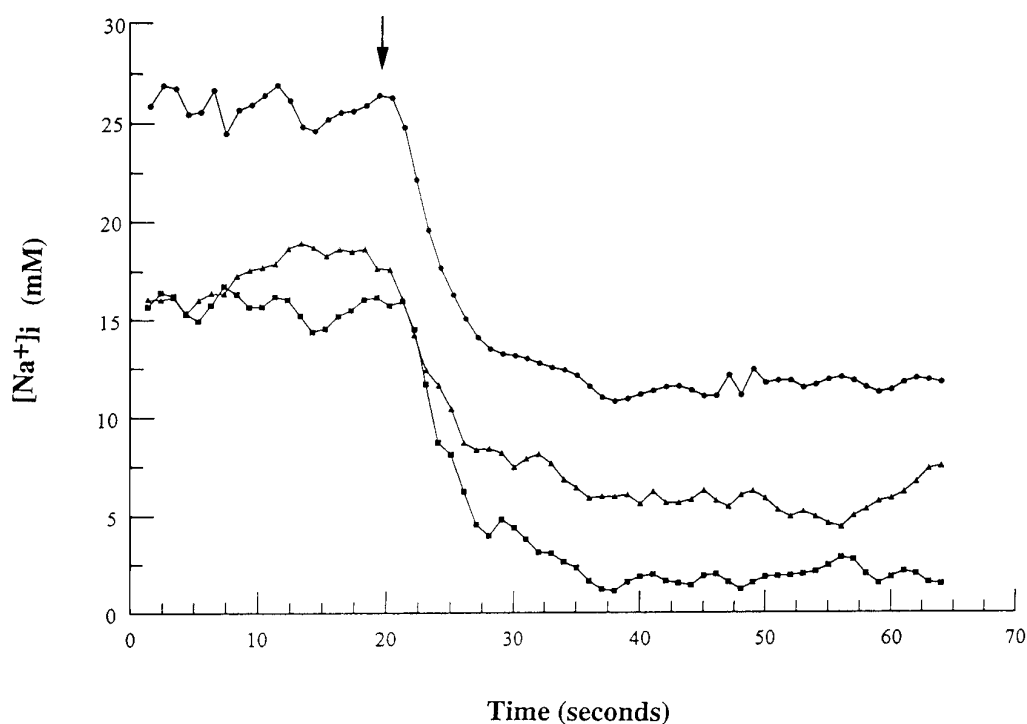
**Fig. 2.** Ouabain-dependent increase of  $[\text{Na}^+]_i$  revealed by digital imaging fluorescence microscopy from OK cells transfected with either the wild-type rodent  $\alpha 1$  cDNA (*top*) or the  $\text{NH}_2$ -deletion mutant  $\alpha 1$  cDNA (*bottom*). OK cells were loaded with the  $\text{Na}^+$ -sensitive fluorescent indicator SBFI, and the level of emitted fluorescence on excitation at 340 and 385 nm was determined. As indicated, images correspond to 0, 5, and 10 min after the addition of 5 mM ouabain. Pseudocolor calibration: *dark pink*, 0–5 mM; *dark blue*, 5–10 mM; *light blue*, 10–15 mM; *green*, 15–20 mM; *yellow*, 20–25 mM; *red*, 25–30 mM; and *white*, >30 mM.



**Fig. 3.** Rapid reduction in  $[\text{Na}^+]_i$  produced by stimulation of PKC with phorbol esters. Cells expressing the wild-type rodent  $\text{Na}^+$  pump  $\alpha$  subunit were loaded with the  $\text{Na}^+$ -sensitive fluorescent indicator SBFI, and the level of emitted fluorescence on excitation at 340 and 385 nm was determined. Reduction in  $[\text{Na}^+]_i$  produced by the addition of  $1 \mu\text{M}$  PMA to the cell medium is illustrated. *Arrow*, point of addition of PMA. *Each point*, data accumulated for 250 msec at each excitation wavelength (340 and 385 nm). At the end of each experiment, calibration of the excitation ratio of SBFI was performed to accurately assess the  $[\text{Na}^+]_i$ .



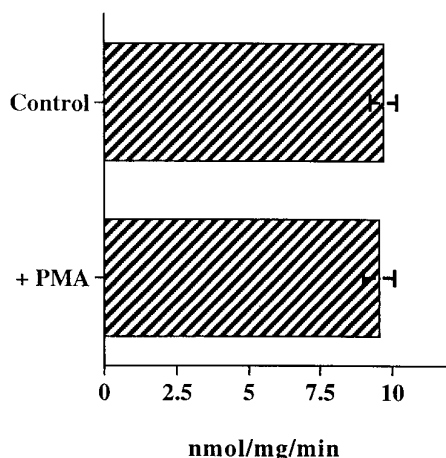
**Fig. 4.** The  $[\text{Na}^+]_i$  of OK cells transfected with the wild-type  $\alpha 1$  cDNA was not affected by treatment with  $1 \mu\text{M}$   $4\alpha$ -PMA, a phorbol ester that does not stimulate PKC. The same result was observed with  $4\alpha$ -PDD. Arrow, point of addition of PMA. Other experimental details are given in the legend to Fig. 3 and in the text.



**Fig. 5.** Inhibition of the  $\text{Na}^+/\text{H}^+$  exchanger reduces the  $[\text{Na}^+]_i$  of OK cells. The  $[\text{Na}^+]_i$  of cells transfected with the wild-type rodent  $\alpha 1$  cDNA was rapidly reduced by treatment with  $8 \mu\text{M}$  MIA. The same result was observed with cells transfected with the  $\text{NH}_2$ -deletion mutant cDNA. Arrow, point of addition of MIA. Other experimental details are given in the legend to Fig. 2 and in the text.

activity. To study this possibility, we transfected OK cells with a  $\text{NH}_2$ -terminal deletion mutant of the rodent  $\text{Na}^+$  pump  $\alpha$  subunit cDNA. This mutant expresses an  $\alpha$  subunit without the 31 first amino acids including Ser11 and Ser18, the putative target for PKC (34–36). Elimination of the  $\alpha$  subunit  $\text{NH}_2$ -terminus did not affect the  $[\text{Na}^+]_i$  of these cells. Thus, Fig. 7 shows that cells transfected with the  $\alpha$  subunit  $\text{NH}_2$ -deletion mutant cDNA have the same  $[\text{Na}^+]_i$  as cells transfected with the wild-type  $\alpha$  subunit cDNA (Fig. 3). However, the PMA-induced drop in  $[\text{Na}^+]_i$  previously seen in cells transfected with the wild-type rodent  $\alpha 1$ -cDNA was com-

pletely abolished in cells expressing the  $\alpha 1$   $\text{NH}_2$ -deletion mutant (Fig. 7). Similar results were observed in every preparation of these cells in which the effect of PMA was tested. This lack of response was not due to the cells being dead or damaged because cells transfected with the  $\alpha 1$   $\text{NH}_2$ -deletion mutant cDNA responded normally to other stimuli. Thus, the  $[\text{Na}^+]_i$  of these cells was increased by inhibition of the  $\text{Na}^+$  pump activity with ouabain (Fig. 2, bottom) and 8-bromo-cAMP, a nonhydrolyzable analog of cAMP produced a reduction in  $[\text{Na}^+]_i$  (Fig. 8). These reagents had the same effect on cells transfected with the wild-type  $\alpha 1$  cDNA. It is known



**Fig. 6.** The total  $^{22}\text{Na}^+$  uptake of cells transfected with the wild-type rodent  $\alpha 1$  cDNA was not affected by treatment with  $1\ \mu\text{M}$  PMA.  $\text{Na}^+$  influx was determined as indicated in the text.

that protein kinase A-mediated effects on the  $\text{Na}^+$  pump activity do not involve the  $\text{NH}_2$ -terminus of the  $\alpha$  subunit (34, 36, 37). Consistent with this, 8-bromo-cAMP reduced  $[\text{Na}^+]_i$  in cells transfected with either wild-type rodent  $\alpha 1$  or  $\text{NH}_2$ -deletion mutant cDNAs, whereas PKC stimulation affected only the  $[\text{Na}^+]_i$  of cells transfected with the wild-type rodent  $\alpha 1$  cDNA.

The difference in response to PMA stimulation between cells transfected with the rodent wild-type and mutant  $\alpha 1$  cDNA is not due to big differences in the level of protein expression. As shown in Fig. 1, transfected cells express the rodent wild-type and mutant  $\alpha 1$  at comparable levels. Taken together, these results indicate that the  $\text{Na}^+$  pump is the protein responsible for the reduced  $[\text{Na}^+]_i$  in response to PMA treatment.

**PKC stimulation increases  $\text{Na}^+$  pump-mediated  $\text{Rb}^+$  uptake.** To reduce the steady state  $[\text{Na}^+]_i$ , PMA had to increase the activity of the  $\text{Na}^+$  pump in intact cells. To demonstrate this, the  $\text{Na}^+$  pump-mediated  $\text{Rb}^+$  transport was determined.  $\text{Rb}^+$  was used as a  $\text{K}^+$  congener to determine the transport activity of the  $\text{Na}^+$  pump. As expected, PMA treatment of cells transfected with the wild-type  $\alpha 1$  cDNA increased the  $\text{Na}^+$  pump-mediated  $\text{Rb}^+$  transport (Table 1). This effect was not observed when the cells were treated with  $4\alpha\text{-PDD}$ , a phorbol ester that does not stimulate PKC (Table 1). Thus, the activation induced by PMA was specific and mediated by PKC.

Consistent with the lack of effect of PMA on  $[\text{Na}^+]_i$  of cells expressing the  $\alpha 1$   $\text{NH}_2$ -deletion mutant, the  $\text{Rb}^+$  transport of these cells was not increased by PMA treatment (Table 1). The lack of response to PMA observed in cells transfected with the  $\alpha$  mutant cDNA was not due to a reduced  $\text{Na}^+$  pump activity. These cells have the same basal level of ouabain-sensitive  $\text{Rb}^+$  transport and maximal  $\text{Na}^+/\text{K}^+$ -ATPase activity as cells transfected with wild-type  $\alpha 1$  cDNA (Table 1). However, in sharp contrast to the endogenous and introduced wild-type enzymes, the activity of the mutant was not significantly modified by treatment with PMA. These results suggest that PMA treatment specifically modified the  $\text{Na}^+$  pump activity with no significant effect on the  $\text{Na}^+/\text{H}^+$  exchanger or other protein(s) that may be involved in  $\text{Na}^+$  transport into the cell.

## Discussion

In this report, we have shown that PMA treatment of OK cells transfected with the rodent  $\alpha 1$  cDNA leads to reduced  $[\text{Na}^+]_i$  and increased  $\text{Na}^+$  pump-mediated  $\text{Rb}^+$  transport. Because PMA treatment has no effect on the  $\text{Na}^+$  entry, the reduction in  $[\text{Na}^+]_i$  and activation of  $\text{Rb}^+$  transport must have been mediated entirely through the  $\text{Na}^+$  pump. This conclusion is further supported by the observation that the  $[\text{Na}^+]_i$  and  $\text{Rb}^+$  transport of cells transfected with the  $\alpha 1$  mutant cDNA were not affected by PMA treatment.

Many of the studies examining the regulation of the  $\text{Na}^+$  pump by hormones and second messengers have been performed in isolated rat proximal tubule segments (8, 9, 12). The OK cells used in our experiments are an established epithelial cell line that is often studied as a physiological model system of renal proximal tubule function (33, 38). The reason we used transfected cells for these experiments was to determine the effect of PMA treatment in cells transfected with the  $\alpha 1$   $\text{NH}_2$ -deletion mutant cDNA. To specifically impair the PKC modulation of the  $\text{Na}^+$  pump was important to further support the conclusion that the reduced  $[\text{Na}^+]_i$  was produced by activation of the  $\text{Na}^+$  pump and not by inhibition of the passive  $\text{Na}^+$  influx. In nontransfected cells, the  $\text{Rb}^+$  transport was increased and the  $[\text{Na}^+]_i$  was decreased by PMA treatment in the same way as cells transfected with the wild-type  $\alpha 1$  cDNA. Thus, the results observed with the introduced pumps reflect the normal activities of the cell and not an artifact produced by transfection of exogenous cDNA.

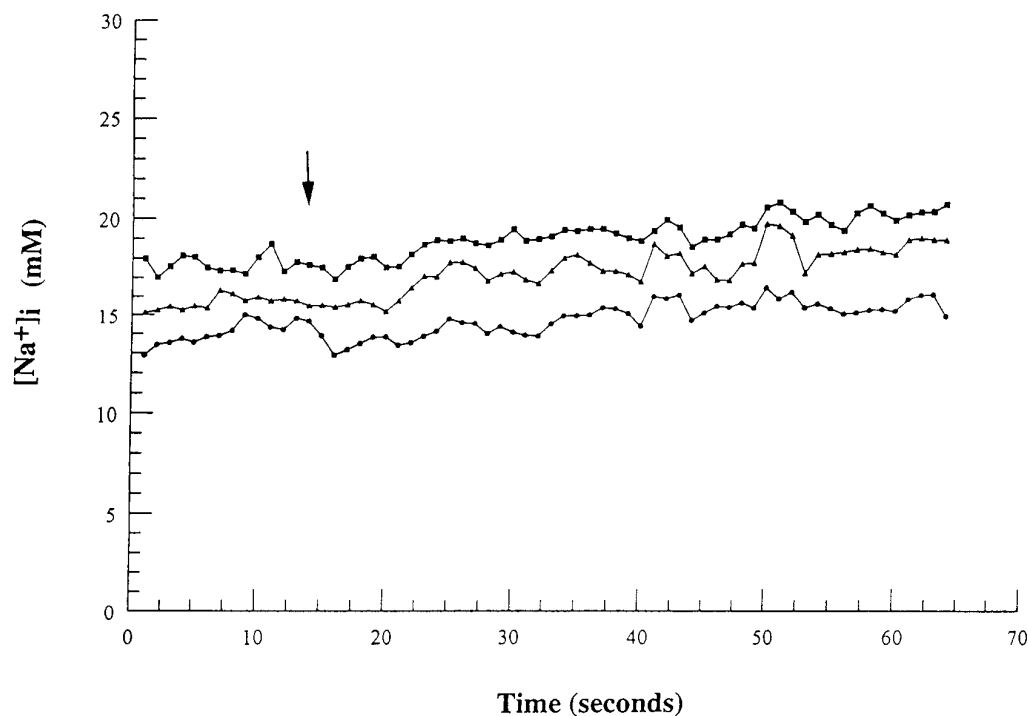
The free  $[\text{Na}^+]_i$  value of  $19.7 \pm 2.4\ \text{mM}$  that we measured in OK cells is similar to that determined by other authors in kidney cells using the fluorescent  $\text{Na}^+$  indicator SBFI (39–41). Moreover, the  $[\text{Na}^+]_i$  we measured is in the range of the  $K_{0.5}$  for  $\text{Na}^+$  determined in toad kidney [12.3 mM (42)], rat kidney [20.4 mM (43)], and human kidney [16 mM (44)]  $\text{Na}^+$  pumps. Thus, the  $[\text{Na}^+]_i$  of kidney cells seems to be maintained at a level that ensures maximum responsiveness of the  $\text{Na}^+$  pump to changes in  $[\text{Na}^+]_i$ .

Inhibition of the  $\text{Na}^+$  pump by ouabain produced a rise in  $[\text{Na}^+]_i$  of  $\sim 7.5\ \text{mM}$  in 10 min. Based on this rate of increase and an intracellular volume of 2.4 pL,<sup>1</sup> the estimated rate for the passive  $\text{Na}^+$  influx is 1.8 fmol/min/cell. With 0.2 ng of protein/cell,<sup>2</sup> the value of  $9.6 \pm 0.5\ \text{nmol/mg/min}$  determined for  $\text{Na}^+$  influx corresponds to  $\sim 1.9\ \text{fmol/min/cell}$ . In steady state, the influx rate of  $\text{Na}^+$  is the same as the rate of efflux through the  $\text{Na}^+$  pump; then, the  $\text{Na}^+$  influx can be independently calculated from the basal  $\text{Rb}^+$  transport we determined. Because the  $\text{Na}^+$  pump transports three  $\text{Na}^+$  and two  $\text{Rb}^+$  ions per cycle, 9.5 nmol/mg/min  $\text{Rb}^+$  corresponds to 14.3 nmol/mg/min  $\text{Na}^+$  transported by the  $\text{Na}^+$  pump. With 0.2 ng of protein/cell, the  $\text{Na}^+$  transport by the  $\text{Na}^+$  pump would be 2.9 fmol/min/cell. The fact that  $\text{Na}^+$  influx is lower in cells poisoned with ouabain is in agreement with reports that  $\text{Na}^+$  influx would decrease with higher  $[\text{Na}^+]_i$  (45). Despite some uncertainties in our estimates, the last value is very close to that calculated for the passive  $\text{Na}^+$  influx from the rise in

<sup>1</sup> In suspension, OK cells have a diameter of  $\sim 17\ \mu\text{m}$ . Using this value and the equation to calculate the volume of a sphere, a volume of 2.4 pL was calculated. It was assumed that attached cells have the same intracellular volume as cells in suspension.

<sup>2</sup> This value was calculated from determinations of protein of a known number of cells.





**Fig. 7.** PMA (1  $\mu$ M) does not affect the  $[\text{Na}^+]_i$  of cells transfected with the  $\text{NH}_2$ -deletion mutant  $\alpha 1$  cDNA. The same result was observed with PDD,  $4\alpha$ -PMA, and  $4\alpha$ -PDD. Arrow, point of addition of PMA. Other experimental details are given in the legend to Fig. 3 and in the text.

$[\text{Na}^+]_i$  by ouabain inhibition and the direct measurement of  $\text{Na}^+$  uptake. These values were calculated from totally independent determinations that were performed with the use of very different techniques (digital imaging fluorescence microscopy and radioisotope flux analysis).

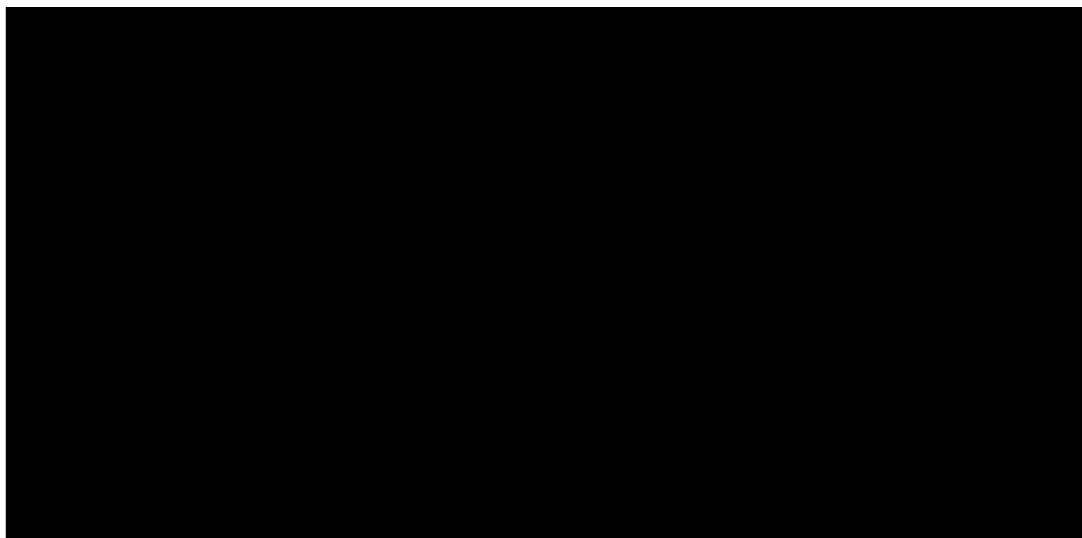
The treatment of cells expressing the rodent  $\alpha 1$  subunit with PMA produced a rapid decrease in  $[\text{Na}^+]_i$  of  $\sim 15$  mM in 10 sec. Considering a cell volume of 2.4 pL, this drop in  $\text{Na}^+$  concentration corresponds to 36 fmol eliminated/cell. To reduce that amount of intracellular  $\text{Na}^+$ , a rate of  $\text{Na}^+$  efflux of 216 fmol/min/cell is required. Because the  $\text{Na}^+$  efflux increased from 1.8–2.9 to 216 fmol/min/cell on the addition of PMA, the  $\text{Na}^+$  pump was increased 74–120-fold. Interestingly, the transient 74–120-fold increase in pump activity is in the same range as the 76-fold increase in  $\text{Na}^+$  pump activity calculated by Moore and Fay (29) for the isoproterenol-induced reduction in  $[\text{Na}^+]_i$  in muscle cells. The predicted stimulation of the  $\text{Na}^+$  pump seems to be in contradiction with the rate of  $\text{Rb}^+$  transport we determined. However, the 74–120-fold increase in pump activity is a transient activation that occurs during 10 sec after the addition of PMA, and we have not measured  $\text{Rb}^+$  transport during this period. The  $\text{Rb}^+$  transport we determined corresponds to the new steady state  $[\text{Na}^+]_i$  reached after 10 sec.

The PMA-induced activation in  $\text{Na}^+$  pump transport capacity can be due to an increase in  $V_{\text{max}}$  and/or the affinity of the  $\text{Na}^+$  pump for a ligand that is at a rate-limiting concentration. Our measurements of  $\text{Rb}^+$  transport and  $[\text{Na}^+]_i$  were performed under *in vivo* conditions in which the  $\text{Na}^+$  pump works at saturating concentrations of its ligands except for intracellular  $\text{Na}^+$  (10). In this condition, the  $\text{Na}^+$  pump rate is about five times slower than its maximal transport capacity because it is limited by the low  $[\text{Na}^+]_i$  (10, 11, 20). Therefore, an increase in  $\text{Na}^+$  pump  $V_{\text{max}}$  should have no effect on steady state  $\text{Na}^+$  transport. It follows that the PMA-induced activation of  $\text{Rb}^+$  transport must result from an increased

affinity of the  $\text{Na}^+$  pump for intracellular  $\text{Na}^+$ . However, pump recruitment should not be ruled out because the number of  $\text{Na}^+$  pump molecules that are normally at the cell membrane may not be sufficient to produce the PMA-induced rapid change of steady state  $[\text{Na}^+]_i$  required by a 74–120-fold transient increase in  $\text{Na}^+$  transport capacity. If  $\text{Na}^+$  pumps are mobilized from intracellular stores to the membrane, these molecules must have an increased affinity for  $\text{Na}^+$  to contribute to the rapid reduction in  $[\text{Na}^+]_i$  as discussed above. That PKC stimulation produces an increase in  $\text{Na}^+$  pump affinity for  $\text{Na}^+$  and not in  $V_{\text{max}}$  has been determined in proximal convoluted tubule cells (46, 47) and may explain the observation that PKC phosphorylation of purified rodent kidney  $\text{Na}^+/\text{K}^+$ -ATPase did not increase the maximal ATP hydrolysis activity (36).

We have demonstrated that stimulation of PKC activates the  $\text{Na}^+$  pump of OK cells. However, other researchers have observed that stimulation of PKC inhibited the  $\text{Na}^+/\text{K}^+$ -ATPase of COS cells (48) and *Xenopus laevis* oocytes (49) transfected with the rodent  $\alpha$  subunit. In each case, phosphorylation of the rodent  $\alpha$  subunit was determined. Interestingly, when the experiments were repeated with cells transfected with an  $\alpha$  subunit mutant cDNA with substitution of Ser18 to Ala, PKC-stimulation neither phosphorylated the mutant  $\alpha$  subunit nor affected the  $\text{Na}^+/\text{K}^+$ -ATPase activity (48, 49). This is very strong evidence of a causal link between PKC-phosphorylation of amino acids at the  $\alpha$  subunit  $\text{NH}_2$ -terminus and modulation of  $\text{Na}^+/\text{K}^+$ -ATPase activity. The apparent discrepancy between activation versus inhibition of the  $\text{Na}^+/\text{K}^+$ -ATPase in response to PKC stimulation may have its origin in the experimental conditions under which the results were obtained. Thus, Feraille *et al.* (46) observed that PKC activation of rodent proximal tubule cells produced stimulation or inhibition of the  $\text{Na}^+/\text{K}^+$ -ATPase-mediated  $\text{Rb}^+$  transport, depending on whether the determination was performed in oxygenated or anoxic





**Fig. 8.** Stimulation of protein kinase A produces a reduction of  $[Na^+]_i$  as revealed by digital imaging fluorescence microscopy. OK cells transfected with the  $NH_2$ -deletion mutant  $\alpha 1$  cDNA were loaded with the  $Na^+$ -sensitive fluorescent indicator SBFI, and the level of emitted fluorescence on excitation at 340 and 385 nm was determined. Images were obtained before and 2 sec after the addition of 1 mM 8-bromo-cAMP. Pseudocolor calibration: dark pink, 0–5 mM; dark blue, 5–10 mM; light blue, 10–15 mM; green, 15–20 mM; yellow, 20–25 mM; red, 25–30 mM; and white, >30 mM.

medium. On the other hand, the  $NH_2$ -terminus of the  $\alpha 1$  isoform has a different amino acid composition in different species (34, 36). For its part, PKC has several isoforms that may be activated by phorbol esters (32); then, whether activation or inhibition of the  $Na^+/K^+$ -ATPase by PKC is observed could depend on the coincident expression of a specific  $\alpha 1$  isoform with a particular isoform of PKC. This hypothesis is supported by the following experimental determinations. Although stimulation of endogenous *X. laevis* oocytes PKC produced inhibition of the  $Na^+/K^+$ -ATPase-mediated  $Rb^+$  transport, microinjection into the oocytes of rodent PKC led to increased  $Rb^+$  transport activity (49). Furthermore, rat PKC inhibited the  $Na^+/K^+$ -ATPase-mediated  $Rb^+$  transport of *X. laevis* oocytes transfected with rodent  $\alpha 1$  but stimulated the endogenous activity (49). The discrepancies observed may have explanations that do not contradict the main conclusion of all these results: the  $Na^+/K^+$ -ATPase activity is modulated by PKC phosphorylation of amino acids located in the  $\alpha$  subunit  $NH_2$ -terminus.

It has been observed that Ser11 and Ser18 of the  $\alpha 1$   $NH_2$ -terminus are phosphorylated by PKC (34, 36, 48, 49). In proximal tubule cells, Carranza *et al.* (47) observed that PKC-dependent stimulation of the  $Na^+$  pump is accompanied by phosphorylation of the  $\alpha$  subunit. Although our results have not explicitly demonstrated phosphorylation of the  $\alpha$  subunit by PKC, such covalent modification seems a likely explanation for the increased  $Na^+$  transport. The observation that  $Rb^+$  transport of cells expressing the  $NH_2$ -deletion mutant was not affected by PMA implicates amino acids at the  $\alpha 1$   $NH_2$ -terminus as the likely targets for phosphorylation. However, we cannot rule out the possibility that there are other proteins that may be substrates for PKC that directly or indirectly affect the activity of the  $Na^+$  pump. Independent of which amino acids or proteins are phosphorylated by PKC, our results clearly indicate that amino acids of the  $\alpha$  subunit  $NH_2$ -terminus are involved in the PKC modulation of the  $Na^+$  pump activity. Although the molecular details remain to be elucidated, the current data show that stimu-

lation of PKC causes a profound decrease in  $[Na^+]_i$  via activation of the  $Na^+$  pump. The magnitude and speed of the effect of PKC stimulation on  $[Na^+]_i$  are consistent with the hypothesis that reduction of  $[Na^+]_i$  mediated by the  $Na^+$  pump increases  $Na^+$  influx into the cell, thereby producing increased  $Na^+$  translocation in kidney epithelial cells. This is consistent with the idea that short term hormonal regulation of the  $Na^+$  pump contributes to the regulation of urinary  $Na^+$  reabsorption.

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