ERK1/2 Controls Na,K-ATPase Activity and Transepithelial Sodium Transport in the Principal Cell of the Cortical Collecting Duct of the Mouse Kidney*

The collecting duct of normal kidney exhibits significant activity of the MEK1/2-ERK1/2 pathway as shown in vivo by immunostaining of phosphorylated active ERK1/2 (pERK1/2). The MEK1/2-ERK1/2 pathway controls many different ion transports both in proximal and distal nephron, raising the question of whether this pathway is involved in the basal and/or hormone-dependent transepithelial sodium reabsorption in the principal cell of the cortical collecting duct (CCD), a process mediated by the apical epithelial sodium channel and the basolateral sodium pump (Na,K-ATPase). To answer this question we used ex vivo micropoisoned CCDs from normal mouse kidney or in vitro cultured mpkCCDcl4 principal cells. Significant basal levels of pERK1/2 were observed ex vivo and in vitro. Aldosterone and vasopressin, known to up-regulate sodium reabsorption in CCDs, did not change ERK1/2 activity either ex vivo or in vitro. Basal and aldosterone- or vasopressin-stimulated sodium transport was down-regulated by the MEK1/2 inhibitor PD98059, in parallel with a decrease in pERK1/2 in vitro. The activity of Na,K-ATPase but not that of epithelial sodium channel was inhibited by MEK1/2 inhibitors in both unstimulated and aldosterone- or vasopressin-stimulated CCDs in vitro. Cell surface biotinylation showed that intrinsic activity rather than cell surface expression of Na,K-ATPase was controlled by pERK1/2. PD98059 also significantly inhibited the activity of Na,K-ATPase ex vivo. Our data demonstrate that the ERK1/2 pathway controls Na,K-ATPase activity and transepithelial sodium transport in the principal cell and indicate that basal constitutive activity of the ERK1/2 pathway is a critical component of this control.
shown that pERK1/2 is expressed in the principal cell and not in the intercalated cells. These in vivo data suggest that basal activity of ERK1/2 may have a specific physiological relevance in the principal cell of the collecting duct, independent of hormonal or ligand-induced stimuli and distinct from the hormone-dependent effects observed in intercalated cells.

To address this question we investigated the role of ERK1/2 activity on transepithelial sodium transport in the principal cell of ex vivo microdissected cortical collecting duct (CCD) tubules or in vitro in mpkCCD14 cells, a well differentiated cell culture model of the principal cell (18). The aim of this study was 2-fold; first, to assess the role of ERK1/2 activity in basal and hormone-stimulated transepithelial sodium transport in the principal cell, and second, to examine whether ENaC and/or Na,K-ATPase activity is controlled by the constitutive or ligand-independent ATPase but has no effect on ENaC and that the activation of Na,K-ATPase by the pERK1/2 pathway is required for both ATPase but has no effect on ENaC and that the activation of Na,K-ATPase by the pERK1/2 pathway is required for both ATPase and transepithelial sodium reabsorption in the principal cell. The equivalent activity of ERK1/2 in vivo data suggest that basal activity of ERK1/2 may have a specific physiological relevance in the principal cell of the collecting duct, independent of hormonal or ligand-induced stimuli and distinct from the hormone-dependent effects observed in intercalated cells.

MATERIALS AND METHODS

Isolation of Different Parts of the Nephron—Experiments were performed on male NMRI mice that had free access to water and standard laboratory chow until anesthesia. Isolated tubules were obtained by microdissection of the left kidney perfused with 40 μg/ml Liberase Blendzyme 2 (Roche Applied Science) dissolved in DMEM/F-12 (1:1) medium (21041 medium, Invitrogen). Thin pyramids cut along the corticomedullary axis were incubated at 37 °C for 40 min in aerated DMEM/F-12 (1:1) medium containing 40 μg/ml Liberase. Microdissection was performed in ice-cold DMEM/F-12 (1:1) medium as described (19). The following structures were isolated: proximal tubule, medullary, and cortical portions of the collecting duct. Tubular length was measured with an ocular micrometer (Zeiss), and pools of CCDs containing 10–20 microdissected tubules with the total tubular length of ~10 mm/pool were transferred to DMEM/F-12 (1:1) medium. Isolated tubules were washed and placed in Ussing chambers. The incubation was performed according to the protocol described by Planes et al. (20).

Western Blotting on Microdissected Nephron Segments—Samples were heated at 95 °C for 5 min and loaded and electrophoresed on a 13% SDS-PAGE. Proteins were transferred for Western blotting, as described above. A pool of active or phosphorylated ERK1/2 was detected using anti-phospho-ERK1/2 monoclonal antibodies (Santa Cruz) and anti-total-ERK1/2 monoclonal antibodies (Cell Signaling). Stimulation with hormones was performed for 5 min. After stripping, the same membrane was used for detection of the total pool of ERK1/2 using anti-ERK1/2 monoclonal antibody (Zymed Laboratories Inc.) at a 1:1000 dilution.

Measurements of Na,K-ATPase Current in Apically Permeabilized mpkCCD14 Cells—After incubation for at least 18 h in Minimal medium, confluent mpkCCD14 cells were transfected to a Ussing chamber in the Na-free buffer containing 116 mM β-gluconate, 1.8 mM CaCl₂, 0.8 mM KH₂PO₄, 2 mM nonessential amino acids (free acids), 2 mM nonessential amino acids (free acids), 0.4 mM glutamine, 25 mM HEPES, 3 mM BaCl₂, pH 7.4 (Buffer A). After a 7-min stabilization period, cells were apically permeabilized with 17.5 μg/ml amphotericin B for 5 min, as described (21). The Na,K-ATPase was activated by apical and basolateral addition of 116 mM Na⁺-gluconate, 56 mM K⁺-gluconate, 1.8 mM CaCl₂, 16 mM (60 mM Na⁺-gluconate, 1.8 mM CaCl₂, 1.6 mM MgCl₂, 0.8 mM KH₂PO₄, 2 mM nonessential amino acids, 2 mM nonessential amino acids, 0.4 mM glutamine, 25 mM HEPES, 3 mM BaCl₂, pH 7.4) (Buffer B). This raised the apical and basolateral Na⁺ concentrations to 30 mM. After a 3-min stabilization of the Na,K-ATPase current, MEKI/2 inhibitors were added to both apical and basolateral sides. The effect of the inhibitors was monitored for 5 min.

Measurements of ENaC Current in Basolaterally Permeabilized mpkCCD14 Cells—After incubation for at least 18 h in Minimal medium, confluent mpkCCD14 cells were transfected to a Ussing chamber in the 100 mM Na⁺-apical buffer containing 100 mM Na⁺-gluconate, 1.8 mM CaCl₂, 1.6 mM MgCl₂, 0.8 mM KH₂PO₄, 2 mM d-glucose, 12 mM essential amino acids, 2 mM nonessential amino acids, 25 mM HEPES, 1.0 μM amiloride, pH 7.4 (Buffer C), and in the 100 mM K⁺-basolateral buffer containing 100 mM K⁺-gluconate, 1.8 mM CaCl₂, 1.6 mM MgCl₂, 0.8 mM KH₂PO₄, 2 mM d-glucose, 12 mM essential amino acids, 2 mM nonessential amino acids, 0.4 mM glutamine, 25 mM HEPES, 2 mM ouabain, pH 7.4 (Buffer D). After stabilization of cell monolayer resistance, cells were basolaterally permeabilized with 17.5 μg/ml amphotericin B. Then the apical amiloride was washed out by replacement of the apical buffer by 50 ml of buffer C, free of amiloride (Buffer E). After stabilization of Isc (~2–3 min) MEKI/2 inhibitors were added to both apical and basolateral sides. At the end of the experiment the ENaC activity was blocked by 10 μM amiloride.

Cell Surface Biotinylation of Na,K-ATPase—Cell surface biotinylation was performed according to protocol described by Qu et al. (22) with minor modifications (22). mpkCCD14 cells grown on Transwell filters (2 filters/condition) were placed on ice and washed three times with ice-cold phosphate-buffered saline solution complemented with 0.1 mM CaCl₂ and 1 mM MgCl₂ (Buffer F). Basolateral membrane proteins were biotinylated by a 1-h incubation at 4 °C with 1.25 mg/ml NHS-sulfo-biotin (Pierce) freshly diluted in the biotinylation buffer (10 mM triethanolamine, 2 mM CaCl₂, 150 mM NaCl, pH 7.5) (Buffer G) and stirred for 1 h at 4 °C. After biotinylation, the membrane was washed with PBS and incubated in blocking buffer (1% BSA, 0.1% Tween-20, 0.1 M sodium phosphate buffer, pH 7.4) for 1 h. The membrane was then incubated for 1 h with a 1:200 dilution of biotinylated rat Na,K-ATPase antibody. After incubation, the membrane was washed with PBS and incubated for 1 h with a 1:1000 dilution of streptavidin–horseradish peroxidase conjugate. After incubation, the membrane was washed with PBS and incubated for 30 min with SuperSignal reagent (Pierce).
Expression of pERK1/2 and ERK1/2 proteins along the nephron. Protein extracts from 10 glomeruli or 10 mm of microdissected nephron segments were loaded and electrophoresed and probed with an anti-pERK1/2 antibody, as described under “Materials and Methods.” The same blot was stripped and re-probed with an anti-ERK1/2 antibody. After a second stripping, the blot was re-probed with an anti-actin antibody. Three independent experiments showed the same distribution of ERK1/2 and pERK1/2 along the nephron. MTAL and CTAL, respectively, medullary and cortical portions of the thick ascending limb; DCT, distal convoluted tubule; CNT, connecting tubule; OMCD, and outer medullary portion of the collecting duct.

Measurement of Na,K-ATPase Activity in Microdissected Cysts—The hydrolytic activity of Na,K-ATPase was determined on pools of 4–6 permeabilized Cysts by a radiolysis microassay, as previously described (24). Tubules were permeabilized by the classical procedure with a freezing-thawing step. Total ATPase activity was measured after the addition of a solution that contained 100 mM NaCl, 5 mM KCl, 10 mM MgCl₂, 1 mM EDTA, 100 mM Tris-HCl, 5 mM EDTA, 100 mM Tris, pH 7.5 (Buffer H), containing protease inhibitors. The lysates were clarified by centrifugation at 14,000 × g for 10 min at 4 °C, and the supernatants were incubated overnight with streptavidinagarose beads to recover biotinylated proteins. The beads were then pelleted by centrifugation, and aliquots of supernatants were taken to determine the unbound, intracellular pool of proteins. Biotinylated intracellular protein biotinylation, the same blots were probed with an anti-actin antibody (Sigma).

FIG. 1. Expression of pERK1/2 and ERK1/2 proteins along the nephron. Protein extracts from 10 glomeruli or 10 mm of microdissected nephron segments were loaded and electrophoresed and probed with an anti-pERK1/2 antibody, as described under “Materials and Methods.” The same blot was stripped and re-probed with an anti-ERK1/2 antibody. After a second stripping, the blot was re-probed with an anti-actin antibody. Three independent experiments showed the same distribution of ERK1/2 and pERK1/2 along the nephron. MTAL and CTAL, respectively, medullary and cortical portions of the thick ascending limb; DCT, distal convoluted tubule; CNT, connecting tubule; OMCD, and outer medullary portion of the collecting duct.

RESULTS

Ex Vivo Expression of pERK1/2 along the Nephron—Mice used in these experiments had free access to water and standard laboratory chow and were not subjected to any special treatment. To assess the pattern of ERK1/2 and pERK1/2 expression along the nephron and to compare it to the previously published immunostaining pattern observed in vivo (14–17), we microdissected different segments of the mouse nephron. Protein extracts from 10 glomeruli or 10 mm of microdissected tubules (~3000–5000 cells) were used for Western blotting with anti-pERK1/2, anti-ERK1/2, and anti-actin antibodies. As shown in Fig. 1, pERK1/2 is expressed at detectable levels in the distal convoluted tubule (DCT), connecting tubule (CNT), and CCD but not in the other more proximal nephron segments. On the same blot, the anti-ERK1/2 antibody detected a strong expression of total (active and inactive) pool of ERK1/2 in the proximal tubule but was not sensitive enough to detect ERK1/2 expression in other nephron segments. In microdissected Cysts, the presence of the active form of ERK1/2 in basal conditions was demonstrated in 15 independent experiments and was consistently detected independently of many possible variables including circadian rhythm (the time of mouse perfusion and of microdissection varied from early morning to late afternoon).

To examine whether the basal activity pERK1/2 observed in Cysts could be further stimulated by hormones known to increase transepithelial sodium transport, we stimulated microdissected Cysts with aldosterone (165 min, 10⁻⁶ M) or vasopressin (90 min, 10⁻⁸ M). As shown in Fig. 2, A and B, respectively, there was no significant change of pERK1/2 expression (as corrected for actin) induced by aldosterone or vasopressin (110.1 ± 17.6% (n = 5, not significant) or 103.2 ± 7.6% (n = 6, not significant) respectively, compared with unstimulated cells). These observations are consistent with the in vivo immunostaining data.

Expression of pERK1/2 in mpkCCDcl4 Cells—To study the role of the pERK1/2 pathway in regulating sodium reabsorption in the principal cell, we used the mpkCCDcl4 cell line derived from the principal cell of the mouse collecting duct (18). This cell line exhibits a high transmural resistance (~3000 Ohm cm²) and a high transepithelial Na⁺ transport, which is up-regulated by aldosterone and vasopressin (18). This phenotype is achieved by changing the culture condition in the function of growth or differentiation. For growth, the mpkCCD requires insulin, dexamethasone, T₃, EGF, and fetal calf serum. For differentiation, the cells are then seeded on Transwell filters to establish polarized monolayers, allowing measurement of transepithelial sodium transport. The medium contained insulin, dexamethasone, T₃, but no serum for 5 days. Finally, for the electromorphological measurement, all factors can be removed. The monolayer can keep its sodium transport properties for days cultured in straight DMEM/F-12 medium without any serum or factors added. It is, therefore, an ideal system to study whether basal ERK 1/2 activity could be maintained in the absence of any potential external stimulus. It permits establishing direct relationships between the sodium transport (measured by the short circuit current method) and the biochemical response (by assessing the level of ERK1/2 and its active form). We first addressed the question of whether pERK1/2 is expressed in mpkCCDcl4 cells in basal conditions.
and whether pERK1/2 expression is regulated by aldosterone or vasopressin. In standard experimental conditions we used polarized mpkCCD cl 4 cells grown on porous filters incubated in serum- and hormone-free “Minimal medium” for at least 18 h before the experiments (see “Materials and Methods”). As shown in Fig. 3, A and B, the mpkCCD cl 4 cells in standard conditions express a detectable basal level of pERK1/2. Up to 3 h of stimulation with aldosterone (10^{-6} M) and up to 4 h of stimulation with vasopressin (10^{-6} M) did not significantly change the levels of pERK1/2 expression (Fig. 3, A and B, respectively) as observed ex vivo.

To examine whether pERK1/2 expression in mpkCCD cl 4 cells could be regulated by other “classical” external stimuli, we incubated cells in three different culture media that contain (Complete medium, see “Materials and Methods”) or do not contain (Light and Minimal media, see “Materials and Methods”) serum and EGF, two known stimulators of ERK1/2 activity. As shown in Fig. 3C, pERK1/2 levels were similar between cells incubated in Light or Minimal media. However, pERK1/2 level was significantly stimulated in cells incubated in the Complete medium (pERK1/2 expression in the Light medium was 148 ± 38% of the pERK1/2 expression in the Minimal medium (n = 4, NS); pERK1/2 expression in the Complete medium was 261 ± 25% of the pERK1/2 expression in the Minimal medium (n = 4, p < 0.05)). These results demonstrate that the absence of regulation of pERK1/2 expression by aldosterone and vasopressin in mpkCCD cl 4 cells is not due to a maximal stimulation of ERK1/2 activity.

PD98059 Reversibly Inhibits Transepithelial Sodium Transport and ERK1/2 Phosphorylation in mpkCCD cl 4 Cell—To further examine the role of the ERK1/2 pathway in transepithelial sodium transport in the principal cell, we assessed the effect of PD98059 (a MEK1/2 inhibitor) on transepithelial short circuit current (I sc). PD98059 is known to inhibit the activation of MEK1 and MEK2 with IC_{50} values of 2–7 and 50 μM, respectively (25). I sc was measured on confluent mpkCCD cl 4 cells grown on Snapwell filters and incubated in the Minimal medium for at least 18 h before experiments. The addition of PD98059 (50 μM) to both basolateral and apical sides of unstimulated mpkCCD cl 4 cells resulted in a rapid (within minutes) inhibition of basal I sc, a decrease in transepithelial potential difference, and an increase in transepithelial resistance (Fig. 4A and Table I). This inhibition of I sc was slowly (within 30 min) but completely reversible without washout of the inhibitor. PD98059 did not produce any effect on I sc when ENaC activity was blocked by 10 μM amiloride (Fig. 4B). The addition of PD98059 to apical or basolateral sides of mpkCCD cl 4 cells produced the same inhibitory effect on I sc as when the inhibitor was added from both sides (data not shown).

Stimulation of mpkCCD cl 4 cells with aldosterone or vasopressin significantly increased I sc with time courses characteristic of these hormones; the first significant induction of I sc with aldosterone was detectable after ~1 h of stimulation, and the maximal increase of I sc was reached after 3 h of stimulation (Figs. 4C and Table I), whereas vasopressin rapidly (within minutes) increased I sc, which reached its maximal level after ~90 min of stimulation with the hormone (Fig. 4E and Table I). PD98059 added from both apical and basolateral sides of cells significantly and rapidly inhibited both aldosterone- and vasopressin-stimulated I sc, decreased the transepithelial potential difference, and increased the transepithelial resistance (Figs. 4, C and E, respectively, and Table I). This inhibition of aldosterone- and vasopressin-stimulated I sc was maintained over 30 min in the presence of PD98059 and was reversible after washout of the inhibitor (Figs. 4, D and F, respectively). As shown in Table I, the inhibitory effects of 50 μM PD98059 were similar between basal and aldosterone- or vasopressin-stimulated I sc (~33–30, and ~38% of inhibition, respectively). Importantly, this reversible inhibition of I sc produced by PD98059 in aldosterone- and vasopressin-stimulated mpkCCD cl 4 cells paralleled a reversible decrease in ERK1/2 phosphorylation, as shown in Figs. 5, A and B, respectively. Collectively, these data demonstrate that both the basal and aldosterone- or vasopressin-stimulated sodium transport in the principal cell are controlled by the ERK1/2 pathway.

I sc Inhibition by PD98059 Correlates with a Decreased Activity of Na,K-ATPase—In the principal cell two major transporters involved in sodium transport are the apical amiloridesensitive sodium channel (ENaC) and the basolateral Na,K-ATPase. Thus, we addressed the question of whether ENaC or Na,K-ATPase activity is controlled by the pERK1/2 pathway. We first tested the effect of PD98059 and two other MEK1/2 inhibitors.
FIG. 4. Effect of PD98059 (50 μM) on $I_{sc}$ in mpkCCD14 cells. 

A, effect of PD98059 (50 μM) on basal $I_{sc}$ in unstimulated mpkCCD14 cells (●). PD98059 was added after a 5-min current stabilization period to both apical and basolateral sides. The $I_{sc}$ in control cells (without PD98059 treatment) of the same experiment is shown as a dotted curve. At the end of the experiment (95 min), amiloride (10 μM) was added to the apical side.

B, effect of PD98059 (50 μM) on basal $I_{sc}$ in unstimulated mpkCCD14 cells (●) in the presence of amiloride (10 μM). The $I_{sc}$ in control cells (unstimulated cells without PD98059 treatment) of the same experiment is shown as a dotted curve.

C, effect of PD98059 (50 μM) on aldosterone-stimulated $I_{sc}$. PD98059 was added to both apical and basolateral sides after 180 min of incubation with the hormone (●). The $I_{sc}$ in aldosterone-stimulated cells but without P98059 addition is shown as a curve with filled squares (■). The $I_{sc}$ in unstimulated cells of the same experiment is shown as a dotted curve. At the end of the experiment (280 min), amiloride (10 μM) was added to the apical side.

D, reversibility of effect of PD98059 on aldosterone-stimulated $I_{sc}$. PD98059 was added to both apical and basolateral sides of aldosterone-stimulated cells (180 min incubation with the hormone). After 30 min of incubation with PD98059, both apical and basolateral incubation medium were replaced by the same medium but without PD98059 (Œ). The $I_{sc}$ in aldosterone-stimulated and PD98059-treated cells but without washout of the inhibitor is shown as a curve with filled circles (●). The $I_{sc}$ in unstimulated cells of the same experiment is shown as a dotted curve. At the end of the experiment (280 min), amiloride (10 μM) was added to the apical side.

E, effect of PD98059 (50 μM) on vasopressin-stimulated $I_{sc}$. PD98059 was added to both apical and basolateral sides after 90 min of incubation with the hormone (●). The $I_{sc}$ in vasopressin-stimulated cells but without the P98059 addition is shown as a curve with filled squares (■). The $I_{sc}$ in unstimulated cells of the same experiment is shown as a dotted curve. At the end of the experiment (140 min), amiloride (10 μM) was added to the apical side.

F, reversibility of the effect of PD98059 on vasopressin-stimulated $I_{sc}$. PD98059 was added to both apical and basolateral sides of vasopressin-stimulated cells (80 min incubation with the hormone). After 30 min of incubation with PD98059, both apical and basolateral incubation medium were replaced by the same medium but without PD98059 (Œ). The $I_{sc}$ in vasopressin-stimulated and PD98059-treated cells but without washout of the inhibitor is shown as a curve with filled circles (●). The $I_{sc}$ in unstimulated cells of the same experiment is shown as a dotted curve. At the end of the experiment (240 min), amiloride (10 μM) was added to the apical side. Arrows indicate the time of addition.
ERK1/2 Controls Na,K-ATPase Activity in the Principal Cell

Table I

Effect of PD98059 (50 μM) on transepithelial resistance (R), transepithelial voltage (V), and transepithelial short circuit current (Isc) in mpkCCD14 cells

Statistical significance was calculated using Student’s t test. The results are the means ± S.E.

|                      | Before PD98059 | With PD98059 | Change | p     |
|----------------------|----------------|--------------|--------|-------|
|                      | R (ohm × cm²)  | V (mV)       | Isc (μA/cm²) |       |       |
| Unstimulated cells   | 1269.7 ± 207.2 | 29.1 ± 8.1   | 23.7 ± 4.6   | +9.1 ± 3.1 | a    |
| Vasopressin-stimulated cells | 1409.0 ± 253.5 | 23.4 ± 7.1   | 15.9 ± 3.4   | -32.8 ± 4.4 | a    |
| Aldosterone-stimulated cells | 697.1 ± 85.4  | 44.0 ± 4.7   | 62.2 ± 6.3   | +6.4 ± 1.9  | a    |

α p < 0.05.
β p < 0.001.

**FIG. 5.** Effect of PD98059 on ERK1/2 phosphorylation in mpkCCD14 cells. A, effect of PD98059 (50 μM) on ERK1/2 phosphorylation in aldosterone-stimulated mpkCCD14 cells. PD98059 was added to both apical and basolateral sides. Protein extracts were made (i) from control or unstimulated cells incubated in the same conditions as aldosterone (Aldo)-stimulated cells, (ii) from cells stimulated with aldosterone (10⁻⁹ M) for 180 min, (iii) from cells stimulated with aldosterone for 180 min and incubated with PD98059 for 30 min, and (iv) from cells stimulated with aldosterone for 180 min incubated with PD98059 that was washed out after 30 min of incubation. B, effect of PD98059 (50 μM) on ERK1/2 phosphorylation in vasopressin (Vaso)-stimulated mpkCCD14 cells. PD98059 was added to both apical and basolateral sides. Protein extracts were made from (i) control or unstimulated cells incubated in the same conditions as vasopressin-stimulated cells, (ii) cells stimulated with vasopressin (10⁻⁸ M) for 90 min, (iii) cells stimulated with vasopressin for 90 min and incubated with PD98059 for 30 min, and (iv) cells stimulated with vasopressin for 90 min incubated with PD98059 that was washed out after 20 min of incubation. Protein extracts were electrophoresed through 13% SDS-PAGE. Western blots were first probed with anti-pERK1/2 antibody and, after stripping, with anti-ERK1/2 and anti-actin antibodies.

Importantly, the Iₛₑ in permeabilized unstimulated cells is inhibited by U0126 with an IC₅₀ value of 26 μM (Fig. 7), which corresponds well with the IC₅₀ of U0126 in in vitro assays (~13 μM) (26).

As shown in Fig. 6A and in Table II, PD98059 (50 μM), U0126 (50 μM), and SL327 (50 μM) significantly inhibited the Iₛₑ in unstimulated cells, albeit to a different extent. Importantly, the Iₛₑ in permeabilized unstimulated cells is inhibited by U0126 with an IC₅₀ value of 26 μM (Fig. 7), which corresponds well with the IC₅₀ of U0126 in in vitro assays (~13 μM) (26). As shown in Fig. 6B and in Table II, pretreatment of cells with aldosterone (180 min) resulted in a significant increase in Iₛₑ (72 ± 11% of increase in n = 9, p < 0.001), whereas Iₛₑ measured in cells pretreated with vasopressin was not significantly different from that in unstimulated cells (Fig. 6C and Table II).

Importantly, the Iₛₑ in aldosterone- or vasopressin-stimulated cells could be blocked by PD98059 and U0126 to a similar extent as in unstimulated cells (Figs. 6, B and C, and Table II), thereby indicating that activity of the Na,K-ATPase is controlled by the MEK1/2-ERK1/2 pathway.

We were concerned by possible nonspecific direct effects of MEK1/2 inhibitors on the Na,K-ATPase. To examine this possibility we tested the effect of U0126 (50 μM) on the endogenous Na,K-ATPase of Xenopus laevis oocytes. It is worth noting that the MEK1/2-ERK1/2 pathway in stage VI immature Xenopus oocytes remains inactive until its stimulation by progesterone, which induces oocyte maturation and activation of cell cycle progression. Therefore, this expression system is convenient for testing the possible direct nonspecific effects of MEK1/2 inhibitors on Na,K-ATPase activity observed in mpkCCD14 cells are, therefore, unlikely to be due to a direct nonspecific inhibition of the Na,K-ATPase.

Effects of PD98059 and U0126 on ENaC Activity in Basolaterally Permeabilized mpkCCD14 Cells—To assess the role of inhibitors, namely U0126 and SL327, on Na,K-ATPase transport activity using a protocol developed by Summa et al. (21). In this protocol the apical membrane of mpkCCD14 cells is selectively permeabilized to monovalent cations with amphotericin B, thus allowing free diffusion of Na⁺ into the cell through the apical membrane. To check the efficiency of apical membrane permeabilization we tested the effect of amiloride (10 μM) on Iₛₑ measured in permeabilized cells. These control experiments demonstrated that mpkCCD14 cells treated from the apical side with amphotericin B (5 min, 17.5 μg/ml) do not express any residual amiloride-sensitive Iₛₑ, thus indicating that most of the cells are efficiently permeabilized (data not shown). To block the basolateral potassium conductance, all the experiments were performed in the presence of 3 mM Ba²⁺ in the basolateral solution. In these conditions most of the Iₛₑ activated by replacement of Na⁺-free by 30 mM Na⁺-containing incubating media (see “Materials and Methods”) is due to the activation of the Na,K-ATPase and is termed the Na⁺ pump current (Iₛₑ).
the pERK1/2 pathway in the control of ENaC activity, we permeabilized the basolateral membrane with amphotericin B (5 min, 17.5 μg/ml) in a high basolateral K⁺ solution (100 mM K⁺ gluconate). Under these experimental conditions, the amiloride-sensitive sodium current (Iₒ) depends only on the large Na⁺ gradient artificially maintained across the apical membrane and not on the Na,K pump activity at the basolateral membrane. This was checked by the addition of 2 mM ouabain to the basolateral solution, which had no detectable effect on the transepithelial current (data not shown). As shown in Fig. 8A, the addition of PD98059 or U0126 to basolaterally permeabilized unstimulated cells did not produce a significant effect on Iₒ. Pretreatment of cells with aldosterone or vasopressin significantly increased the Iₒ (326 ± 50% increase with aldosterone (n = 9, p < 0.001) and 169 ± 7% increase with vasopressin (n = 8, p < 0.001), Fig. 8B and C, respectively). No change in Iₒ was observed upon treatment of aldosterone- or vasopressin-stimulated cells with PD98059 or U0126 (Fig. 8B and C, respectively). These data indicate that the apical membrane Na⁺ conductance, and thus, ENaC activ-
biotinylation of unstimulated mpkCCDcl4 cells treated or not with inhibitors decrease intrinsic Na,K-ATPase pump activity or its cell surface expression. We performed basolateral cell surface biotinylation of unstimulated mpkCCDcl4 cells to address the question of whether MEK1/2 Na,K-ATPase activity is not controlled by the ERK1/2 pathway in permeabilized mpkCCDcl4 cells.

Effects of PD98059 and U0126 on Cell Surface Expression of Na,K-ATPase—To address the question of whether MEK1/2 inhibitors decrease intrinsic Na,K-ATPase pump activity or its cell surface expression, we performed basolateral cell surface biotinylation of unstimulated mpkCCDcl4 cells treated or not with PD98059, U0126, or SL327 for 10 min and with PD98059 or U0126 for 30 min. The biotinylation was performed on cells grown on two Transwell filters, and total biotinylated proteins were precipitated with streptavidin-agarose beads and electrophoresed through SDS-PAGE. In parallel, one-third of intracellular proteins recovered after precipitation of biotinylated proteins were loaded on the same gel. The proteins were Western-probed with an anti-α1 Na,K-ATPase subunit antibody. As shown in Fig. 9 (upper panel), none of these three inhibitors induced any significant changes in Na,K-ATPase expressed at cell surface (lanes 1–6) or in intracellular pools of Na,K-ATPase (lanes 7–12). Interestingly, the quantitation of cell surface versus intracellular pool of Na,K-ATPase demonstrated that in control cells a majority (63 ± 2%, n = 10) of Na,K-ATPase is expressed at the cell surface. As a control for the biotinylation procedure we re-probed the same Western blot with an anti-actin antibody. As shown in Fig. 9 (lower panel), only intracellular protein extracts were positive for anti-actin staining (lanes 7–12), thus demonstrating that only cell surface-expressed proteins were biotinylated in our experiments. Collectively, these data indicate that inhibition of Na,K-ATPase current by PD98059, U0126, or SL327 results from a decreased intrinsic activity of Na,K-ATPase and not from a decrease in its cell surface expression.

Effect of PD98059 on Na,K-ATPase Activity and pERK1/2 Expression in Microdissected CCDs—We also checked whether the MEK1/2-ERK1/2 pathway controls the activity of the Na,K-ATPase in microdissected CCDs ex vivo. In these experiments we used microdissected CCDs preincubated or not with cAMP, a mediator of intracellular vasopressin signaling in the principal cell. As shown in Fig. 10A, 50 µM PD98059 significantly inhibited the Na,K-ATPase activity in both untreated and cAMP-treated CCDs. This decrease in Na,K-ATPase activity was in parallel with a decreased ERK1/2 phosphorylation as determined by Western blot (Fig. 10B). Thus, the MEK1/2-ERK1/2 pathway is also involved in the control of Na,K-ATPase activity in microdissected CCDs.

### DISCUSSION

In the present study we describe a novel mechanism that controls sodium reabsorption in the principal cell by a basal, hormone-independent activation of Na,K-ATPase by the ERK1/2 MAP kinase pathway.

| Inhibitor | Ip before inhibitor (% of control) | Ip with inhibitor (% of control) | Change (%) | n | p |
|-----------|----------------------------------|----------------------------------|------------|---|---|
| Unstimulated cells | | | | | |
| Control | 13.4 ± 0.3 | 13.4 ± 0.2 | +0.2 ± 2.0 | 6 | Not shown |
| PD98059 | 14.1 ± 0.7 | 9.3 ± 0.5 | −34.5 ± 0.4 | 4 | a |
| U0126 | 13.6 ± 0.5 | 4.1 ± 0.2 | −69.6 ± 1.7 | 6 | a |
| SL327 | 13.5 ± 0.7 | 9.2 ± 0.3 | −30.9 ± 5.6 | 3 | a |
| Vasopressin-stimulated cells | | | | | |
| Control | 11.9 ± 1.8 | 11.0 ± 1.8 | −8.1 ± 3.2 | 4 | Not shown |
| PD98059 | 12.1 ± 0.5 | 8.6 ± 0.7 | −29.5 ± 3.1 | 4 | a |
| U0126 | 11.2 ± 2.2 | 4.9 ± 0.5 | −52.0 ± 7.8 | 3 | a |
| Aldosterone-stimulated cells | | | | | |
| Control | 17.0 ± 1.5 | 14.6 ± 1.2 | −13.7 ± 3.3 | 3 | Not shown |
| PD98059 | 13.7 ± 1.6 | 6.5 ± 2.2 | −54.3 ± 10.8 | 3 | a |
| U0126 | 19.4 ± 0.8 | 5.4 ± 0.3 | −72.2 ± 2.4 | 3 | a |

* p < 0.05.
** p < 0.001.
basal activation of ERK1/2. Indeed, activated ERK1/2 (pERK1/2) are usually rapidly dephosphorylated after removal of external stimuli, whereas in our experimental conditions incubation of microdissected CCDs in hormone-free medium for up to 3 h did not reduce the basal ERK1/2 phosphorylation. A possible mechanism for this basal activation of the ERK1/2 pathway in the principal cell could be the previously reported basal activation of G protein-coupled receptors or tyrosine kinase receptors (27, 28). Second, significant basal expression of pERK1/2 was also detected in mpkCCDcl4 cells after 6 days of deprivation from serum and EGF. This strongly suggests that basal ERK1/2 activity in mpkCCDcl4 cells results from a ligand-independent basal activation of the cascade. Third, neither aldosterone nor vasopressin significantly affected ERK1/2 activity in microdissected CCDs or in mpkCCDcl4 cells. These results are different from those obtained by Hendron and Stoc kand (29) in A6 cells derived from X. laevis kidney in which aldosterone significantly increased the level of ERK1/2 phosphorylation via stimulation of expression and activity of K-RasA. The reason for the difference is not known. A possible explanation is that Ras-dependent activation of ERK1/2 could be specific for amphibian cells, and the expression of Ras proteins in mammalian principal cells may not be regulated by aldosterone.

Our data are similar to recent observations made in tissues or cells showing that the basal activation of MAP kinase pathways may be responsible for sustained stimulation or inhibition of various ion transport processes. For example, in hepatocytes the basal activity of p38 MAP kinase is required for maintenance of cell volume through tonic inhibition of Na’-permeable ion channels (30). In dendrites of pyramidal neurons, the basal activity of ERK1/2 is responsible for sustained down-regulation of A-type outward potassium currents (31, 32).

Control of Transepithelial Sodium Reabsorption in the Principal Cell by the pERK1/2 Pathway—We provide the following lines of evidence that the basal activity of ERK1/2 directly controls the transepithelial sodium transport in mpkCCDcl4 cells.

First, PD98059, a MEK1/2 inhibitor, significantly and reversibly inhibits short circuit current ($I_{sc}$) in both unstimulated and aldosterone- or vasopressin-stimulated cells; this inhibition of $I_{sc}$ correlates with a small increase in transepithelial resistance and a decrease in transepithelial potential difference. Second, the inhibitory effect of PD98059 is absent in cells incubated in the presence of amiloride in the apical medium, thus demonstrating that sodium transport is the major target for the ERK1/2 pathway in the principal cell. Third, the inhibition of sodium transport by PD98059 correlates with a decrease in ERK1/2 phosphorylation. Fourth, the effect of PD98059 is specific for the ERK1/2 pathway. It is independent of aldosterone.

"Materials and Methods." Ctrl, control. PD98059 (50 μM) (●) or U0126 (50 μM) (▲) were added to both apical and basolateral sides. The $I_{sc}$ in control cells (MEK1/2 inhibitors treatment) is shown as a dotted curve. B, aldosterone (Aldo)-stimulated cells. mpkCCDcl4 cells were stimulated for 180 min with aldosterone (10⁻⁶ M). PD98059 (50 μM) (▲) or U0126 (50 μM) (●) were added to both apical and basolateral sides. The $I_{sc}$ recording from aldosterone-stimulated cells that were not treated with MEK1/2 inhibitors is shown with filled squares (■). The $I_{sc}$ in control cells (without aldosterone and MEK1/2 inhibitor treatment) is shown as a dotted curve. C, vasopressin (Vaso)-stimulated cells. mpkCCDcl4 cells were stimulated for 90 min with vasopressin (10⁻⁸ M). The vasopressin-stimulated mpkCCDcl4 cells were permeabilized with amphotericin B from the basolateral side. PD98059 (50 μM) (▲) or U0126 (50 μM) (●) were added to both apical and basolateral sides. The $I_{sc}$ in vasopressin-stimulated cells that were not treated with MEK1/2 inhibitors is shown with filled squares (■). The $I_{sc}$ in control cells (without vasopressin and MEK1/2 inhibitor treatment) is shown as a dotted curve.
of whether the inhibitor is added to the apical or basolateral incubating medium, indicating that inhibition of $I_{sc}$ by PD98059 is not a direct nonspecific effect of the inhibitor on apical or basolateral channels and/or transporters. Moreover, MEK1/2 inhibitors have been reported to have several nonspecific targets. For example, PD98059 has been shown to inhibit the activity of COX-1, an enzyme involved in prostaglandin synthesis in the principal cell (33, 34). Because prostaglandins are paracrine autacoids causing local regulation of sodium reabsorption in the principal cell, we were concerned by possible MEK1/2-unrelated effects of PD98059 on $I_{sc}$. We, therefore, tested whether the effect of PD98059 on $I_{sc}$ is additive to that of resveratrol, a specific COX-1 inhibitor. These experiments demonstrated that resveratrol induces a small but significant decrease in $I_{sc}$ in mpkCCDcl4 cells that is fully additive to the decrease in $I_{sc}$ provoked by PD98059 (data not shown). Thus, the effect of PD98059 on $I_{sc}$ is not due to a nonspecific effect of this inhibitor on COX-1.

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