

Aldosterone stimulates vacuolar H⁺-ATPase activity in renal acid-secretory intercalated cells mainly via a protein kinase C-dependent pathway

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Winter C, Kampik NB, Vedovelli L, Rothenberger F, Păunescu TG, Stehberger PA, Brown D, John H, Wagner CA. Aldosterone stimulates vacuolar H⁺-ATPase activity in renal acid-secretory intercalated cells mainly via a protein kinase C-dependent pathway. *Am J Physiol Cell Physiol* 301: C1251–C1261, 2011. First published August 10, 2011; doi:10.1152/ajpcell.00076.2011.—Urinary acidification in the collecting duct is mediated by the activity of H⁺-ATPases and is stimulated by various factors including angiotensin II and aldosterone. Classically, aldosterone effects are mediated via the mineralocorticoid receptor. Recently, we demonstrated a nongenomic stimulatory effect of aldosterone on H⁺-ATPase activity in acid-secretory intercalated cells of isolated mouse outer medullary collecting ducts (OMCD). Here we investigated the intracellular signaling cascade mediating this stimulatory effect. Aldosterone stimulated H⁺-ATPase activity in isolated mouse and human OMCDs. This effect was blocked by suramin, a general G protein inhibitor, and GP-2A, a specific G_{αq} inhibitor, whereas pertussis toxin was without effect. Inhibition of phospholipase C with U-73122, chelation of intracellular Ca²⁺ with BAPTA, and blockade of protein kinase C prevented the stimulation of H⁺-ATPases. Stimulation of PKC by DOG mimicked the effect of aldosterone on H⁺-ATPase activity. Similarly, aldosterone and DOG induced a rapid translocation of H⁺-ATPases to the luminal side of OMCD cells *in vivo*. In addition, PD098059, an inhibitor of ERK1/2 activation, blocked the aldosterone and DOG effects. Inhibition of PKA with H89 or KT2750 prevented and incubation with 8-bromoadenosine-cAMP mildly increased H⁺-ATPase activity. Thus, the nongenomic modulation of H⁺-ATPase activity in OMCD-intercalated cells by aldosterone involves several intracellular pathways and may be mediated by a G_{αq} protein-coupled receptor and PKC. PKA and cAMP appear to have a modulatory effect. The rapid nongenomic action of aldosterone may participate in the regulation of H⁺-ATPase activity and contribute to final urinary acidification.

collecting duct; kidney; receptor; signaling cascade

THE KIDNEY PLAYS A MAJOR ROLE in the control of systemic acid-base homeostasis by reabsorbing filtered bicarbonate, generating new bicarbonate, and secreting protons largely bound to buffers such as phosphate and ammonia. The final step of urinary acidification occurs along the cortical and outer medullary collecting duct (OMCD) mainly through the action of vacuolar H⁺-ATPases expressed in the luminal membrane of acid-secretory type A intercalated cells (2, 8, 15, 27, 69–71).

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Renal acid secretion and urinary acidification are tightly regulated by a number of factors including several hormones such as endothelin, angiotensin II, and aldosterone (15, 27, 70–71). The influence of the renin-angiotensin II-aldosterone system (RAAS) on the ability of the kidney to excrete protons is well recognized. Both angiotensin II and aldosterone have been demonstrated to directly and indirectly influence transport pathways involved in acid secretion such as Na⁺/H⁺ exchangers and vacuolar H⁺-ATPases in different nephron segments (1, 16, 19–23, 30, 51–52, 58, 75). Aldosterone deficiency or defects in its receptor activation are causative for the hyperkalemic form of distal renal tubular acidosis (type IV dRTA) (15).

In the connecting segment and cortical collecting duct, aldosterone stimulates the activity of the electrogenic vacuolar H⁺-ATPase, an effect that has been proposed to be of a primarily indirect nature. Increased reabsorption of Na⁺ through the epithelial Na⁺ channel ENaC in neighboring principal cells renders the tubule lumen more negative and could, thus, facilitate proton pumping (15, 27). We have recently demonstrated that this interaction occurs mainly in the connecting tubule (40). Thus, the exact mechanism by which Na⁺ absorption, H⁺ secretion, and aldosterone effects are linked in the cortical collecting duct is not fully understood. In contrast, in the OMCD, aldosterone stimulates proton secretion and this effect occurs even in the absence of significant Na⁺ absorption, suggesting a direct effect on vacuolar H⁺-ATPase activity (65). Consistent with these observations, long-term stimulatory effects of aldosterone on vacuolar H⁺-ATPase activity have been described in rabbit and rat OMCDs (14, 19, 30, 52).

Cellular responses to aldosterone in the classic long-term range (i.e., responses requiring several hours or days) are mediated by alterations in gene expression driven by the binding of the steroid hormone to a cytosolic or nuclear receptor acting as a transcription factor (50, 64, 68). However, not all biological effects of aldosterone can be explained by transcriptional or genomic effects. Over the past years an increasing number of studies have identified nongenomic effects of aldosterone in both epithelial and nonepithelial tissues (for review, see refs. 17, 46–50, 64). Taken together, these studies suggest the existence of novel steroid hormone receptors or possibly classical receptors embedded in the membrane that initiate nongenomic signaling cascades (17–18, 35, 49). Activation of these putative receptors initiates several intracellular signaling cascades that appear to be tissue or cell specific,

and the involvement of phospholipase C, inositol 1,4,5-trisphosphate (IP₃), protein kinase C pathways as well as adenylyl cyclases, cAMP and PKA-dependent pathways has been described (17, 48–49). In addition, activation of ERK1/2 MAPK kinases has been observed (20, 41, 57). However, most of these studies were performed in cell culture models and only little information exists if similar pathways are activated in freshly isolated cells and tissues.

Recently, we described the direct stimulation of vacuolar H⁺-ATPase activity in freshly isolated mouse acid-secretory intercalated cells by aldosterone. In OMCD intercalated cells, aldosterone increased vacuolar H⁺-ATPase activity in the presence of the mineralocorticoid receptor blocker spironolactone and did not require transcription or translation. The stimulatory effect of aldosterone was associated with a transient rise in intracellular Ca²⁺ and required intact protein kinase C (PKC) (75). Here we further investigated the mechanism by which aldosterone stimulates vacuolar H⁺-ATPase activity. The results present pharmacological evidence supporting the hypothesis that aldosterone acts through both PLC/Ca²⁺/PKC- and cAMP/PKA-dependent pathways and requires ERK1/2 activity. Furthermore, the rapid effects of aldosterone may require a G protein-coupled membrane receptor sensitive to suramin and GP-2A but not pertussis toxin.

MATERIALS AND METHODS

Animals. C57BL/6J mice (Jackson Laboratory) (male, 12–15 wk old, 30–35 g) were housed in climate- and humidity-controlled light-cycled rooms, fed standard rodent chow, and allowed free access to water. All studies carried out in Zurich were approved by the local Swiss Veterinary Authority (Veterinäräm, Zurich, Switzerland) and were performed according to Swiss Animal Welfare laws.

Human kidney samples. Human kidney samples were collected from patients undergoing nephrectomy for tumor removal (Department of Urology, University of Zurich, Switzerland). Informed consent was obtained from all patients and all studies were approved by the local ethics committee. Kidney samples were taken from the apparently healthy part of the removed kidneys and placed into ice-cold medium (MEM + Earle's + L-glutamine + 25 mM HEPES, GIBCO) for transport.

Isolation of OMCDs. OMCDs were prepared from human and mouse kidneys with a small modification, as described (72, 75). Briefly, mice were anesthetized and perfused through the left ventricle with a phosphate/sucrose buffer (140 mM sucrose/140 mM NaH₂PO₄, pH 7.4) with 6 mg/ml trypsin inhibitor type II-S and 250 µg/ml collagenase (Sigma). Kidneys were harvested and transferred to a dissection microscope, coronal slices 1–2 mm in thickness were prepared, and the outer medulla was dissected. Outer medulla pieces were transferred to a digestion buffer and incubated at 37°C for 30 min, washed several times with ice-cold HEPES buffer, and stored on ice until further use. Human kidney samples were cut into coronal slices, the outer medulla were identified and prepared, and small cubic pieces of about 2–3 mm in side length were incubated in the digestion buffer at 37°C for 45–60 min. After several washes with ice-cold HEPES buffer, tubules were stored on ice until further use. Human and mouse OMCDs were identified under the dissection microscope, transferred on coverslips precoated with Cell-Tak (BD Biosciences, Franklin Lakes, NJ), and allowed to adhere.

Intracellular pH measurements. Coverslips with isolated OMCDs were transferred to a thermostatically controlled perfusion chamber (≈3 ml/min flow rate) maintained at 37°C on an inverted microscope (Zeiss Axiovert 200, Carl Zeiss, Feldbach, Switzerland) equipped with a video imaging system (Visitron, Munich, Germany) for the duration of the experiment. The isolated OMCDs were incubated in a

HEPES-buffered Ringer solution containing the pH-sensitive dye BCECF-AM [2',7'-bis(2-carboxylethyl)-5(6)-carboxyfluorescein ester] (10 µM, Molecular Probes) for 15 min and were washed to remove all nondeesterified dye.

Intracellular pH (pH_i) was measured microfluorometrically by alternately exciting the dye with light at 495 and 440 nm while monitoring the emission at 532 nm with a video imaging system. Each experiment was calibrated for pH_i using the nigericin/high-K⁺ method, and the obtained ratios were converted to pH_i as described (66, 72, 75). All experiments were performed in the nominal absence of bicarbonate. The initial solution was a HEPES-buffered Ringer solution (in mM: 125 NaCl, 3 KCl, 1 CaCl₂, 1.2 MgSO₄, 2 KH₂PO₄, 32.2 HEPES, pH 7.4). Cells were acidified by using the NH₄Cl (20 mM) prepulse technique and washed into a Na⁺-free solution [Na⁺ was replaced by equimolar concentrations of *N*-methyl-D-glucamine (NMDG)]. Intercalated cells were identified by their rapid uptake of BCECF as described previously (72). The rate of H⁺-ATPase activity was determined as the concanamycin-sensitive pH_i alkalinization rate in the absence of Na⁺. Rates were calculated over the same range of pH_i (6.55–6.75) for all cells studied. All chemicals were of highest purity and were purchased from Sigma (Buchs, Switzerland) and Calbiochem (Merck, Darmstadt, Germany). Chemicals were added to the solutions from stock solutions at the given concentrations for each experimental protocol (Table 1).

Intrinsic buffering power and calculation of proton fluxes. The intrinsic buffering power (β_i) is defined by the amount of acid or base that has to be added to a solution to change its pH by one unit. The β_i of OMCD cells was measured as described previously (7, 73), but changes in the pH_i were induced by superfusion with a weak acid and the exclusion of Cl⁻ from the bath. The cellular H⁺-dependent regulatory mechanisms were excluded as a buffering system by using the inhibitors concanamycin A (200 µM) and Sch28080 (2-methyl-8-(phenylmethoxy)imidazo[1,2-*a*]pyridine-3-acetonitrile) (Tocris Biosciences, Bristol, UK) (100 µM). The tubules were incubated either with 10 µM BCECF alone or with 10 nM aldosterone for 20 min in HEPES-buffered Ringer solution, pH 7.3. Afterward, the tubules were equilibrated with an acetate- and Cl⁻-free solution (in mM: 15 Ca-gluconate, 50 NMDG gluconate, 98 K-gluconate, 10 HEPES, 1.2 MgSO₄, 1 K₂HPO₄, pH 7.3). The superfusion solution was switched to an acetate-containing but Cl⁻-free solution (in mM: 15 Ca-gluconate, 20 K-acetate, 50 NMDG gluconate, 98 K-gluconate, 10 HEPES, 1.2 MgSO₄, 1 K₂HPO₄, pH 7.3) resulting in a decrease of pH_i. When the cell acidification reached a plateau, the experiment was calibrated to pH 6.5 and 7.0 using the nigericin/high-K⁺ technique (66, 72). The β_i was calculated as the

Table 1. Pharmacological agents used to modulate the activity of intracellular signaling molecules

Substance	Target
Pertussis toxin	G _i and G _o , G protein inhibitor
Suramin	G protein inhibitor. Also directly blocks purinergic and adrenergic receptors.
G2A	G _{αq} inhibitor
U-73122	Inhibitor of phospholipase C
U-73343	Inactive analog to U-73122
BAPTA-AM	Chelator of intracellular calcium
Chelerythrine	Pan-PKC inhibitor
DOG (1,2-dioctanoyl- <i>sn</i> -glycerol)	Diacylglycerol analog, PKC activator
PD098059	Prevents activation of mitogen-activated protein kinase kinase-1
H89 [<i>N</i> -[2-(<i>p</i> -bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride]	Inhibitor of protein kinase A
KT5720	Reversible and ATP-competitive inhibitor of protein kinase A

ratio of the change in intracellular acetate concentration to the change in pH_i : $\beta_i = \Delta A/\Delta pH_i$, where ΔpH_i is the decrease in pH_i resulting from weak acid addition and ΔA is the change in intracellular acetate concentration calculated from its pK_a (4.74 at 37°C) and pH_i .

Proton fluxes J_H (in $\text{pmol}\cdot\text{min}^{-1}\cdot\text{mm}^{-1}$) were calculated following the equation: $J_H = dpH_i/dt \times \beta_i \times V$, where dpH_i/dt is the rate of H^+ -ATPase activity and V is cell volume. Net proton efflux is indicated by positive J_H values.

Tissue preparation and treatment of rats with aldosterone and DOG. Adult male Sprague-Dawley rats (Charles River, Wilmington, MA) were housed under standard conditions and fed a standard rodent diet. Animal studies were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care, in accordance with the National Institutes of Health, Department of Agriculture, and Association for Assessment and Accreditation of Laboratory Animal Care International requirements.

Rats were anesthetized with pentobarbital sodium (Nembutal, Abbott Laboratories, Abbott Park, IL, 50 mg/kg body wt ip) and treated using a modification of the protocol we described previously (55). A 200- μl bolus injection of 200 nM or 200 μM aldosterone (Sigma-Aldrich, St. Louis, MO) or 20 μM DOG (Cayman Chemical, Ann Arbor, MI), a cell membrane-permeable diacylglycerol analog and activator of protein kinase C, dissolved in physiological buffer was injected intravenously into the tail vein. Immediately following the bolus injection, a steady infusion of the respective drug at the same concentration was made for 15 min into the tail vein. After the infusion, the animal was perfused via the left cardiac ventricle with phosphate-buffered saline (PBS, 0.9% NaCl in 10 mM phosphate buffer, pH 7.4) followed by paraformaldehyde-lysine-periodate fixative (PLP) as previously described (54). Three control rats were injected and infused with buffer alone, and three rats each were injected and infused with aldosterone at both concentrations or with DOG as described above. Following the PLP perfusion, the kidneys were dissected, sliced, and further fixed by immersion in PLP for 4 h at room temperature and then overnight at 4°C. Kidneys were rinsed extensively in PBS the following day and stored at 4°C in PBS containing 0.02% sodium azide.

Immunofluorescence and confocal microscopy. PLP-fixed kidney slices were cryoprotected in PBS containing 0.9 M sucrose, embedded in Tissue-Tek OCT compound 4583 (Sakura Finetek USA, Torrance, CA) and frozen at -20°C, and 5- μm sections were cut on a Leica CM3050 S cryostat (Leica Microsystems, Bannockburn, IL). Sections were rehydrated in PBS and treated with 1% (wt/vol) SDS for 4 min for retrieval of antigenic sites (9). We subsequently followed a previously described immunostaining protocol which includes a 20-min blocking step in 1% (wt/vol) bovine serum albumin in PBS, a 90-min incubation with a primary antibody against the V-ATPase 70-kDa "A" subunit (34) diluted in Dako antibody diluent (Dako, Carpinteria, CA), and a 60-min incubation with Alexa Fluor 594 goat anti-rabbit IgG (H+L) (Invitrogen, Carlsbad, CA) at 2.5 $\mu\text{g}/\text{ml}$ as secondary antibody, all at room temperature (55).

Digital images were acquired using a Nikon 80i epifluorescence microscope (Nikon Instruments, Melville, NY) equipped with an Orca 100 CCD camera (Hamamatsu, Bridgewater, NJ). Images were acquired using IPLab version 3.2.4 (Scanalytics, Fairfax, VA) and final figures were produced using Adobe Photoshop version CS2 image editing software (Adobe Systems, San Jose, CA) (55).

Statistics. Data are presented as means \pm SE. One-way ANOVA with Bonferroni post test was performed when comparing means across three or more groups. All other data were tested for significance using the unpaired Student's *t*-test, and only results with $P < 0.05$ were considered as statistically significant.

RESULTS

Rapid stimulation of H^+ -ATPase activity in mouse OMCD intercalated cells by aldosterone. In type A acid-secretory intercalated cells of mouse OMCD, the mean initial pH_i was 7.31 ± 0.01 (Table 2). pH_i acidified after removal of sodium from the bath and alkalinized after the addition of an NH_4Cl pulse (20 mM) (Fig. 1A). After removal of NH_4Cl from the perfusion chamber, the OMCD intercalated cells were acidified to a mean pH 6.42 ± 0.01 . In the continued absence of Na^+ the intracellular pH_i recovered slowly. The observed intracellular alkalinization rate was 0.031 ± 0.002 pH_i units/min. This alkalinization is mainly due to the activity of H^+ -ATPases and is mostly inhibited by the specific inhibitor concanamycin as previously shown (72, 75). Readdition of Na^+ to the bath led to a rapid increase of pH_i to the initial value mediated by Na^+/H^+ exchange (Fig. 1A).

Preincubation of mouse OMCDs with 10 nM aldosterone for 20 min at 37°C increased the Na^+ -independent alkalinization rate 2–3 fold to 0.069 ± 0.002 pH_i units/min as observed previously (75). To test for the immediate onset of stimulation, 10 nM aldosterone was added to the bath 3 min before the solution was switched to Na^+ -free conditions (75). The stimulation of H^+ -ATPase activity was similar to the 20-min preincubation (0.081 ± 0.006 pH units/min) (Table 2 and Fig. 1), suggesting that the nongenomic stimulation of H^+ -ATPase activity occurs within a very short time frame. Addition of 1 nM aldosterone in the same experimental series (3-min preincubation) led to a small but significant increase of the Na^+ -independent alkalinization rate (0.041 ± 0.004 pH units/min, Table 2 and Fig. 1). The steroid hormone hydrocortisone had no effect at similar concentrations (10 nM) with preincubation periods of 20 min (0.030 ± 0.002 pH units/min, Table 2 and Fig. 1). Thus, aldosterone specifically stimulates H^+ -ATPase activity in OMCD intercalated cells in a concentration-dependent manner and this effect occurs very rapidly, consistent with a rapid and nongenomic effect (75).

We also measured intrinsic buffering power (β_i) in OMCD intercalated cells. Incubation with 10 nM aldosterone for 20 min decreased β_i significantly from 42.8 ± 1.5 ($n = 61$) to 34.0 ± 1.4 ($n = 58$) ($P < 0.001$). Net proton fluxes were calculated and found to be significantly stimulated by aldosterone (Fig. 1C).

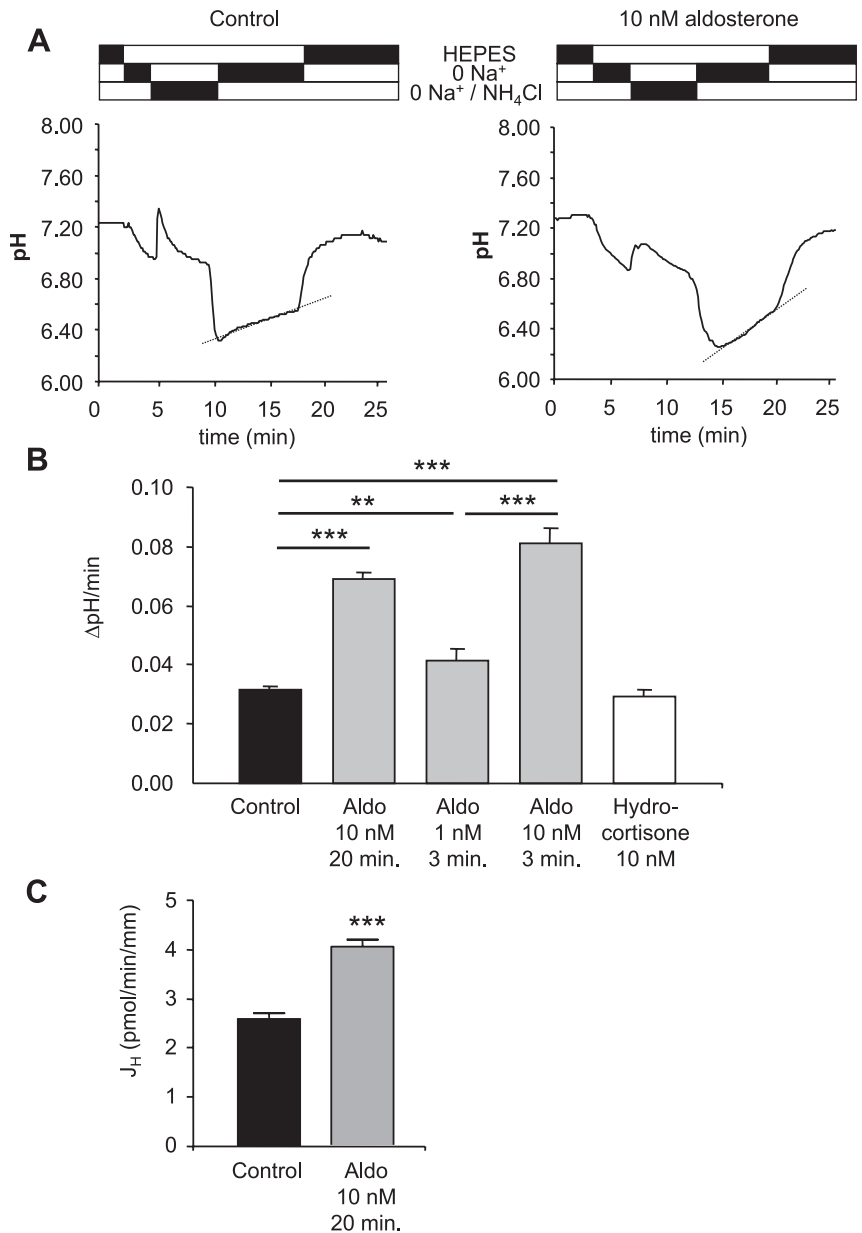
Aldosterone stimulates H^+ -ATPases in human OMCD cells. Similar to the effect observed in freshly isolated mouse OMCD

Table 2. Summary of Na^+ -independent pH_i alkalinization rates of single intercalated cells in freshly isolated mouse outer medullary collecting ducts

	Initial pH_i	Na^+ -Independent pH_i Recovery, $\Delta pH/\text{min}$	Number of Cells, OMCDs, Mice
Control	7.31 ± 0.01	0.031 ± 0.002	122 (11/6)
Aldosterone 10 nM (20 min)	7.30 ± 0.02	$0.069 \pm 0.002^\dagger$	123 (15/13)
Aldosterone 1 nM (3 min)	7.39 ± 0.03	$0.041 \pm 0.004^*$	32 (3/3)
Aldosterone 10 nM (3 min)	7.29 ± 0.01	$0.081 \pm 0.006^\dagger$	72 (4/3)
Hydrocortisone	7.26 ± 0.02	0.030 ± 0.002	82 (5/3)

Values are means \pm SE. pH_i , intracellular pH; OMCDs, outer medullary collecting ducts. * $P < 0.01$, $^\dagger P < 0.001$ as compared with the respective control.

Fig. 1. Aldosterone (Aldo) stimulates vacuolar H⁺-ATPase activity in freshly isolated mouse outer medullary collecting duct (OMCD) intercalated cells. *A*: original intracellular pH (pH_i) tracing of single intercalated cells in freshly isolated mouse OMCDs. OMCDs were incubated with 10 nM aldosterone for 20 min or left untreated (control). The dotted line marks the increase in pH_i measured and used to calculate ΔpH/min values. The bars above the tracing indicate solutions used. Initially, OMCD fragments were bathed in a solution containing sodium; after removal of sodium, an initial pH_i acidification could be observed. Addition of NH₄Cl to the bath caused a transient alkalinization, and upon its removal from the bath, a strong acidification. pH_i recovered slowly in the absence of sodium and more rapidly after readdition of sodium to the bath. *B*: summary bar graph of intracellular Na⁺-independent pH_i alkalinization rates of mouse single intercalated cells. OMCDs were either incubated for 20 min with 10 nM aldosterone or aldosterone was added to the superfusate during the measurement 3 min before switching to Na⁺-free solutions after the NH₄Cl-prepulse. Hydrocortisone (10 nM) was incubated for 20 min. *C*: summary of net proton fluxes (J_H) calculated from intracellular buffering power and ΔpH/min for control and aldosterone incubated OMCD intercalated cells. Values are presented as means ± SE. ***P* < 0.01, ****P* < 0.001.



intercalated cells, aldosterone also stimulated H⁺-ATPase activity in freshly isolated human OMCDs prepared from human kidney samples after nephrectomy. The basal Na⁺-independent pH_i recovery rate was 0.028 ± 0.002 pH_i units/min (Table 3 and Fig. 2). After preincubation with 10 nM aldosterone for 20 min the intracellular pH_i recovery rate increased twofold to

Table 3. Summary of Na⁺-independent pH_i alkalinization rates of single intercalated cells in freshly isolated human outer medullary collecting ducts

	Initial pH _i	Na ⁺ -Independent pH _i Recovery, ΔpH/min	Number of Cells, OMCDs, Patients
Human OMCD, control	7.20 ± 0.01	0.028 ± 0.002	65 (5/4)
Human OMCD, aldosterone (10 nM)	7.19 ± 0.02	0.067 ± 0.004*	39 (3/1)

Values are means ± SE. **P* < 0.001 as compared with control.

0.067 ± 0.004 pH_i units/min (Table 3 and Fig. 2). Because of the limited access to human tissue, further studies were performed with mouse tissues. However, the results demonstrate that the stimulatory effect of aldosterone occurs also in human renal tissue and is not restricted to mouse OMCD intercalated cells.

The rapid stimulatory effect of aldosterone requires G_{αq} proteins and phospholipase C activity. Aldosterone has been shown to require phospholipase C activity for its rapid effects in other tissues (6, 11). To investigate whether G proteins might be involved in the aldosterone-induced stimulation of H⁺-ATPase activity in mouse OMCDs, suramin (200 μM), a general and relatively unspecific inhibitor of G proteins (32), was used. Preincubation with suramin (200 μM) alone had no effect on the basal rates of alkalinization, whereas it prevented completely the stimulatory effect of aldosterone (Table 4 and Fig. 3). Pertussis toxin (200 ng/ml), which inactivates more

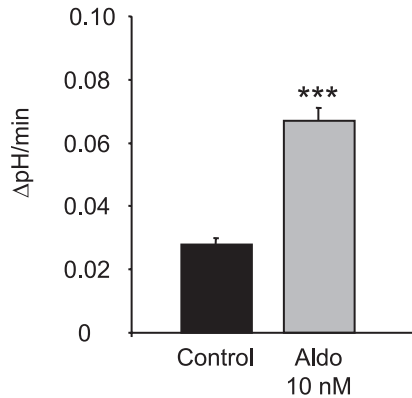


Fig. 2. Aldosterone stimulates vacuolar H^+ -ATPase activity in freshly isolated human OMCD intercalated cells. OMCDs were incubated with 10 nM aldosterone for 20 min or were left untreated (control). The summary of Na^+ -independent pH_i alkalization rates in single intercalated cells in freshly isolated human OMCDs in the absence and presence of aldosterone (10 nM, 20 min preincubation) is shown. Values are presented as means \pm SE. *** $P < 0.001$.

specifically G_i and G_o G proteins, had no effect alone and did not prevent the stimulatory effect of aldosterone (Table 4 and Fig. 3). In contrast, GP-2A (20 μ M), a small peptide inhibitor of $G_{\alpha q}$ proteins (33), abolished the stimulatory effect of aldosterone, whereas it did not affect the basal activity (Table 4 and Fig. 3). Inhibition of phospholipase C activity with U-73122 (10 μ M) completely impeded the stimulation by aldosterone (Table 4 and Fig. 3B). In contrast, the inactive analog U-73343 (10 μ M) was unable to prevent the stimulatory effect of aldosterone (Table 4 and Fig. 3C). Thus, the rapid nongenomic action of aldosterone may require a $G_{\alpha q}$ protein-coupled membrane receptor and phospholipase C activity.

Intracellular calcium, protein kinase C, and ERK1/2 participate in the stimulatory action of aldosterone. We had previously shown that aldosterone acutely and transiently raised intracellular Ca^{2+} in mouse OMCD intercalated cells (75). Therefore, we tested whether intracellular Ca^{2+} is involved in the stimulatory effect of aldosterone. Chelation of intracellular Ca^{2+} by preincubation of isolated OMCDs with BAPTA-AM abolished the stimulation of H^+ -ATPase activity without altering the basal activity (Table 4 and Fig. 4).

Inhibition of PKC activity with chelerythrine (1 μ M) prevented completely the stimulatory effect of aldosterone in mouse OMCDs. On the other hand, incubation with the PKC activator DOG (1 μ M) mimicked the effect of aldosterone and increased H^+ -ATPase activity twofold (Table 4 and Fig. 4).

We had previously shown that acute application of aldosterone increased luminal localization of H^+ -ATPases in vivo (75). Here, collecting duct A-type intercalated cells of rats exhibited a similar response to aldosterone as after exposure to the cell-permeable cAMP analog CPT-cAMP (55). H^+ -ATPase localization following aldosterone became more polarized, and apical H^+ -ATPase immunostaining intensity increased markedly in most kidney regions, especially in the outer medulla (Fig. 5, A and C). Infusion of DOG had a similar effect as aldosterone alone (Fig. 5B). As previously reported for cAMP, the brighter apical staining was associated in many cells with the development of extensive microvilli that were easily detectable by conventional wide field fluorescence microscopy (Fig. 5C, see *inset*). The effect of aldosterone is

augmented upon increasing its concentration from 200 nM to 200 μ M (Fig. 5, see *inset*).

Aldosterone can interact in a nongenomic fashion with the mitogen-activated protein kinase (MAPK) signaling pathway involving ERK1/2 (20, 41, 51, 57). The stimulating effect of aldosterone on H^+ -ATPase activity in intercalated cells of OMCDs was completely inhibited when the activation of ERK1/2 was prevented using PD098059 (20 μ M), a specific inhibitor. Preincubation with PD098059 alone had no effect on H^+ -ATPase activity (Table 4 and Fig. 6). To investigate the interaction between PKC and ERK1/2, we performed experiments using the PKC activator DOG (1 μ M) together with the ERK1/2 inhibitor PD098059 (20 μ M). The stimulation of H^+ -ATPase activity was attenuated (Table 4 and Fig. 6), suggesting that ERK1/2 may act downstream of PKC.

Protein kinase A participates in aldosterone signaling. Conflicting reports exist concerning the role of cAMP and protein kinase A (PKA) activity in the rapid effects of aldosterone, suggesting that this pathway may be cell and target specific (12, 28, 38, 62). Moreover, we have previously shown that cAMP stimulates H^+ -ATPase activity and luminal accumulation in type A intercalated cells (55). Thus, in a last set of experiments, the role of cAMP and PKA in the aldosterone-

Table 4. Summary of Na^+ -independent pH_i alkalization rates of single intercalated cells in freshly isolated mouse outer medullary collecting ducts in the absence and presence of different inhibitors of intracellular signaling molecules

	Initial pH_i	Na^+ -Independent pH_i Recovery, $\Delta pH/min$	No. of Cells, OMCDs, Mice
Suramin	7.39 \pm 0.02	0.029 \pm 0.002	74 (5/3)
Suramin + aldosterone	7.40 \pm 0.02	0.030 \pm 0.002§	51 (4/3)
Pertussis toxin	7.20 \pm 0.01	0.031 \pm 0.001	84 (5/3)
Pertussis toxin + aldosterone	7.28 \pm 0.01	0.084 \pm 0.004†	83 (5/3)
GP-2A	7.13 \pm 0.01	0.024 \pm 0.002	68 (4/2)
GP-2A + aldosterone	7.26 \pm 0.01	0.017 \pm 0.001*§	92 (5/2)
U-73122	7.27 \pm 0.01	0.031 \pm 0.002	53 (3/3)
U-73122 + aldosterone	7.26 \pm 0.01	0.026 \pm 0.001§	116 (8/3)
U-73343 + aldosterone	7.22 \pm 0.01	0.078 \pm 0.004†	112 (12/8)
BAPTA-AM	7.42 \pm 0.02	0.036 \pm 0.002	68 (4/3)
BAPTA-AM + aldosterone	7.35 \pm 0.02	0.032 \pm 0.002§	60 (5/4)
Chelerythrine	7.23 \pm 0.02	0.028 \pm 0.001	68 (4/3)
Chelerythrine + aldosterone	7.25 \pm 0.01	0.026 \pm 0.002§	77 (4/3)
DOG	7.38 \pm 0.02	0.060 \pm 0.003†	123 (10/5)
DOG + PD098059	7.42 \pm 0.01	0.050 \pm 0.003†§	87 (5/3)
DOG + H89	7.33 \pm 0.03	0.069 \pm 0.005†	39 (3/3)
PD098059	7.23 \pm 0.02	0.042 \pm 0.003	86 (7/4)
PD098059 + aldosterone	7.27 \pm 0.01	0.039 \pm 0.003§	73 (6/3)
H89	7.16 \pm 0.03	0.028 \pm 0.003	26 (2/2)
H89 + aldosterone	7.26 \pm 0.03	0.030 \pm 0.003§	63 (5/3)
KT5720	7.16 \pm 0.09	0.031 \pm 0.002	82 (7/3)
KT5720 + aldosterone	7.22 \pm 0.01	0.036 \pm 0.005§	114 (7/4)
8-Br-cAMP	7.43 \pm 0.01	0.047 \pm 0.002†‡	110 (7/3)
8-Br-cAMP + chelerythrine	7.10 \pm 0.08	0.024 \pm 0.002§	92 (7/3)

Values are means \pm SE. Incubation with aldosterone (10 nM) was for 20 min before measurement of alkalization rates. * $P < 0.05$ and † $P < 0.001$, significantly different from control; ‡ $P < 0.01$ and § $P < 0.001$, significantly different from aldosterone alone.

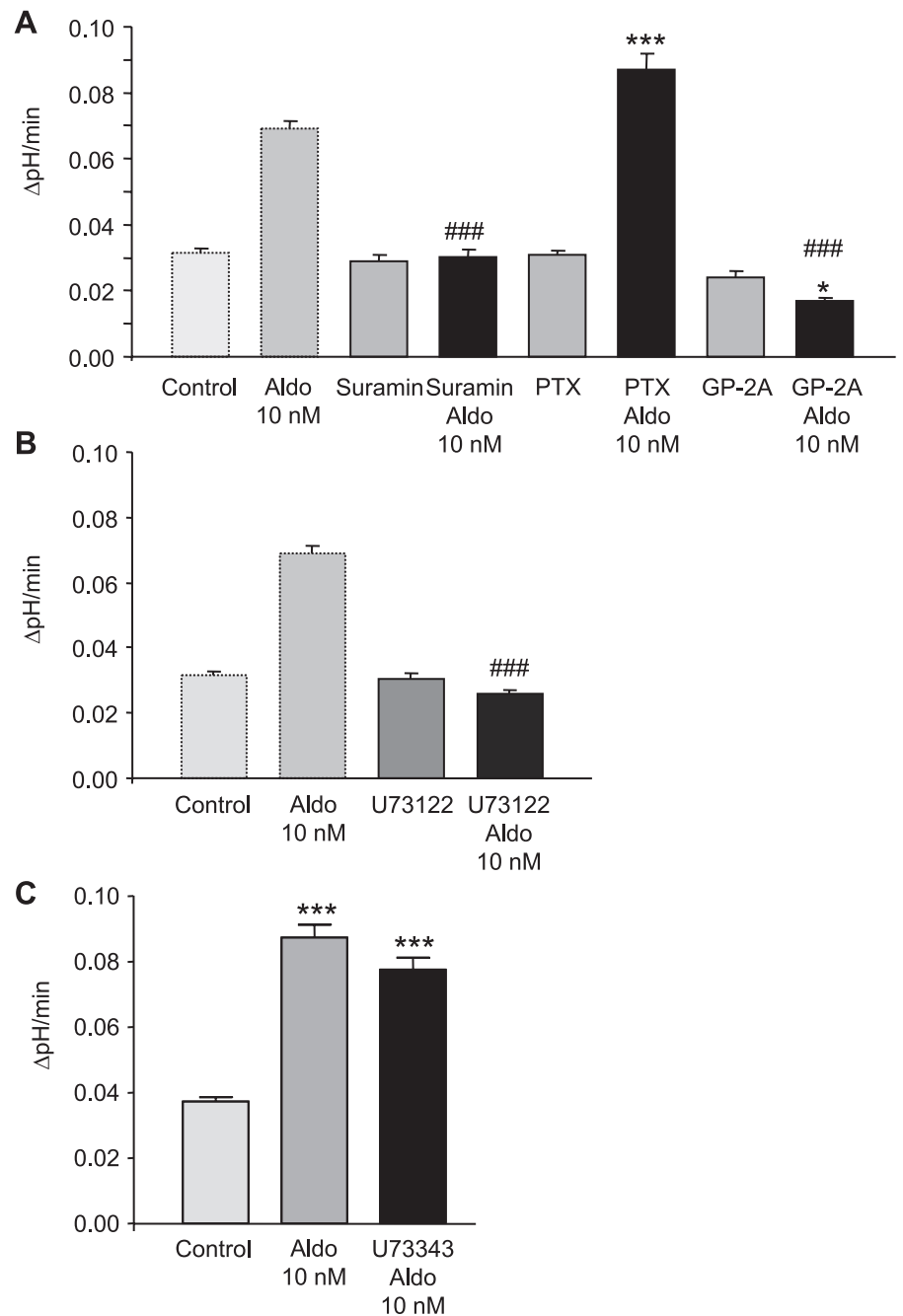


Fig. 3. The stimulatory effect of aldosterone requires $G_{\alpha q}$ proteins and phospholipase C activity. **A:** OMCDs were incubated with the inhibitors of small G proteins suramin (200 μ M), pertussis toxin (PTX, 200 ng/ml), or GP-2A (20 μ M) in the absence and presence of aldosterone (10 nM). Suramin and GP-2A prevented the stimulatory effect of aldosterone, whereas PTX failed to have any effect on the stimulation. **B:** U-73122 (10 μ M), a specific inhibitor of phospholipase C activity, prevented the effect of aldosterone. The values of control and aldosterone-treated OMCDs are shown again for comparison. **C:** the inactive U-73122 analog U-73343 (10 μ M) could not prevent the stimulatory effect of aldosterone. Control and aldosterone values were obtained from a separate set of experiments but yielded qualitatively similar values to the previous experiments listed in Table 2 ($n = 5-7$ OMCDs from at least three different mice and 50–70 intercalated cells examined per condition). Values are presented as means \pm SE. * $P < 0.05$ and *** $P < 0.001$ significantly different from control, ### $P < 0.001$ significantly different from aldosterone alone.

induced stimulation of H^+ -ATPase activity in OMCD intercalated cells was examined. Inhibition of PKA activity with H89 (10 μ M) or KT5720 (1 μ M) completely prevented the aldosterone-induced stimulation of H^+ -ATPase activity (Fig. 7 and Table 4). In addition, incubation with the cell-permeable cAMP analog 8-bromoadenosine-cAMP (8-Br-cAMP, 50 μ M) increased H^+ -ATPase activity but to a lesser extent than aldosterone or DOG (Table 4 and Fig. 7). Inhibition of PKA with H89 in the presence of the PKC activator DOG (1 μ M) did not prevent the robust stimulation of vacuolar H^+ -ATPase activity that reached a similar level as with DOG alone (Fig. 7B). In contrast, activation of PKA with 8-Br-cAMP in the presence of the PKC inhibitor chelerythrine (1 μ M) did not stimulate H^+ -ATPase activity. These results suggest that the

cAMP/PKA-dependent pathway is involved in the aldosterone action and may act upstream of PKC.

DISCUSSION

Nongenomic effects of several steroid hormones including vitamin D₃, sex hormones, glucocorticoids, and aldosterone have been identified in various tissues and cells (for review, see refs. 25–26, 48–50, 59, 74). Aldosterone is mainly involved in maintaining extracellular Na⁺ and K⁺ homeostasis by regulating intestinal and renal Na⁺ absorption and K⁺ excretion (68). Moreover, in the kidney, aldosterone also regulates acid secretion by acting on proximal tubular Na⁺/H⁺ exchange and most importantly by stimulating H⁺-secretion along the collecting

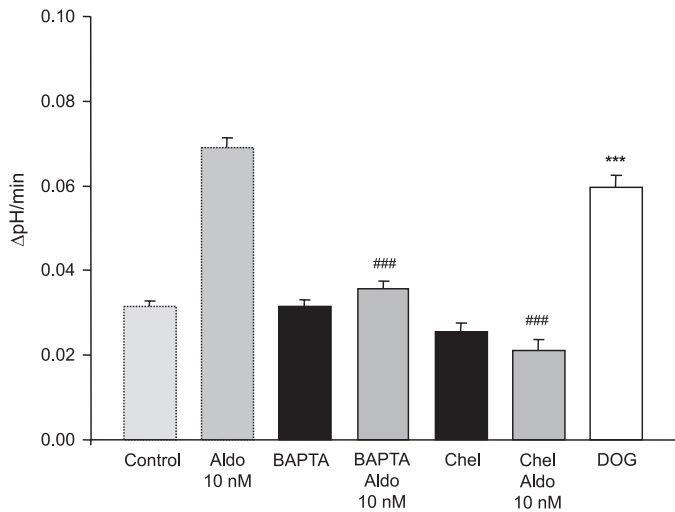


Fig. 4. Aldosterone acts via intracellular Ca^{2+} and protein kinase C. Chelation of intracellular Ca^{2+} with BAPTA-AM (100 μM) prevented the aldosterone (10 nM) effect. Similarly, inhibition of protein kinase C activity with chelerythrine (Chel, 1 μM) completely blocked the stimulation by aldosterone. On the other hand, stimulation of PKC activity with DOG (1 μM) mimicked the effect of aldosterone. The values of control and aldosterone-treated OMCDs are shown again for comparison. Values are presented as means \pm SE. *** P < 0.001 significantly different from control, ### P < 0.001 significantly different from aldosterone alone.

duct. Several studies have documented the chronic effects of aldosterone and aldosterone deficiency in rat and rabbit animal models as well as in cell culture and freshly isolated turtle bladder on H^+ -ATPase activity, the $\text{AE1 Cl}^-/\text{HCO}_3^-$ exchanger, and the ability of the collecting duct to excrete protons (1, 13–14, 16, 19, 30, 36, 39, 42, 52, 61, 65). Some of these effects are likely mediated by changes in transcription and translation of proteins involved in acid-base transport or acting on these transporters (36).

The biological actions of these steroid hormones are mediated not only by changes in transcription but also by much faster nongenomic mechanisms. Distinct rapid effects of aldosterone on acid-base transport in several nephron segments and kidney-derived cell lines have been described. H^+ -ATPase activity is stimulated by aldosterone in freshly isolated rat late proximal tubule and mouse OMCD intercalated cells (44, 75), and Na^+/H^+ exchanger activity increased in the rat late proximal tubule (45), MDCK, and M1 cell lines (20, 22, 51). Similarly, aldosterone stimulates Na^+/H^+ exchange in freshly

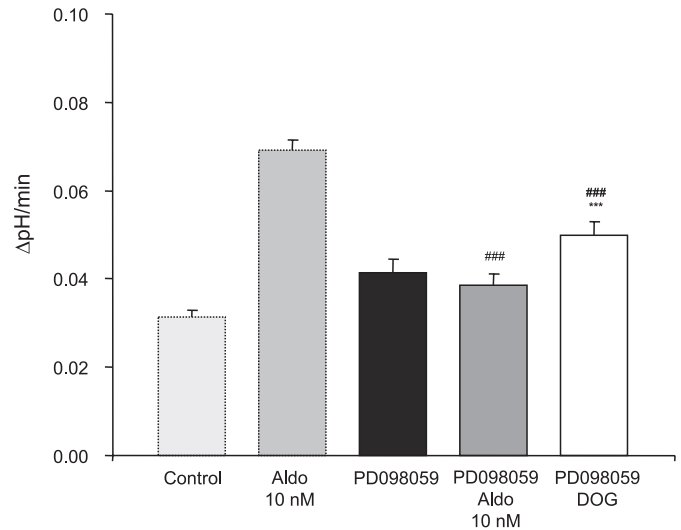


Fig. 6. The stimulatory effect of aldosterone is mediated via ERK1/2 kinases. OMCDs were incubated with the ERK1/2 inhibitor PD098059 (20 μM) in the absence and presence of aldosterone (10 nM). PD098059 abolished the stimulatory effect of aldosterone. Coincubation of OMCDs with the PKC activator DOG (1 μM) and the ERK1/2 inhibitor PD098059 showed a small stimulation which was less pronounced than with DOG alone (see also Fig. 4). The values of control and aldosterone-treated OMCDs are shown again for comparison. Values are presented as means \pm SE. *** P < 0.001 significantly different from control, ### P < 0.001 significantly different from aldosterone alone.

isolated colonic crypts (76). In contrast, in the thick ascending limb, aldosterone acutely inhibits bicarbonate absorption (23).

The intracellular signaling cascades mediating these nongenomic effects of aldosterone have been partly investigated and appear to differ between different cell types. However, most work has been done in cell culture models such as M1, MDCK, or vascular smooth muscle cells but much less information is available from freshly isolated cells and tissues. This may be important because it is well documented that cell lines harbor or loose characteristics not found or present in the native tissues (77). Thus, we investigated the signaling cascade mediating the stimulatory effect of aldosterone on H^+ -ATPase activity in freshly isolated mouse OMCD intercalated cells. We demonstrate that aldosterone stimulates within minutes H^+ -ATPase activity in mouse and human outer medullary collecting duct cells. Moreover, we show that in rat outer medullary collecting duct, aldosterone induces

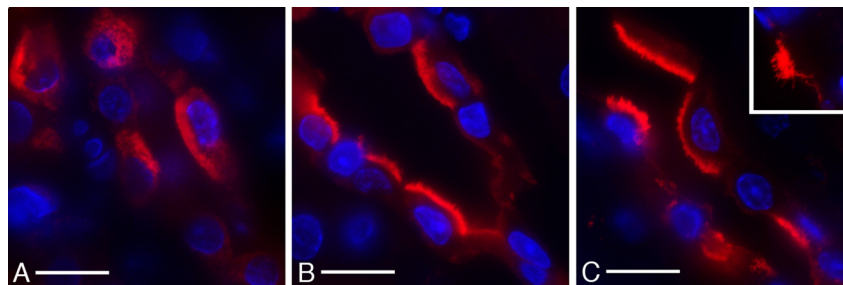


Fig. 5. Aldosterone or activation of protein kinase C enhances luminal H^+ -ATPase. Immunofluorescence labeling for the A subunit of the V-ATPase (red) in control (A), 20 μM DOG- (B), and 200 nM aldosterone-treated (C) rat OMCD intercalated cells. The nuclei are identified by DAPI staining (blue). While intercalated cells from control animals (A) exhibit extensive cytoplasmic staining and their apical membranes have a smooth appearance, extensively stained apical microvilli are readily detectable in the treated rats, and the cytosolic domain staining is less intense. An increased response is seen in the inset of C, which shows an intercalated cell from a rat treated with a higher concentration of aldosterone (200 μM). Bar = 20 μm .

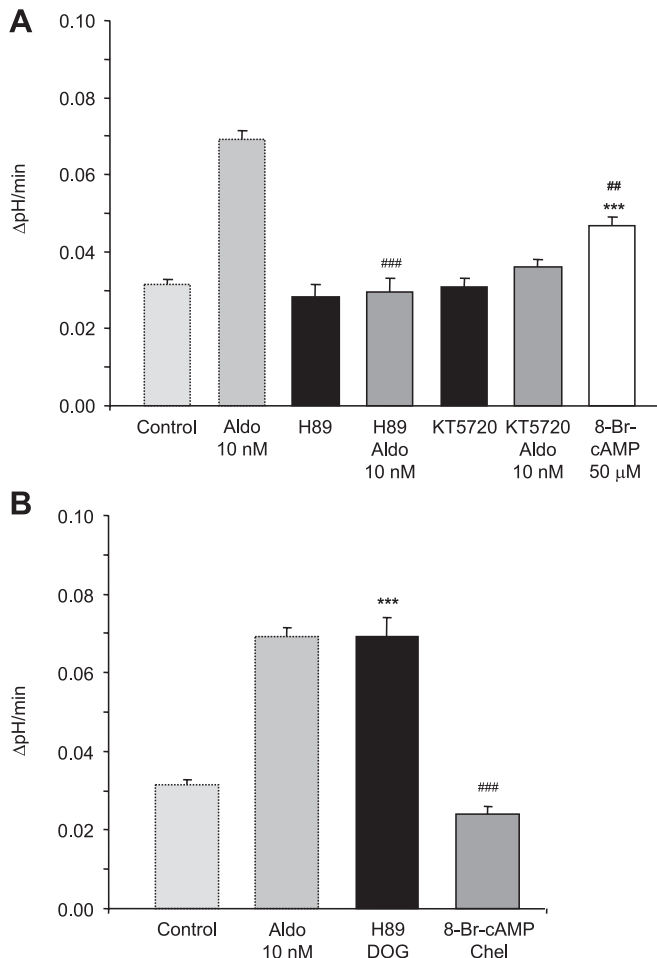


Fig. 7. Aldosterone signaling involves the cAMP-protein kinase A pathway. *A*: inhibition of protein kinase A activity with H89 (10 μM) or KT5720 (1 μM) completely prevented the effect of aldosterone whereas H89 or KT5720 alone had no effect on the pH_i recovery rates. Incubation of OMCDs with the PKA activator 8-Bromoadenosine-cAMP (8-Br-cAMP; 50 μM and 300 μM) stimulated pH_i recovery but to a lesser extent than aldosterone. *B*: coincubation of OMCDs with the PKC activator DOG (1 μM) and the PKA inhibitor H89 (10 μM) resulted in a robust stimulation similar to aldosterone alone demonstrating that PKC activation alone is sufficient for stimulation and that PKA does not act downstream of PKC. Activation of PKA with 8-Br-cAMP (300 μM) in the presence of the PKC inhibitor chelerythrine (1 μM) failed to stimulate H^+ -ATPase activity. The values of control and aldosterone treated OMCDs are shown again for comparison. Values are presented as means \pm SE. *** P < 0.001 significantly different from control, ## P < 0.01 and ### P < 0.001 significantly different from aldosterone alone.

a rapid translocation of H^+ -ATPase to the luminal pole in vivo. These data suggest that the effect is not species specific and may also be relevant in humans.

We had recently shown that the stimulatory effect of aldosterone persists in the presence of the partial mineralocorticoid receptor antagonist spironolactone and does not require the mineralocorticoid receptor, or transcription and translation (75). The stimulation was observed after incubation with 10 nM aldosterone for 20 min. Here we showed that stimulation could be observed even after immediate application and at a concentration as low as 1 nM. The effect of aldosterone was specific and not observed with the corticosteroid hydrocortisone.

It has been postulated that specific receptors for steroid hormones may exist that initiate their nongenomic effects (6, 46). Some of the nongenomic effects of aldosterone and corticosterone involve membrane-bound mineralocorticoid receptors (25–26, 35). Moreover, GPR30, an orphan G protein-coupled receptor, has been recently identified as a receptor for estrogens and shown to mediate some of its nongenomic effects (31, 56, 67). GPR30 may also mediate the nongenomic effects of aldosterone in some tissues (24). Consistently, we found that the rapid stimulatory effect of aldosterone was inhibited by suramin and GP-2A, inhibitors of small G proteins. Suramin is an unspecific inhibitor of G proteins, whereas GP-2A specifically blocks $\text{G}_{\alpha\text{q}}$ proteins (33). Pertussis toxin was without effect, suggesting that G_o and G_i proteins are not involved and that the aldosterone effect involves $\text{G}_{\alpha\text{q}}$ proteins. In addition to G protein inhibition, blockade of phospholipase C activity by U-73122 also prevented the stimulatory effect of aldosterone. This effect of U-73122 was not achieved with the inactive analog U-73343. Recently, a direct stimulatory effect of U-73122 on human PLC has been described (37); however, it is unlikely that this occurred in our experiments since U-73122 had no effect on untreated OMCDs. Similarly, other groups have described evidence for a nongenomic receptor-mediated activation of intracellular signaling cascades by aldosterone that were abolished by inhibitors of G proteins and PLC (11, 22, 43, 76).

The effect of aldosterone on H^+ -ATPase activity was further mediated by Ca^{2+} and protein kinase C stimulation as evident

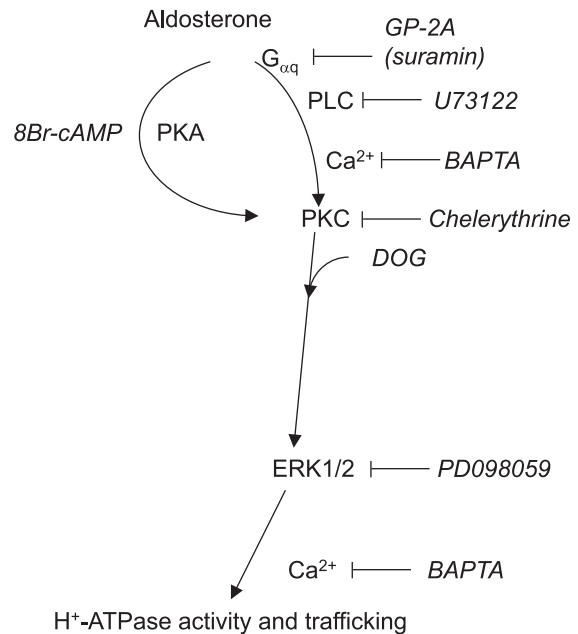


Fig. 8. Model of nongenomic signaling of aldosterone in mouse OMCD intercalated cells to stimulate H^+ -ATPase activity. Aldosterone stimulates H^+ -ATPase activity through a major pathway requiring $\text{G}_{\alpha\text{q}}$ proteins, phospholipase C, intracellular Ca^{2+} , PKC, and ERK1/2. A second interlinked pathway involves cAMP and PKA. The PKA-dependent pathway is likely upstream of PKC and ERK1/2 because inhibition of this pathway blocks the cAMP/PKA-dependent stimulation of H^+ -ATPase activity. Chelation of Ca^{2+} may interfere at several stages because Ca^{2+} may be involved in stimulation of PKC isoforms as well as in the exocytic insertion of H^+ -ATPases into the membrane, which has been shown earlier to occur during stimulation of H^+ -ATPase activity (58, 75). Inhibitors and activators of signaling molecules used in this study are shown in italics.

from 1) the lack of stimulation after chelation of intracellular Ca^{2+} with BAPTA, 2) the previous observation that aldosterone induces an acute and transient rise in intracellular Ca^{2+} (75), 3) the inhibition of stimulation with the PKC inhibitor chelerythrine, and 4) the fact that the diacylglycerol analog DOG mimicked the stimulatory effect of aldosterone. Direct interactions of aldosterone with protein kinase C have been shown (3) but are unlikely to mediate the effect in OMCD intercalated cells since upstream inhibition of PLC completely abolishes the stimulatory effect. In some tissues and cells, the nongenomic effects of aldosterone involve activation of ERK1/2 MAPK kinases (20, 41, 57). In agreement with these observations, blockade of ERK1/2 phosphorylation by PD098059 strongly reduced the stimulation of H^{+} -ATPase activity. Furthermore, it appears that ERK1/2 acts downstream of PKC, because the concomitant incubation of OMCDs with DOG and PD098059 failed to stimulate H^{+} -ATPases. This blockade, however, was not complete, which may indicate either that ERK was not fully inhibited by PD098059 or that an alternative ERK-independent pathway may exist. Thus, H^{+} -ATPase activity in OMCD acid-secretory type A intercalated cells is stimulated by a PLC/ Ca^{2+} /PKC-dependent pathway.

We recently described a very similar stimulatory pathway for angiotensin II in mouse OMCD cells (58), suggesting that it may represent a common pathway of H^{+} -ATPase stimulation in these cells. Stimulation of H^{+} -ATPase activity has also been demonstrated in a cell line derived from the rat inner medullary collecting duct requiring intracellular Ca^{2+} , PLC, and PKC activity (53, 60, 63). Similar observations have been made in the epididymis (5). The activation of protein kinase C is sufficient to induce the rapid translocation of H^{+} -ATPases to the luminal side of type A intercalated cells as demonstrated by the DOG in vivo experiments. Consistently, we have previously shown that disruption of the microtubular network with colchicine abolishes the stimulatory effect of aldosterone (75) and colchicine administration in vivo disrupts H^{+} -ATPase trafficking (10). Whether calcium is also directly required for the trafficking and insertion of H^{+} -ATPases into the membrane remains to be further examined.

We and others have recently shown that the cAMP-dependent pathway stimulates H^{+} -ATPase activity in renal intercalated cells and induces its translocation into the apical membrane (4, 55). Similarly, blockade with two different inhibitors of a protein kinase A-dependent pathway reduced the stimulatory effect of aldosterone. Incubation of OMCDs with the cAMP analog 8-Br-cAMP stimulated H^{+} -ATPase activity but to a lesser extent than the PKC-dependent pathway. The stimulation produced by 50 μM 8-Br-cAMP could not be increased with higher concentrations, indicating that maximal stimulation had been reached. However, pharmacologic stimulation of the PKC-dependent cascade appears to be sufficient for the increase in H^{+} -ATPase activity because DOG in the presence of H89 enhanced vacuolar H^{+} -ATPase activity. In contrast, activation of PKA with 8-Br-cAMP and concomitant inhibition of PKC with chelerythrine prevented the stimulation of H^{+} -ATPase activity, suggesting that PKA requires intact PKC to be effective and that PKA may act upstream of PKC (see hypothetical model Fig. 8). Taken together, it appears that PKA may rather have a modulatory effect and that PKC may transmit the main signal. A mild stimulatory effect of a PKA-dependent pathway on bicarbonate absorption has been de-

scribed previously in perfused rabbit outer medullary collecting duct and may thus reflect a physiologically relevant route of regulation of vacuolar H^{+} -ATPase activity (29).

Besides its well-documented effects on renal Na^{+} and K^{+} transport, aldosterone is an important regulator of renal acid secretion acting mainly on H^{+} -ATPases along the collecting duct. The significance of the stimulatory effect of aldosterone is highlighted by the fact that various inborn or acquired syndromes associated either with aldosterone deficiency or with insufficient aldosterone signaling in the kidney are invariably associated with distal renal tubular acidosis and hyperkalemia (15). It is possible that the rapid nongenomic and slower genomic stimulatory effects of aldosterone on H^{+} -ATPase activity may interact in several ways as is becoming increasingly clear for the effects of aldosterone on the major Na^{+} -transporting mechanisms in the collecting duct (43, 64). Thus, mechanisms identified here may not only lead to the rapid stimulation of H^{+} -ATPase activity but may also contribute subsequently to the activation of genomic events.

In summary, aldosterone rapidly stimulates H^{+} -ATPase activity in acid-secretory OMCD intercalated cells from mouse and human kidney and induces the net translocation of pumps to the luminal membrane. This nongenomic effect is mediated via a PLC/ Ca^{2+} /PKC-dependent pathway but is interconnected with the cAMP-PKA cascade. ERK1/2 kinases are essential for stimulation. A membrane $\text{G}_{\alpha q}$ protein-coupled receptor may be involved in initiating these cascades. The rapid nongenomic stimulation of H^{+} -ATPases by aldosterone may be part of the biological action of aldosterone in the kidney and contribute to the increase in acid secretion and control of systemic acid-base homeostasis.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES

1. Al-Awqati Q, Norby LH, Mueller A, Steinmetz PR. Characteristics of stimulation of H^{+} transport by aldosterone in turtle urinary bladder. *J Clin Invest* 58: 351–358, 1976.
2. Alper SL, Natale J, Gluck S, Lodish HF, Brown D. Subtypes of intercalated cells in rat kidney collecting duct defined by antibodies against erythroid band 3 and renal vacuolar H^{+} -ATPase. *Proc Natl Acad Sci USA* 86: 5429–5433, 1989.
3. Alzamora R, Harvey BJ. Direct binding and activation of protein kinase C isoforms by steroid hormones. *Steroids* 73: 885–888, 2008.
4. Alzamora R, Thali RF, Gong F, Smolak C, Li H, Baty CJ, Bertrand CA, Auchli Y, Brunisholz RA, Neumann D, Hallows KR, Pastor-Soler NM. PKA regulates vacuolar H^{+} -ATPase localization and activity via direct phosphorylation of the A subunit in kidney cells. *J Biol Chem* 285: 24676–24685, 2010.
5. Beaulieu V, Da Silva N, Pastor-Soler N, Brown CR, Smith PJ, Brown D, Breton S. Modulation of the actin cytoskeleton via gelsolin regulates vacuolar H^{+} -ATPase recycling. *J Biol Chem* 280: 8452–8463, 2005.
6. Boldyreff B, Wehling M. Rapid aldosterone actions: from the membrane to signaling cascades to gene transcription and physiological effects. *J Steroid Biochem Mol Biol* 85: 375–381, 2003.

7. Bourgeois S, Meer LV, Wootla B, Bloch-Faure M, Chambrey R, Shull GE, Gawenis LR, Houillier P. NHE4 is critical for the renal handling of ammonia in rodents. *J Clin Invest* 120: 1895–1904, 2010.
8. Brown D, Hirsch S, Gluck S. An H⁺-ATPase in opposite plasma membrane domains in kidney epithelial cell subpopulations. *Nature* 331: 622–624, 1988.
9. Brown D, Lydon J, McLaughlin M, Stuart-Tilley A, Tyszkowski R, Alper S. Antigen retrieval in cryostat tissue sections and cultured cells by treatment with sodium dodecyl sulfate (SDS). *Histochem Cell Biol* 105: 261–267, 1996.
10. Brown D, Sabolic I, Gluck S. Colchicine-induced redistribution of proton pumps in kidney epithelial cells. *Kidney Int Suppl* 33: S79–S83, 1991.
11. Christ M, Douwes K, Eisen C, Bechtner G, Theisen K, Wehling M. Rapid effects of aldosterone on sodium transport in vascular smooth muscle cells. *Hypertension* 25: 117–123, 1995.
12. Christ M, Gunther A, Heck M, Schmidt BM, Falkenstein E, Wehling M. Aldosterone, not estradiol, is the physiological agonist for rapid increases in cAMP in vascular smooth muscle cells. *Circulation* 99: 1485–1491, 1999.
13. Damasco MC, Ansaldo M, Malnic G. Effects of adrenalectomy and acute replacement by corticosteroids on distal acidification. *Can J Physiol Pharmacol* 67: 607–614, 1989.
14. DuBose TD Jr, Caffisch CR. Effect of selective aldosterone deficiency on acidification in nephron segments of the rat inner medulla. *J Clin Invest* 82: 1624–1632, 1988.
15. DuBose TD Jr, Alpern RJ. Renal tubular acidosis. In: *The Metabolic and Molecular Bases of Inherited Disease* (8th ed.), edited by Scriver CR, Beaudet AL, Sly WS, and Valle D. New York: McGraw-Hill, 2001, p. 4983–5021.
16. Eiam-Ong S, Kurtzman NA, Sabatini S. Regulation of collecting tubule adenosine triphosphatases by aldosterone and potassium. *J Clin Invest* 91: 2385–2392, 1993.
17. Falkenstein E, Christ M, Feuring M, Wehling M. Specific nongenomic actions of aldosterone. *Kidney Int* 57: 1390–1394, 2000.
18. Falkenstein E, Tillmann HC, Christ M, Feuring M, Wehling M. Multiple actions of steroid hormones—a focus on rapid, nongenomic effects. *Pharmacol Rev* 52: 513–556, 2000.
19. Garg LC, Narang N. Effects of aldosterone on NEM-sensitive ATPase in rabbit nephron segments. *Kidney Int* 34: 13–17, 1988.
20. Gekle M, Freuding R, Mildnerberger S, Schenk K, Marschitz I, Schramek H. Rapid activation of Na⁺/H⁺-exchange in MDCK cells by aldosterone involves MAP-kinase ERK1/2. *Pflügers Arch* 441: 781–786, 2001.
21. Gekle M, Golenhofen N, Oberleithner H, Silbernagl S. Rapid activation of Na⁺/H⁺ exchange by aldosterone in renal epithelial cells requires Ca²⁺ and stimulation of a plasma membrane proton conductance. *Proc Natl Acad Sci USA* 93: 10500–10504, 1996.
22. Gekle M, Silbernagl S, Oberleithner H. The mineralocorticoid aldosterone activates a proton conductance in cultured kidney cells. *Am J Physiol Cell Physiol* 273: C1673–C1678, 1997.
23. Good DW, George T, Watts BA III. Aldosterone inhibits HCO₃⁻ absorption via a nongenomic pathway in medullary thick ascending limb. *Am J Physiol Renal Physiol* 283: F699–F706, 2002.
24. Gros R, Ding Q, Sklar LA, Prossnitz EE, Arterburn JB, Chorazyczewski J, Feldman RD. GPR30 expression is required for the mineralocorticoid receptor-independent rapid vascular effects of aldosterone. *Hypertension* 57: 442–451, 2011.
25. Grossmann C, Gekle M. New aspects of rapid aldosterone signaling. *Mol Cell Endocrinol* 308: 53–62, 2009.
26. Grossmann C, Ruhs S, Seiferth A, Gekle M. Interaction between mineralocorticoid receptor and cAMP/CREB signaling. *Steroids* 75: 539–543, 2010.
27. Hamm LL, Alpern RJ. Cellular mechanisms of renal tubular acidification. In: *The Kidney: Physiology and Pathophysiology* (3rd ed.), edited by Seldin DW and Giebisch G. Philadelphia, PA: Lippincott Williams & Wilkins, 2000, p. 1935–1979.
28. Haseroth K, Gerdes D, Berger S, Feuring M, Gunther A, Herbst C, Christ M, Wehling M. Rapid nongenomic effects of aldosterone in mineralocorticoid-receptor-knockout mice. *Biochem Biophys Res Commun* 266: 257–261, 1999.
29. Hays S, Kokko JP, Jacobson HR. Hormonal regulation of proton secretion in rabbit medullary collecting duct. *J Clin Invest* 78: 1279–1286, 1986.
30. Hays SR. Mineralocorticoid modulation of apical and basolateral membrane H⁺/OH⁻/HCO₃⁻ transport processes in the rabbit inner stripe of outer medullary collecting duct. *J Clin Invest* 90: 180–187, 1992.
31. Hewitt SC, Deroo BJ, Korach KS. Signal transduction. A new mediator for an old hormone? *Science* 307: 1572–1573, 2005.
32. Hohenegger M, Waldhoer M, Beindl W, Boing B, Kreimeyer A, Nickel P, Nanoff C, Freissmuth M. Gα-selective G protein antagonists. *Proc Natl Acad Sci USA* 95: 346–351, 1998.
33. Hunt RA, Bhat GJ, Baker KM. Angiotensin II-stimulated induction of sis-inducing factor is mediated by pertussis toxin-insensitive G(q) proteins in cardiac myocytes. *Hypertension* 34: 603–608, 1999.
34. Hurtado-Lorenzo A, Skinner M, El Annan J, Futai M, Sun-Wada GH, Bourgoin S, Casanova J, Wildeman A, Bechoua S, Ausiello DA, Brown D, Marshansky V. V-ATPase interacts with ARNO and Arf6 in early endosomes and regulates the protein degradative pathway. *Nat Cell Biol* 8: 124–136, 2006.
35. Karst H, Berger S, Turiault M, Tronche F, Schutz G, Joels M. Mineralocorticoid receptors are indispensable for nongenomic modulation of hippocampal glutamate transmission by corticosterone. *Proc Natl Acad Sci USA* 102: 19204–19207, 2005.
36. Khadouri C, Marsy S, Barlet-Bas C, Cheval L, Doucet A. Short-term effect of aldosterone on NEM-sensitive ATPase in rat collecting tubule. *Am J Physiol Renal Fluid Electrolyte Physiol* 257: F177–F181, 1989.
37. Klein RR, Bourdon DM, Costales CL, Wagner CD, White WL, Williams JD, Hicks SN, Sondek J, Thakker DR. Direct activation of human phospholipase C by its well known inhibitor u73122. *J Biol Chem* 286: 12407–12416, 2011.
38. Koppel H, Christ M, Yard BA, Bar PC, van der Woude FJ, Wehling M. Nongenomic effects of aldosterone on human renal cells. *J Clin Endocrinol Metab* 88: 1297–1302, 2003.
39. Kornandakietti C, Tannen RL. H⁺ transport by the aldosterone-deficient rat distal nephron. *Kidney Int* 25: 629–635, 1984.
40. Kovacicova J, Winter C, Loffing-Cueni D, Loffing J, Finberg KE, Lifton RP, Hummler E, Rossier B, Wagner CA. The connecting tubule is the main site of the furosemide-induced urinary acidification by the vacuolar H⁺-ATPase. *Kidney Int* 70: 1706–1716, 2006.
41. Krug AW, Grossmann C, Schuster C, Freuding R, Mildnerberger S, Govindan MV, Gekle M. Aldosterone stimulates epidermal growth factor receptor expression. *J Biol Chem* 278: 43060–43066, 2003.
42. Krug AW, Papavassiliou F, Hopfer U, Ullrich KJ, Gekle M. Aldosterone stimulates surface expression of NHE3 in renal proximal brush borders. *Pflügers Arch* 446: 492–496, 2003.
43. Le Moellic C, Ouvrard-Pascaud A, Capurro C, Cluzeaud F, Fay M, Jaisser F, Farman N, Blot-Chaubaud M. Early nongenomic events in aldosterone action in renal collecting duct cells: PKCα activation, mineralocorticoid receptor phosphorylation, and cross-talk with the genomic response. *J Am Soc Nephrol* 15: 1145–1160, 2004.
44. Leite-Dellova DC, Malnic G, Mello-Aires M. Genomic and nongenomic stimulatory effect of aldosterone on H⁺-ATPase in proximal S3 segments. *Am J Physiol Renal Physiol* 300: F682–F691, 2011.
45. Leite-Dellova DC, Oliveira-Souza M, Malnic G, Mello-Aires M. Genomic and nongenomic dose-dependent biphasic effect of aldosterone on Na⁺/H⁺ exchanger in proximal S3 segment: role of cytosolic calcium. *Am J Physiol Renal Physiol* 295: F1342–F1352, 2008.
46. Losel R, Feuring M, Wehling M. Non-genomic aldosterone action: from the cell membrane to human physiology. *J Steroid Biochem Mol Biol* 83: 167–171, 2003.
47. Losel R, Schultz A, Wehling M. A quick glance at rapid aldosterone action. *Mol Cell Endocrinol* 217: 137–141, 2004.
48. Losel R, Wehling M. Nongenomic actions of steroid hormones. *Nat Rev Mol Cell Biol* 4: 46–55, 2003.
49. Losel RM, Falkenstein E, Feuring M, Schultz A, Tillmann HC, Rossol-Haseroth K, Wehling M. Nongenomic steroid action: controversies, questions, and answers. *Physiol Rev* 83: 965–1016, 2003.
50. Losel RM, Feuring M, Falkenstein E, Wehling M. Nongenomic effects of aldosterone: cellular aspects and clinical implications. *Steroids* 67: 493–498, 2002.
51. Markos F, Healy V, Harvey BJ. Aldosterone rapidly activates Na⁺/H⁺ exchange in M-1 cortical collecting duct cells via a PKC-MAPK pathway. *Nephron Physiol* 99: p1–p9, 2005.
52. Mujais SK. Effects of aldosterone on rat collecting tubule N-ethylmaleimide-sensitive adenosine triphosphatase. *J Lab Clin Med* 109: 34–39, 1987.

53. Nicoletta JA, Ross JJ, Li G, Cheng Q, Schwartz J, Alexander EA, Schwartz JH. Munc-18-2 regulates exocytosis of H⁺-ATPase in rat inner medullary collecting duct cells. *Am J Physiol Cell Physiol* 287: C1366–C1374, 2004.
54. Paunescu TG, Da Silva N, Marshansky V, McKee M, Breton S, Brown D. Expression of the 56-kDa B2 subunit isoform of the vacuolar H⁺-ATPase in proton-secreting cells of the kidney and epididymis. *Am J Physiol Cell Physiol* 287: C149–C162, 2004.
55. Paunescu TG, Ljubojevic M, Russo LM, Winter C, McLaughlin MM, Wagner CA, Breton S, Brown D. cAMP stimulates apical V-ATPase accumulation, microvillar elongation, and proton extrusion in kidney collecting duct A-intercalated cells. *Am J Physiol Renal Physiol* 298: F643–F654, 2010.
56. Revankar CM, Cimino DF, Sklar LA, Arterburn JB, Prossnitz ER. A transmembrane intracellular estrogen receptor mediates rapid cell signaling. *Science* 307: 1625–1630, 2005.
57. Rossol-Haseroth K, Zhou Q, Braun S, Boldyreff B, Falkenstein E, Wehling M, Losel RM. Mineralocorticoid receptor antagonists do not block rapid ERK activation by aldosterone. *Biochem Biophys Res Commun* 318: 281–288, 2004.
58. Rothenberger F, Velic A, Stehberger PA, Kovacicova J, Wagner CA. Angiotensin II stimulates vacuolar H⁺-ATPase activity in renal acid-secreting intercalated cells from the outer medullary collecting duct. *J Am Soc Nephrol* 18: 2085–2093, 2007.
59. Schmidt BM. Rapid non-genomic effects of aldosterone on the renal vasculature. *Steroids* 73: 961–965, 2008.
60. Schwartz JH, Masino SA, Nichols RD, Alexander EA. Intracellular modulation of acid secretion in rat inner medullary collecting duct cells. *Am J Physiol Renal Fluid Electrolyte Physiol* 266: F94–F101, 1994.
61. Sebastian A, Sutton JM, Hulter HN, Schambelan M, Poler SM. Effect of mineralocorticoid replacement therapy on renal acid-base homeostasis in adrenalectomized patients. *Kidney Int* 18: 762–773, 1980.
62. Shearer EA, Wargent ET, Ashton N, Balment RJ. Rapid stimulation of cyclic AMP production by aldosterone in rat inner medullary collecting ducts. *J Endocrinol* 175: 343–347, 2002.
63. Slotki I, Schwartz JH, Alexander EA. Interrelationship between cell pH and cell calcium in rat inner medullary collecting duct cells. *Am J Physiol Cell Physiol* 265: C432–C438, 1993.
64. Stockand JD. New ideas about aldosterone signaling in epithelia. *Am J Physiol Renal Physiol* 282: F559–F576, 2002.
65. Stone DK, Seldin DW, Kokko JP, Jacobson HR. Mineralocorticoid modulation of rabbit medullary collecting duct acidification. A sodium-independent effect. *J Clin Invest* 72: 77–83, 1983.
66. Thomas JA, Buchsbaum RN, Zimniak A, Racker E. Intracellular pH measurements in Ehrlich ascites tumor cells utilizing spectroscopic probes generated in situ. *Biochemistry* 18: 2210–2218, 1979.
67. Thomas P, Pang Y, Filardo EJ, Dong J. Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells. *Endocrinology* 146: 624–632, 2005.
68. Verrey F. Early aldosterone action: toward filling the gap between transcription and transport. *Am J Physiol Renal Physiol* 277: F319–F327, 1999.
69. Wagner CA, Devuyst O, Bourgeois S, Mohebbi N. Regulated acid-base transport in the collecting duct. *Pflügers Arch* 458: 137–156, 2009.
70. Wagner CA, Finberg KE, Breton S, Marshansky V, Brown D, Geibel JP. Renal vacuolar H⁺-ATPase. *Physiol Rev* 84: 1263–1314, 2004.
71. Wagner CA, Geibel JP. Acid-base transport in the collecting duct. *J Nephrol Suppl* 5: S112–S127, 2002.
72. Wagner CA, Lukewille U, Valles P, Breton S, Brown D, Giebisch GH, Geibel JP. A rapid enzymatic method for the isolation of defined kidney tubule fragments from mouse. *Pflügers Arch* 446: 623–632, 2003.
73. Watts BA, 3rd, Good DW. Apical membrane Na⁺/H⁺ exchange in rat medullary thick ascending limb. pH-dependence and inhibition by hyperosmolality. *J Biol Chem* 269: 20250–20255, 1994.
74. Wehling M. Specific, nongenomic actions of steroid hormones. *Annu Rev Physiol* 59: 365–393, 1997.
75. Winter C, Schulz N, Giebisch G, Geibel JP, Wagner CA. Nongenomic stimulation of vacuolar H⁺-ATPases in intercalated renal tubule cells by aldosterone. *Proc Natl Acad Sci USA* 101: 2636–2641, 2004.
76. Winter DC, Schneider MF, O’Sullivan GC, Harvey BJ, Geibel JP. Rapid effects of aldosterone on sodium-hydrogen exchange in isolated colonic crypts. *J Membr Biol* 170: 17–26, 1999.
77. Zhuang Z, Marshansky V, Breton S, Brown D. Is caveolin involved in normal proximal tubule function? Presence in model PT systems but absence in situ. *Am J Physiol Renal Physiol* 300: F199–F206, 2011.