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**Mechanism underlying flow-stimulation of sodium absorption in the mammalian collecting
duct**

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ABSTRACT

Vectorial Na^+ absorption across the aldosterone-sensitive distal nephron plays a key role in the regulation of extracellular fluid volume and blood pressure. Within this nephron segment, Na^+ diffuses from the urinary fluid into principal cells through an apical, amiloride-sensitive, epithelial Na^+ channel (ENaC), which is considered to be the rate-limiting step for Na^+ absorption. We have reported that increases in tubular flow rate in microperfused rabbit cortical collecting ducts (CCDs) lead to increases in net Na^+ absorption, and that increases in laminar shear stress activate ENaC expressed in oocytes by increasing channel open probability. We therefore examined whether flow stimulates net Na^+ absorption (JNa) in CCDs by increasing channel open probability or by increasing the number of channels at the apical membrane. Both baseline and flow-stimulated JNa in CCDs were mediated by ENaC, as JNa was inhibited by benzamil. Flow-dependent increases in JNa were observed following treatment of tubules with reagents that altered membrane trafficking by disrupting microtubules (colchicine) or Golgi (brefeldin A). Furthermore, reducing luminal $[\text{Ca}^{2+}]$ or chelating intracellular $[\text{Ca}^{2+}]$ with BAPTA did not prevent the flow-dependent increase in JNa. Extracellular trypsin has been shown to activate ENaC by increasing channel open probability, and we observed that trypsin significantly enhanced JNa when tubules were perfused at a slow flow rate. However, trypsin did not further enhance JNa in CCDs perfused at fast flow rates. Similarly, the shear-induced increase in benzamil-sensitive INa in oocytes expressing protease resistance ENaC mutants was similar to that of controls. Our results suggest the rise in JNa accompanying increases in luminal flow rates reflects an increase in channel open probability.

Keywords: ENaC, *in vitro* microperfusion, protein trafficking, mechanoregulation, laminar shear, principal cell

INTRODUCTION

The distal convoluted tubule (DCT), connecting tubule (CNT) and collecting duct (CD) contribute to the final regulation of renal Na^+ reabsorption (13-16, 21, 24, 26, 28, 29, 31, 32), a process that plays a key role in modifying extracellular fluid volume and blood pressure. Within the rabbit cortical collecting duct (CCD), a segment that has been utilized extensively for functional analysis by *in vitro* microperfusion, Na^+ absorption is considered to be electrogenic and mediated by Na^+ diffusion from the urinary fluid into the cell through the apical amiloride-sensitive epithelial Na^+ channel (ENaC).

We (32, 33) and others (13, 27, 38) have previously reported that increases in tubular fluid flow rate stimulate net Na^+ absorption in the mammalian CCD. We speculated that hydrodynamic forces associated with increases in urinary flow rate either directly activate ENaC, or activate cell signaling pathways that indirectly activate ENaC. We have also shown that oocytes expressing $\alpha\beta\gamma$ ENaC respond to increases in laminar shear stress (LSS) with a dose-dependent and reversible stimulation of benzamil-sensitive whole cell Na^+ currents (INa) (9, 33). A flow-mediated increase in net Na^+ absorption in CCDs or INa in oocytes expressing ENaC can result from an increase in the number of apical channels and/or channel open probability. Mutant ENaC channels ($\alpha\beta\text{S518K}\gamma$ or $\alpha\text{S580C}\beta\gamma$ following activation with a sulfhydryl reactive reagent) that have a high intrinsic open probability do not respond to LSS, suggesting that LSS activates ENaC by increasing channel open probability (9). We recently reported that mutations within a key region of the channel that encompasses both the selectivity filter and an amiloride-binding site affect both the rate and magnitude of channel activation in response to LSS,

providing evidence that LSS induced conformational changes within the channel that affect channel gating (10).

Based on these studies, we hypothesized that the increase in net Na^+ absorption in the mammalian CCD that is elicited by an increase in the rate of tubular perfusion reflects ENaC activation as a result of an increase in channel open probability. To test this, we used a pharmacologic approach applied to *in vitro* microperfused rabbit CCDs to examine the contributions of membrane trafficking and/or increases in open probability to flow-stimulation of net Na^+ absorption. Our results suggest that flow stimulates net Na^+ absorption in CCDs by increasing open probability of resident Na^+ channels at the membrane, rather than by recruiting channels from intracellular compartments to the plasma membrane.

METHODS

Animals

Adult (>6 wk) female New Zealand White rabbits obtained from Covance (Denver, PA) were housed in the Mount Sinai School of Medicine Center for Comparative Medicine. All animals were allowed free access to water and chow. Adult female *Xenopus leavis* were purchased from Xenopus Express (Plant City, FL). Animals were euthanized in accordance with the National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals*. Animal protocols were approved by IACUC committees at the Mount Sinai School of Medicine and the University of Pittsburgh.

Microperfusion of isolated rabbit CCDs

Kidneys were removed via a midline incision, and single tubules dissected freehand in cold (4°C) Ringer's solution containing (in mM): 135 NaCl, 2.5 K₂HPO₄, 2.0 CaCl₂, 1.2 MgSO₄, 4.0 lactate, 6.0 L-alanine, 5.0 HEPES, and 5.5 D-glucose, pH 7.4, 290±2 mOsm/kg, as previously described (23). A single tubule was studied from each animal.

Isolated collecting ducts were microperfused *in vitro* as previously described (23, 44). Briefly, each isolated tubule was immediately transferred to a temperature and O₂/CO₂-controlled specimen chamber, mounted on concentric glass pipettes, and perfused and bathed at 37°C with Burg's perfusate containing (in mM): 120 NaCl, 25 NaHCO₃, 2.5 K₂HPO₄, 2.0 CaCl₂, 1.2 MgSO₄, 4.0 Na lactate, 1.0 Na₃ citrate, 6.0 L-alanine, and 5.5 D-glucose, pH 7.4, 290±2 mOsm/kg (23). During the 45 min equilibration period and thereafter, the perfusion chamber was continuously suffused with a gas mixture of 95% O₂-5% CO₂ to maintain pH of the Burg's solution at 7.4 at 37°C.

The bathing solution was continuously exchanged at a rate of 10 ml/hr using a syringe pump (Razel, Stamford, CT).

Transport measurements were performed in the absence of transepithelial osmotic gradients and thus water transport was assumed to be zero. Three to four samples of tubular fluid were collected under water-saturated light mineral oil by timed filling of a calibrated 30 nl volumetric constriction pipette at each perfusion rate (slow and fast). To determine the concentration of Na^+ delivered to the tubular lumen, ouabain (100 μM) was added to the bath at the conclusion of each experiment to inhibit all active transport, and an additional three to four samples of tubular fluid were obtained for analysis. The Na^+ concentrations of perfusate and collected tubular fluid were determined by helium glow photometry and the rates of net cation transport (in pmol/min.mm tubular length) were calculated using standard flux equations, as previously described (32). The calculated ion fluxes were averaged to obtain a single mean rate of ion transport for the CCD at each flow rate. The flow rate was varied by adjusting the height of the perfusate reservoir. The sequence of flow rates was randomized within each group of tubules to minimize any bias induced by time-dependent changes in ion transport.

In four experiments, tubular fluid collections were performed in collecting ducts perfused with Burg's solution prepared without Ca^{2+} (Ca^{2+} -free perfusate), with ($n = 2$) or without ($n = 2$) 100 μM EGTA (23). In other experiments, as indicated, tubules were pretreated with luminal benzamil (5 μM) or trypsin (1 $\mu\text{g}/\text{ml}$), or basolateral lumicolchicine or colchicine (10 μM ; (42)), brefeldin A (5 $\mu\text{g}/\text{ml}$; (4)), or BAPTA-AM (20 μM). All inhibitors were added to the luminal or bathing solution, as indicated, after the 45 min equilibration period and were present for at least 30 min before tubular fluid samples were first obtained. Note that a 30 min exposure to colchicine or BFA has been reported to be effective in inhibiting microtubule function or protein trafficking from the Golgi

complex to the cell membrane in distal nephron cells, respectively (22, 41, 42). Samples of tubular fluid for measurement of net Na^+ absorption were collected in the continuous presence of the inhibitors.

Oocyte expression

cRNAs for wild-type or mutant α , β and γ mENaC subunits were synthesized with T3 or T7 mMessage mMachineTM (Ambion, Austin, TX). Stage V-VI *Xenopus laevis* oocytes were pretreated with 1.5 mg/ml type IV collagenase and injected with 0.5-2 ng of cRNA/subunit. Injected oocytes were maintained at 18°C in modified Barth's saline (88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO_3 , 15 mM HEPES, 0.3 mM $\text{Ca}(\text{NO}_3)_2$, 0.41 mM CaCl_2 , 0.82 mM MgSO_4 , pH 7.4) supplemented with 10 $\mu\text{g/ml}$ sodium penicillin, 10 $\mu\text{g/ml}$ streptomycin sulfate and 100 $\mu\text{g/ml}$ gentamicin sulfate.

Two-electrode voltage clamp

Two-electrode voltage clamp (TEV) was performed at 23-26°C using a GeneClamp 500B amplifier (Axon Instruments, Union City, CA). Data were acquired through Clampex 8.0 using a DigiData 1200 interface and stored on the hard disk of the computer. Pipettes filled with 3 M KCl had resistances of 0.5-5 M Ω . The extracellular solution (TEV solution) was (in mM): 110 NaCl, 2 KCl, 1.54 CaCl_2 , 10 HEPES, pH 7.4, unless indicated otherwise. In selected experiments, oocytes were pretreated with 2 $\mu\text{g/ml}$ trypsin for 5 min. The recording chamber was perfused at a rate of 3.5 ml/min. Laminar shear stress was applied by perfusing TEV solution through a vertical pipette localized above the oocyte surface at a rate of 1.6 ml/min, corresponding to 0.137 dynes/cm² of shear stress as previously described (9). Bath perfusion was

maintained during application of laminar shear stress. Following the stimulation process, whole cell Na^+ currents were determined following bath perfusion with TEV solution supplemented with 5 μM benzamil. The benzamil-sensitive component of the whole cell Na^+ current at -60 mV was used to determine ENaC-mediated whole cell Na^+ current.

Confocal immunofluorescence microscopy

Microdissected CCDs were transferred to a Petri dish containing phosphate buffered saline (PBS) with or without colchicine (10 μM) or BFA (5 $\mu\text{g/ml}$) for 1 hr and then fixed for 30 min at room temperature in PBS containing 2.5% paraformaldehyde. Fixed CCDs were rinsed in PBS three times for 5 min, blocked for 3 h at room temperature in incubation solution (1X PBS containing 1% BSA and 0.1% Triton X-100), and then incubated overnight at 4°C with a 1:250 dilution of mouse monoclonal anti- α tubulin (clone DM1A; Sigma-Aldrich) or anti-giantin (gift from Adam Linstedt, Carnegie Mellon University) antibody prepared in incubation solution. After rinsing four times with PBS, CCDs were incubated for 80 min at room temperature with a 1:500 dilution of a fluorescein goat anti-mouse IgG (H+L) secondary antibody (Molecular Probes, Eugene, OR) prepared in incubation solution (without Triton X-100). CCDs were rinsed four times, and then mounted on coverslips using Prolong Gold (Molecular Probes) mounting medium.

Imaging of immunolabeled CCDs was performed on a TCS-SL confocal microscope equipped with argon and green and red helium-neon lasers (Leica, Dearfield, IL). Images were acquired by sequential scanning using a 100 \times (1.4 numerical aperture) planapochromat oil objective and the appropriate filter combination. Settings were as follows: photomultipliers set to 500-600 V, 1 Airy disk, and Kalman filter ($n = 3$). Serial (z) sections were captured with a 0.30-

μm step size. The images (512×512 pixels) were saved as TIFF files. The Volocity program (Improvision, Lexington, MA) was used to project the serial sections into one image. The contrast level of the final images was adjusted in Photoshop, and the contrast-corrected images were imported into Macromediate FreeHand (Adobe, Mountain View, CA). Staining for tubulin and giantin was not observed in the absence of primary antibody (data not shown).

Reagents

Benzamil hydrochlorothiazide, trypsin, colchicine and its inactive structural analogue lumicolchicine were obtained from Sigma-Aldrich. Stock solutions of brefeldin A (BFA; Calbiochem, La Jolla, CA) were prepared in DMSO and diluted 1000-fold to yield the final concentration to which the tubule was exposed. BAPTA-AM was purchased from Molecular Probes.

Statistics

All results are expressed as means \pm SE; n equals the number of animal or tubule samples used for *in vitro* microperfusion or number of oocytes used in TEV studies. Comparisons were made by paired and unpaired t -tests as appropriate, using commercially available statistical software for the calculations (SPSS Inc. Chicago, IL). Data comparisons among multiple groups of tubules were performed by ANOVA. Significance was asserted if $p < 0.05$.

RESULTS

Flow-stimulated Na⁺ absorption mediated by ENaC

We confirmed that an increase in tubular fluid perfusion rate from 1.1 ± 0.1 to 5.3 ± 0.3 nl/min.mm led to an increase in net Na⁺ absorption from 15.4 ± 2.6 to 68.5 ± 6.9 pmol/min.mm ($n = 9$; $p < 0.001$; Fig. 1) and no significant change in V_{te} from $(-8.6 \pm 3.9$ to -3.8 ± 2.7 mV; $p = \text{NS}$). To test whether flow-stimulated Na⁺ absorption is mediated by ENaC, the effect of luminal benzamil (5 μM) on flow-stimulated net Na⁺ absorption was measured in 5 rabbit CCDs (Fig. 1). Luminal benzamil completely inhibited net Na⁺ absorption at slow (-5.3 ± 6.6 pmol/min.mm at 1.0 ± 0.1 nl/min.mm) and fast (-12.1 ± 8.4 pmol/min.mm at 4.4 nl/min.mm) flow rates ($p < 0.05$ compared to control CCDs). V_{te} in the benzamil-treated CCDs was 3.0 ± 0.9 and 2.4 ± 1.0 mV at the two flow rates ($p = \text{NS}$), respectively. These data suggest that a benzamil-sensitive pathway, presumably ENaC, mediates both basal and flow-stimulated net Na⁺ absorption in mammalian CCDs.

Flow-induced increases in net Na⁺ transport do not require an increase in $[\text{Ca}^{2+}]_i$

We have previously shown that increases in the rate of tubular perfusion are associated with a large transient high peak and lower sustained elevation in $[\text{Ca}^{2+}]_i$ in principal cells as well as intercalated cells (23, 43, 44). While large increases in $[\text{Ca}^{2+}]_i$ are predicted to inhibit ENaC at the plasma membrane (30, 36), more modest increases in $[\text{Ca}^{2+}]_i$ might facilitate exocytic insertion of channels from an intracellular pool into the plasma membrane (8, 45). To determine whether luminal Ca²⁺ entry and the consequent rise in $[\text{Ca}^{2+}]_i$ associated with high tubular flow rates in the CCD is required for flow-stimulated net Na⁺ absorption, isolated tubules were

perfused in the absence of luminal Ca^{2+} (either with or without luminal EGTA) or after loading with the permeant intracellular Ca^{2+} -chelator BAPTA-AM (20 μM). We have previously shown that removal of luminal Ca^{2+} does not affect resting $[\text{Ca}^{2+}]_i$ but markedly attenuates the flow-induced rise in $[\text{Ca}^{2+}]_i$ (23).

In the absence of luminal Ca^{2+} , an increase in flow rate from 1.0 ± 0.2 to 5.7 ± 0.2 nl/min.mm induced a significant increase in net Na^+ absorption from 12.9 ± 2.6 to 63.1 ± 16.9 pmol/min.mm ($p < 0.05$; $n = 4$; Fig. 2), indicating that flow-stimulation of net Na^+ absorption does not require luminal Ca^{2+} entry. Similarly, chelation of $[\text{Ca}^{2+}]_i$ did not inhibit flow-stimulation of net Na^+ absorption. An increase in flow rate from 1.4 ± 0.3 to 5.4 ± 0.1 nl/min.mm in BAPTA-loaded CCDs induced a significant increase in net Na^+ absorption from 10.1 ± 3.1 to 48.6 ± 6.3 pmol/min.mm ($p < 0.05$; $n = 3$; Fig. 2).

Flow-induced increases in net Na^+ absorption reflect an increase in channel open probability

Microtubule-dependent vesicle transport plays an important role in specific membrane trafficking events, including exocytosis (17). To test whether the flow-induced increase in net Na^+ absorption requires intact microtubule function, CCDs were incubated with 10 μM colchicine ($n = 6$) or the inactive analogue lumicolchicine ($n = 4$) for 1 hr and the effect of an increase in luminal flow rate on net Na^+ absorption was measured in the continued presence of the agent. Localization of tubulin with a monoclonal anti-tubulin antibody demonstrated that the microtubular architecture was disrupted in colchicine-treated tubules (Figs. 3A and 3B). An increase in luminal flow rate in colchicine-treated CCDs from 1.0 ± 0.2 to 4.5 ± 0.3 nl/min.mm was associated with an increase in net Na^+ absorption from 14.7 ± 3.8 to 41.8 ± 6.5 pmol/min.mm ($p < 0.01$; Fig. 4), an increase similar to that detected in untreated control and lumicolchicine-

treated ($n = 4$) CCDs perfused at similar flow rates ($p = \text{NS}$; Fig. 4). These results suggest that flow-activation of ENaC is not dependent on intact microtubules.

To further explore whether an increase in luminal flow rate stimulates trafficking of newly synthesized channels from the trans-Golgi network to the plasma membrane, we examined the effect of BFA (5 $\mu\text{g/ml}$) on flow-stimulated net Na^+ absorption in the CCD ($n = 4$). BFA treatment results in an inhibition of delivery of channels from the intracellular pool to the plasma membrane (4, 11). If ENaC activation by flow is dependent on exocytic insertion of channels into the plasma membrane, BFA treatment should block the flow-dependent increase in net Na^+ absorption. We observed that net Na^+ absorption increased from 12.2 ± 3.4 to 32.4 ± 8.5 pmol/min.mm ($p < 0.05$) in BFA-treated tubules as the tubular flow rate was increased from 1.0 ± 0.1 to 5.0 ± 0.3 nl/min.mm (Fig. 4). Net Na^+ absorption in BFA-treated CCDs did not significantly differ from that measured in untreated control, lumicolchicine- and colchicine-treated CCDs perfused at the slow and fast flow rates in this set of experiments ($p = \text{NS}$). While protein trafficking to plasma membranes has been reported to be sensitive to BFA, some cells have been shown to be relatively resistant to the disruption of Golgi architecture by BFA (3, 25). In fact, we observed that the perinuclear localization of giantin, a Golgi marker, was similar in control and BFA-treated tubules (Figs. 3C and 3D). A lack of effect of low concentrations of BFA, sufficient to disrupt trafficking to the plasma membrane in MDCK and mouse CCD cells, on the distribution of selected Golgi markers has been observed in MDCK cells (3, 4, 25).

Recent studies suggest that ENaC extracellular domains are processed by proteases (18, 20). Channels that have not been processed by proteases appear to have a very low open probability (7, 18, 34). Furthermore, channels with an intrinsically low open probability respond to external trypsin with a dramatic increase in channel open probability, such that channels

exhibit “normal” gating behavior with characteristically long open and closed times (7, 34). If both flow and trypsin-dependent proteolysis activate ENaC by increasing channel open probability, the effects of flow and trypsin on net Na⁺ absorption might not be additive. We first measured net Na⁺ absorption at a slow tubular flow rate before and after luminal perfusion with trypsin (1 µg/ml). At a slow flow rate of 1.3±0.2 nl/min.mm, the rate of net Na⁺ absorption in trypsin-treated CCDs (25.8±3.0 pmol/min.mm; n = 5) significantly exceeded that measured in control tubules (15.4±2.6, n = 9; p=0.03; Fig. 5), consistent with protease activation of resident ENaCs in the apical membrane. However, trypsin did not alter the rate of net Na⁺ absorption measured at fast flow rates (68.5±6.9 pmol/min.mm at 5.3±0.3 nl/min.mm in the absence of trypsin vs. 76.3±7.3 pmol/min.mm at 5.7±0.4 nl/min.mm in presence of trypsin; p=0.48; Fig. 5). In CCDs pre-treated with trypsin, the 3.2±0.6 fold increase in net Na⁺ absorption elicited by an increase in tubular flow rate was significantly lower than the 5.9±1.6-fold increase observed in control CCDs (p<0.05).

Flow activates channels that have not been processed by proteases

Our previous observations, as well as work from other groups, suggest that both non-cleaved channels as well as channels that have been processed by proteases are expressed at the apical plasma membrane of epithelia (2, 6, 19, 20). Channels that have not been processed by proteases respond to trypsin with a large increase in open probability (6, 7, 18). Our observation that trypsin treatment did not significantly enhance the rate of Na⁺ absorption under high flow conditions raised the possibility that non-cleaved channels are activated by flow. We have previously shown that ENaCs with mutations at key sites in the α (RtripleA (R205A/R028A/R231A)) and γ (R143A) subunits are not processed by proteases in oocytes (18).

We examined whether α RtripleA β γ R143A channels expressed in oocytes were activated by laminar shear stress, which was generated by perfusing TEV solution through a vertical pipette localized above the oocyte surface at a rate of 1.6 ml/min, corresponding to 0.137 dynes/cm² of shear stress. The fold-increase in benzamil (5 μ M)-sensitive whole cell Na⁺ currents (INa) in oocytes expressing α RtripleA β γ R143A in response to laminar shear stress was 0.38 \pm 0.05 fold (n=14; Fig. 6), nearly identical to the 0.36 \pm 0.04 fold (n = 16) increase in INa observed in oocytes expressing wild-type ENaC (p=0.72, unpaired *t* test).

Flow activates channels that have been processed by proteases

Net Na⁺ absorption in trypsin-treated CCDs perfused at slow flow rates (25.8 \pm 3.0 pmol/min.mm; n = 5) was significantly less than that measured at fast flow rates (76.3 \pm 7.3 pmol/min.mm; n=5, p < 0.01). These results suggest that flow activates channels that have been previously activated by proteases. We therefore examined whether wild type channels expressed in oocytes, following trypsin treatment, were activated by laminar shear stress. The fold-increase in benzamil (5 μ M)-sensitive whole cell INa in oocytes expressing wild type channels in response to laminar shear stress was 0.37 \pm 0.07 fold (n=8; Fig. 7), similar to the 0.52 \pm 0.13 fold (n = 6) increase in INa observed in oocytes expressing wild type ENaC that were pre-treated with trypsin (p=0.31, unpaired *t* test). Our results suggest that the open probability of protease-activated channels can be further increased by laminar shear stress.

DISCUSSION

Renal epithelial cells in the distal nephron are subject to continuous variations in urinary flow rate. We have previously reported that an increase in luminal flow rate from 1 to ~5 nl/min.mm stimulates net Na⁺ absorption as well as K⁺ secretion (32, 33, 44). The purpose of the present study was to confirm that flow-stimulated net Na⁺ absorption is mediated by ENaC and, if so, examine whether the flow-dependent activation of Na⁺ transport reflects an increase in open probability or density of apical resident ENaCs in the mammalian CCD.

The observation that benzamil inhibited net Na⁺ absorption both at slow and fast flow rates (Fig. 1) is consistent with a major role of ENaC in mediating flow-stimulated net Na⁺ absorption. ENaC activity can be regulated by two distinct mechanisms: changes in open probability of channels resident at the apical membrane or changes in number of apical conducting channels due to recruitment of ENaCs from subapical storage pools (37). We have previously shown that a rapid increase in luminal flow rate in the microperfused rabbit CCD elicits an increase in [Ca²⁺]_i that reflects both release of Ca²⁺ from internal, phosphoinositol-sensitive stores and external Ca²⁺ entry, processes that are mutually dependent on each other (23). While increases in [Ca²⁺]_i might increase ENaC activity by enhancing the trafficking of ENaC channels from an intracellular pool to the plasma membrane, Palmer *et al.* (30, 36) have suggested that increases in [Ca²⁺]_i from basal levels to concentrations > 500 nM inhibit ENaC activity by reducing channel open probability. The effect of modest increases in [Ca²⁺]_i noted in response to an increase in flow on Na⁺ channel open probability is not known. Our studies demonstrate that flow-dependent increases in ENaC activity are not dependent on increases in [Ca²⁺]_i (Fig. 2). We observed flow-dependent increases in net Na⁺ absorption in tubules

pretreated with BAPTA to chelate intracellular Ca^{2+} , as well as tubules perfused with a luminal buffer nominally free of Ca^{2+} .

Acute stimulation of ENaC in Na^+ absorptive epithelia by forskolin (or cAMP) is mediated by channel recruitment to the apical membrane from a subapical vesicle-based recycling pool (4). Both colchicine, which disrupts microtubules, as well as BFA, which disrupts Golgi and inhibits antegrade trafficking from the TGN to the apical membrane, prevented forskolin-stimulated increases in ENaC activity in cultured CCD cells (4, 5). In contrast, we observed flow-dependent increases in net Na^+ absorption in CCDs treated with either colchicine or BFA (Fig. 4), suggesting that flow-dependent activation of ENaC is not due recruitment of channels from an intracellular pool to the plasma membrane.

ENaC open probability is regulated by a number of factors. Recent studies suggest that proteolytic processing of ENaC subunits by proteases, including furin, prostatic, and other serine proteases activates ENaC by increasing channel open probability (1, 6, 7, 18, 39, 40). Channel activation by proteases appears to be associated with a conversion of channels that have a very low open probability, referred to as "near-silent channels", to channels that exhibit "normal" gating behavior with long mean open and closed times (7). Analysis of the effects of trypsin on flow-stimulated Na^+ absorption provides insight into mechanisms mediating flow-dependent increases in net Na^+ absorption. We noted that net Na^+ absorption in trypsin-treated CCDs perfused at a slow luminal flow rate of ~ 1 nl/min.mm was approximately twice that measured in control (non-trypsin-treated) CCDs perfused at the same flow rate (Fig. 5). This result is consistent with a protease-mediated increase in open probability of resident channels and suggests that a sizeable pool of non-cleaved channels is present at the CCD plasma membrane. We also observed that Na^+ absorption in trypsin-treated CCDs at slow flow rates was

significantly less than that observed in trypsin-treated CCDs at high flow rates. Furthermore, Na^+ currents measured in oocytes expressing wild type ENaC that were pre-treated with trypsin increase in response to laminar shear. These results suggest that flow activates channels that have been previously activated by proteases. In contrast, when tubular segments were perfused at fast flow rates, a significant enhancement of net Na^+ absorption was not observed following trypsin treatment. We interpret these results to indicate that (i) flow-stimulation of net Na^+ absorption is predominantly due to an increase in ENaC open probability, and (ii) both non-cleaved and cleaved channels are activated by flow. We observed that the fold-increase in whole cell Na^+ currents in oocytes expressing non-cleaved channels ($\alpha\text{R205A/R208A/R231A}\beta\gamma\text{R143A}$) in response to LSS was similar that observed in oocytes expressing wild-type channels (Fig. 5). These data provide additional evidence that non-cleaved channels are activated by flow.

In summary, our results suggest that increases in tubular flow rates activate ENaC primarily by increasing channel open probability. Once channels are activated by flow, they do not exhibit further activation in response to proteases. Although the mechanisms by which ENaC senses mechanical forces in the distal nephron have not been elucidated, we propose that variations in flow rates in the distal nephron induce conformational changes in the channel's gate that alter channel open probability (10).

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FIGURE LEGENDS

1. Effect of benzamil (BZ) on flow-stimulated net Na⁺ absorption in microperfused rabbit CCDs. Net Na⁺ absorption was measured at tubular flow rates of ~1 and 5 nl/min.mm in the absence (control; n = 9) or presence of 5 μM BZ (n = 5), a selective inhibitor of ENaC. *, p<0.05 vs. transport rate at 1 nl/min.mm in the same CCDs; #, p<0.05 vs. control at same flow rate.

2. Effect of removal of luminal Ca²⁺ (Ca²⁺ free perfusate) or intracellular Ca²⁺ chelation on flow-stimulated net Na⁺ absorption in microperfused rabbit CCDs. Net Na⁺ absorption was measured at tubular flow rates of ~1 and 5 nl/min.mm in the absence of luminal Ca²⁺ (±EGTA, as indicated in METHODS section; n = 4) or presence of 20 μM BAPTA-AM, a chelator of intracellular Ca²⁺ (n = 3). *, p<0.05 vs. transport rate at 1 nl/min.mm in the same CCDs.

3. Effect of colchicine and brefeldin-A (BFA) on microtubular and Golgi architecture.

Isolated rabbit CCDs were treated with 10 μM colchicine, 5 μg/ml BFA, or vehicle alone for 1 h and then fixed. Microtubules were localized with a monoclonal anti-tubulin antibody in control (A) and colchicine-treated (B) CCDs. Giantin, a Golgi marker, was localized with a monoclonal anti-giantin antibody in control (C) and BFA-treated (D) CCDs.

4. Effect of lumicolchicine (LCO), colchicine (CO), and brefeldin-A (BFA) on flow-stimulated net Na⁺ absorption in microperfused rabbit CCDs. Net Na⁺ absorption was measured at tubular flow rates of ~1 and 5 nl/min.mm in the absence (C for control; n = 4) or presence of 10 μM colchicine (n = 6), a microtubule inhibitor, the same concentration of its

inactive structural analogue lumicolchicine ($n = 4$), or 5 $\mu\text{g/ml}$ BFA ($n = 4$), an agent that disrupts Golgi and inhibits delivery of channels from the intracellular pool to the plasma membrane (4, 35). *, $p < 0.05$ vs. transport rate at 1 nl/min.mm in the same CCDs.

5. Effect of trypsin on flow-stimulated net Na^+ absorption in microperfused rabbit CCDs.

Net Na^+ absorption was measured at tubular flow rates of ~ 1 and 5 nl/min.mm in the absence (control; $n = 9$) or presence of 1 $\mu\text{g/ml}$ trypsin ($n = 5$), which increases the open probability of the channel (7, 12, 18). *, $p < 0.05$ vs. transport rate at 1 nl/min.mm in the same CCDs; #, $p < 0.05$ vs. control at same flow rate.

6. Effect of laminar shear stress (LSS) on whole cell Na^+ currents in oocytes expressing non-cleaved ENaC channels ($\alpha\text{R205A/R208A/R231A}\beta\gamma\text{R143A}$). Oocytes were injected with cRNAs for wild-type $\alpha\beta\gamma$ or for $\alpha\text{RtripleA}\beta\gamma\text{R143A}$. The α and γ subunits had N-terminal HA tag and a C-terminal V5 tags. The fold-increase in benzamil (5 μM)-sensitive whole cell Na^+ currents (I_{Na}) in oocytes expressing wild-type ENaC ($n = 16$) or $\alpha\text{RtripleA}\beta\gamma\text{R143A}$ ($n = 14$) was measured in response to a LSS rate of 0.137 dynes/cm^2 .

7. Effect of trypsin on laminar shear stress-induced increase in whole cell Na^+ currents in oocytes expressing wild-type ENaC. Oocytes were injected with cRNAs for wild-type $\alpha\beta\gamma$ ENaC. LSS was generated as described in the Methods. The extracellular solution contained (in mM): 110 Na gluconate, 1.54 CaCl_2 , 2 BaCl_2 , 10 tetraethylammonium chloride, 10 HEPES, pH 7.4 The fold-increase in benzamil (5 μM)-sensitive whole cell Na^+ currents (I_{Na}) in ENaC-expressing oocytes studied in the absence ($n = 8$) or presence of trypsin (2 $\mu\text{g/ml}$; $n = 6$) was

measured in response to a LSS rate of 0.137 dynes/cm^2 .

Fig. 1

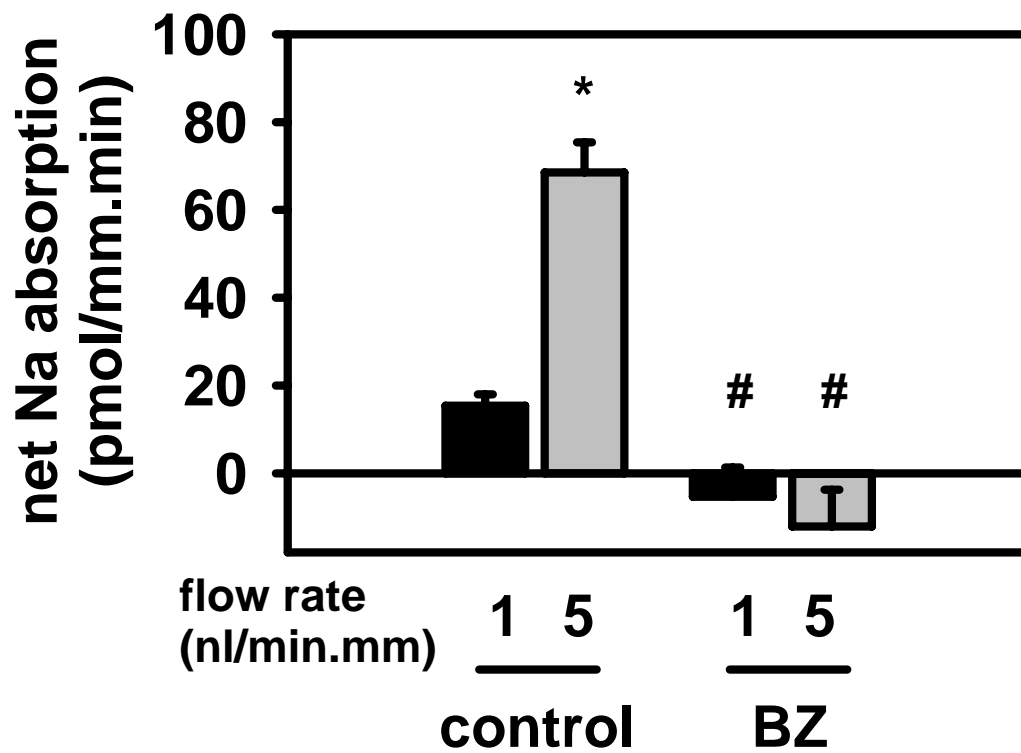


Fig. 2

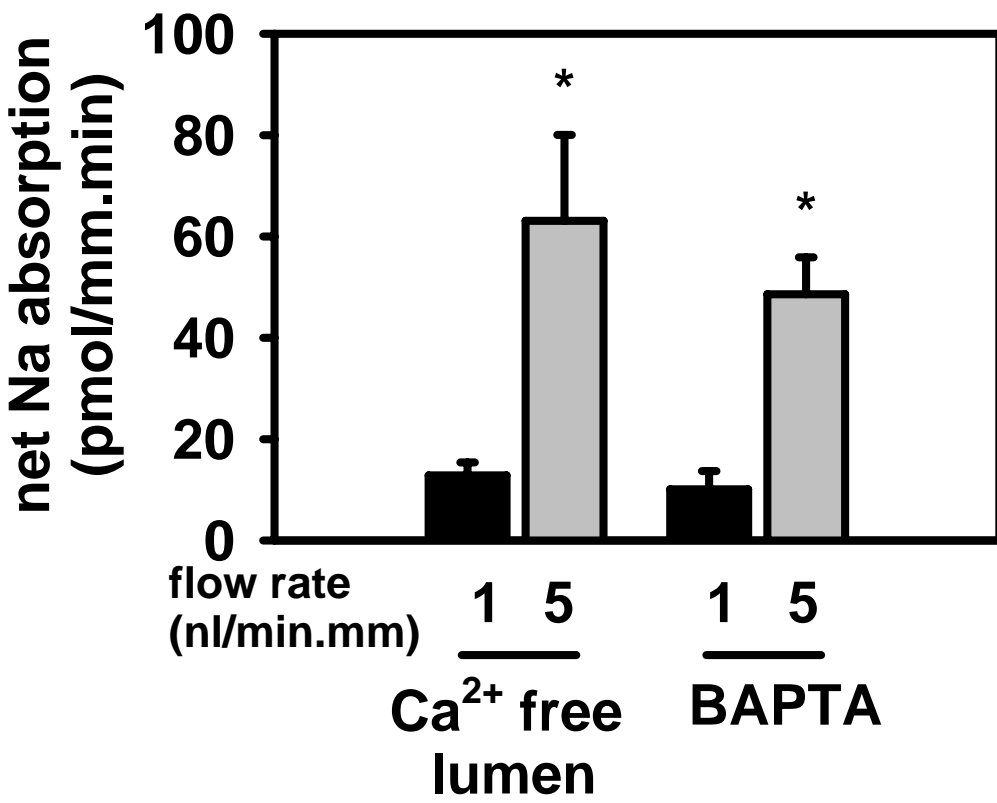


Fig. 3

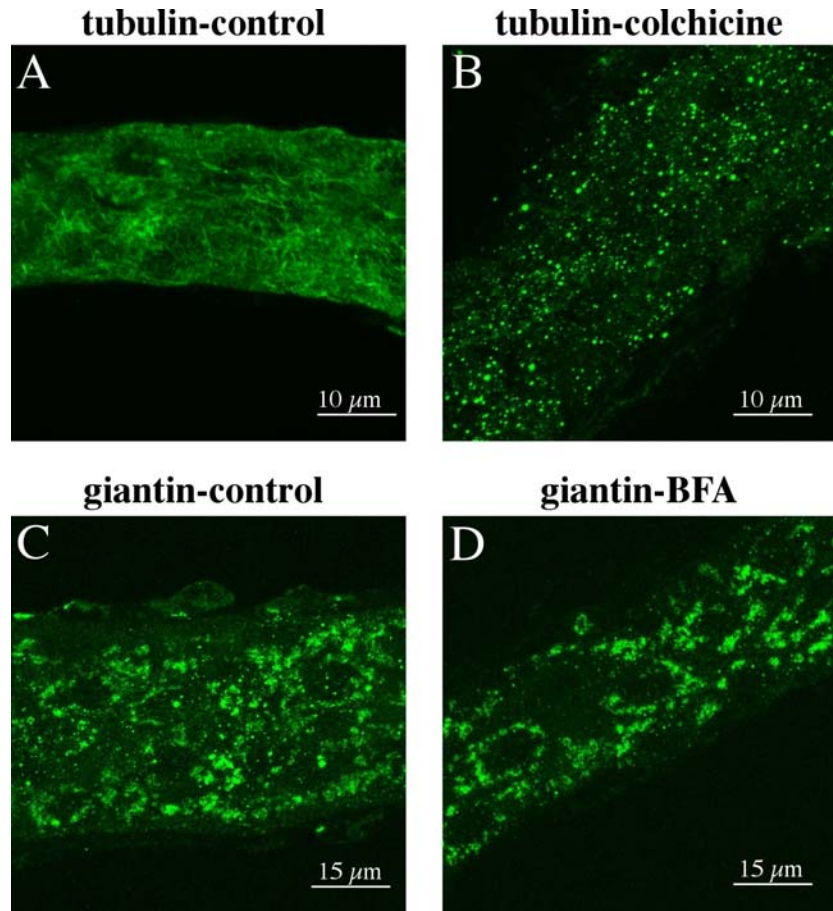


Fig. 4

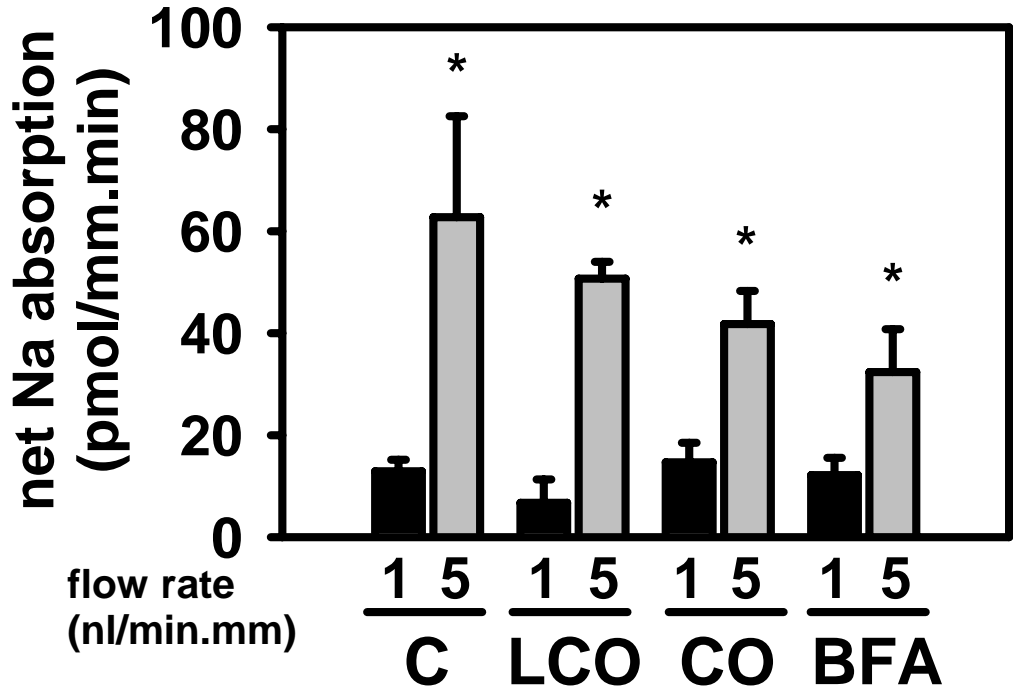


Fig. 5

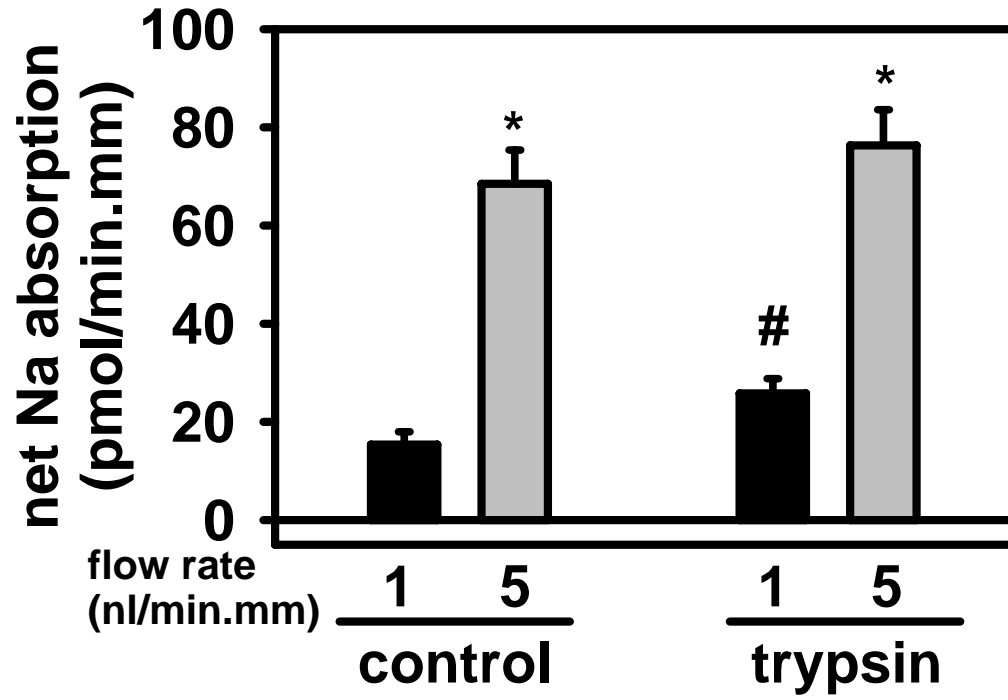


Fig. 6

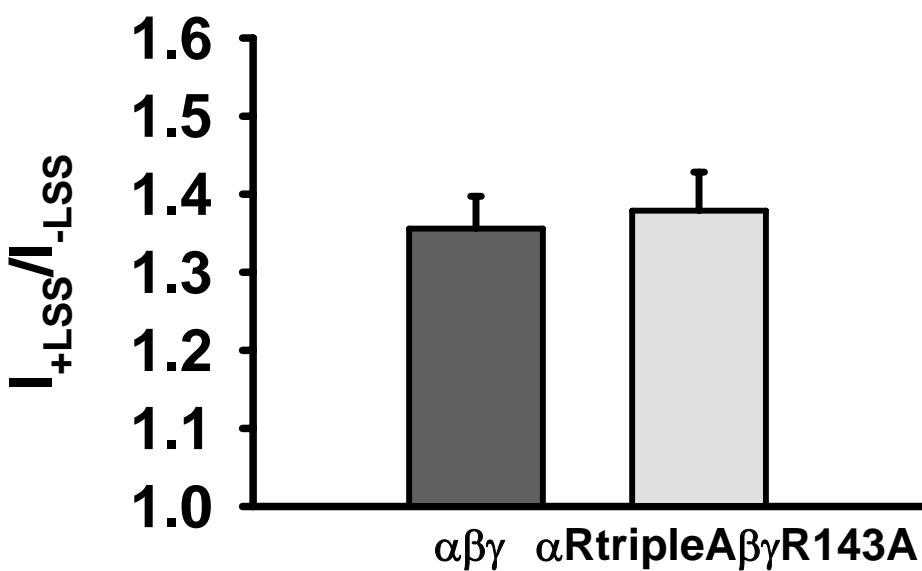


Fig. 7

