Epithelial Na⁺ Channels Are Activated by Laminar Shear Stress*

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Marcelo D. Carattino‡§, Shaohu Sheng‡, and Thomas R. Kleyman‡¶

From the Renal-Electrolyte Division, Departments of ‡Medicine and ¶Cell Biology and Physiology, University of Pittsburgh, Pittsburgh, Pennsylvania 15261

The degenerin/epithelial Na⁺ channel (ENaC) superfamily is a group of structurally related ion channels that are involved in diverse biological processes, including responses to mechanical stimuli. In renal cortical collecting ducts, changes in rates of perfusion affect Na⁺ reabsorption through an amiloride-sensitive pathway, suggesting that ENaC may be a mechanosensitive channel. In this study, we examined whether ENaC expressed in oocytes is regulated by laminar shear stress (LSS). A 1.8-mm (internal diameter) perfusion pipette was placed within 0.5–1.0 mm of the oocyte to provide laminar flow across the oocyte surface. LSS induced a dose-dependent and reversible increase in benzamil-sensitive whole cell Na⁺ currents in oocytes expressing αβγ ENaC. The half-time for activation by LSS was ~5 s. Repetitive stimulation by LSS of oocytes expressing ENaC was associated with a reduction in the response to LSS. Oocytes expressing αβS518Kγ, a pore region mutant with a high open probability, were insensitive to LSS. We demonstrated previously that channels with a Cys residue introduced at position αSer-380 had a low open probability, but, following modification by [2-(trimethylammonium)ethyl]methanethiosulfonate bromide (MTSET), channels exhibited a high open probability. Oocytes expressing αS580Cβγ ENaC respond to LSS similar to wild type; however, covalent modification by MTSET largely eliminated the response to LSS. Our results suggest that shear stress activates ENaC by modifying the gating properties of the channel.

Genetic and molecular analyses of Caenorhabditis elegans with a touch-insensitive phenotype led to the identification of several genes whose products were postulated to be part of a touch-transducing complex. Dominant mutations in MEC-4, MEC-10, UNC-8, UNC-105, and DEG-1 result in the deaths of specific mechanosensory neurons where these proteins are expressed, which is thought to result from an increase in cation channel activity (2, 6). Mutations within three distinct regions appear to generate hyperactive channels, including a region following the first transmembrane domain, a tract of 22 amino acids within the extracellular loop present only in degenerins that could be part of a gating domain, and a single amino acid substitution in the pre-second membrane-spanning (M2) domain that appears to be within a conserved gating domain (degenerin site) (2). Functional channels were observed when gain-of-function mutations in selected members of the putative touch-transducing complex were expressed in Xenopus oocytes or human embryonic kidney 293 cells, although a functional response to a mechanical stimulus has not been demonstrated (7).

Cells are potentially exposed to variety of mechanical stimuli, including indentations, high frequency vibrations, osmotic pressure gradients, hydrodynamic pressure, and fluid shear stress. In the specific case of ion channels, modifications in channel kinetics could result from changes in membrane tension, thickness, local curvature, and extension or by direct tugging on the protein by cytoskeletal or extracellular tethers (8). Based on its structure homology with other members of the DEG/ENaC family that are presumed to be activated by mechanical stimuli (as postulated for degenerins in C. elegans), it was suggested that ENaC could be a mechanosensitive channel (9, 10). Various studies have examined this hypothesis with conflicting results. Awayda et al. (9) showed that in vitro translated bovine α-ENaC, reconstituted in lipid bilayers, is activated by membrane distension produced by a hydrostatic pressure gradient. Later studies showed that rat αβγ ENaC is mechanosensitive when it is reconstituted in lipid bilayers (10); however conflicting results were reported when oocytes expressing rat ENaC were subjected to an osmotic stimulus (11, 12). When channels were examined in principal cells using a cell attached patch configuration, application of negative pressure led to an increase in single channel activity in >30% of patches (13). A recent study suggested that a lack of reproducibility of stretch-mediated ENaC activation may reflect stretch-induced ATP release from cells that inhibits ENaC by activating purinergic receptors, thus masking stretch-induced ENaC activation (14).

We recently reported that increasing rates of perfusion of isolated rabbit cortical collecting ducts increase the rates of tubular Na⁺ reabsorption via an amiloride-sensitive pathway, suggesting that ENaC is activated in response to increases in tubular flow rates (15). However, this study did not address the question of whether mechanical forces, such as shear stress,
directly modulate gating of the Na\(^+\) channel. In this report, we used a submerged jet to expose oocytes expressing ENaC to laminar shear stress (LSS). We observed that wild type ENaC activity increased in response to LSS, whereas mutant or chemically modified Na\(^+\) channels that exhibit a high intrinsic open probability did not respond to laminar flow. These data suggest that mechanical forces directly modulate ENaC gating.

**MATERIALS AND METHODS**

**DNA Constructs**—Point mutations and truncations of mouse ENaC (mENaC) subunits were generated by site-directed mutagenesis with the sequential polymerase chain reaction method using Phusion DNA polymerase (Thermo Scientific, Waltham, MA). The PCR-amplified fragments containing the desired mutations were ligated into wild type mENaC cDNA. PCR-amplified fragments were sequenced by automated DNA sequencing at the University of Pittsburgh’s sequencing facility to confirm the desired mutation.

**Oocyte Expression**—cRNAs for α, β, and γ mENaC subunits were synthesized with T3 mMessage mMachine \(\text{TM}\) (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 mg subunit mENaC cRNAs. 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 Ca\(_{\text{NO}}\)\(_3\), 0.41 mM CaCl\(_2\), and 0.82 mM MgSO\(_4\); pH 7.4) supplemented with 10 mM sodium CHAPS, 10 μM streptomycin sulfate, and 100 μg/ml gentamicin sulfate.

**Two-electrode Voltage Clamp** (TEV)—Two-electrode voltage clamp was performed at room temperature (20–25 °C) using a GeneClamp 500B amplifier (Axon Instruments, Union City, CA). Data were acquired through Clamplex 8.9 using a DigiData 1200 interface and stored in a program that is the programmer for Clampfit 8.1 (Axon Instruments, Union City, CA). To determine the rate of bath perfusion from 0 to 5.6 ml/min, the bath solution was brought to a constant volume of 1 ml and inspected. The extracellular solution (TEV solution) was 110 mM NaCl, 2 mM KCl, 1.54 mM CaCl\(_2\), and 10 mM HEPES, pH 7.4. Oocytes were kept in a recording chamber (20-mm diameter and 6-mm-deep petri dish) with a constant volume of ~1.5 ml of TEV solution. The recording chamber was perfused with a rate of 3.5 ml/min. Shear stress was applied by perfusing TEV solution through a vertical pipette (1.6-mm internal diameter) that was placed near the surface of the oocyte at a distance of <1 mm as described previously (17). Bath perfusion was either maintained (Fig. 2A) or stopped (Figs. 1, 2B, 3, and 4) using a low-shear perfusion system (see “Results”).

The approximate average shear stress on the oocyte surface was estimated as the relation of the drag force and the oocyte surface area (\(F_d\)/\(A\) surface area of the oocytes) (17). The magnitude of the drag force was determined according to the following equation:

\[ F_d = \frac{1}{2} \rho \, V^2 \, C_d \, A \]

where \(\rho\) is the density of the fluid, \(V\) is the average fluid velocity, and \(C_d\) is the drag coefficient (1–2 for spheres with a Reynolds number within the range of ~3–25). The Reynolds number was determined as described previously (17). To determine a dose-response relationship, vertical pipette perfusion rate was varied between 0.5 and 3.7 ml/min, corresponding to a shear stress of between 0.013 and 0.753 dynes/cm\(^2\). For most studies, the vertical pipette perfusion rate was 1.6 ml/min, corresponding to 0.137 dynes/cm\(^2\) of shear stress.

Whole cell currents were determined using a series of voltage steps (500 ms) from −140 to 60 mV in 20-mV increments in the absence or presence of benamil (5 μM) in the bath solution. ENaC-mediated whole cell Na\(^+\) currents were defined as benzamil-sensitive currents that were measured in the absence of LSS and were referred to as \(I_{\text{LSS}}\). Whole cell currents recorded in the presence of LSS were referred to as \(I_{\text{LSS}}\). To determine mean times of activation, oocytes were clamped at a holding potential of −60 mV, and the vertical pipette perfusion was controlled through the computer using a protocol generated in pClamp 8.0.

**Statistical Analyses**—Data were expressed as the mean ± S.E. (n), unless otherwise indicated, where n equals the number of independent experiments analyzed. Experiments were repeated with a minimum of two batches of oocytes obtained from different frogs. Electrophysiological data were analyzed with Clampfit 8.1 (Axon Instruments, Union City, CA), SigmaPlot 8.02 (SPSS Inc., Chicago, IL), and GraphPad Prism 6.02 (GraphPad Software, San Diego, CA). Statistical comparisons were performed using GraphPad Instant (GraphPad Software, San Diego, CA). Mean time of activation was determined by fitting experimental data with an exponential function \(y = y_0 + a \cdot e^{-x/b}\), where \(y_0\) is the plateau, \(a\) is the difference between the signal at time zero and the plateau, and \(b\) is the time constant. The mean time of activation was calculated as 0.69302/time constant. The dose-response curve was fit with the following equation: \(y = 1/(1 + 10^{(\log_{10}EC_{50} - x)})\), where \(\log_{10}EC_{50}\) is the logarithm of the dose (shear stress) that elicited 50% of the maximal response.

**RESULTS**

**Laminar Flow Increases Benzamil-sensitive Na\(^+\) Currents in Oocytes Expressing αβγ mENaC**—We reported previously that the rates of Na\(^+\) reabsorption in renal cortical collecting tubules perfused in vitro increase in response to increases in the rates of tubular perfusion (15). We also observed increases in whole cell Na\(^+\) currents measured in oocytes expressing αβγ mENaC that were centered in a petri dish in response to a change in the rate of bath perfusion from 0 to 5–6 ml/min, although the increases in currents were highly variable and, on occasion, no increase was observed. To explore whether Na\(^+\) channels are activated in response to increases in LSS, we employed a perfusion system similar to the fluid jet used to stimulate sensory hair cells (19). Oocytes expressing αβγ were positioned in the chamber and exposed to laminar fluid flow from a vertical pipette, as described previously by Hoger et al. (17). Whole cell Na\(^+\) currents were determined before changing from perfusion of the petri dish to perfusion via a vertical pipette that was placed in close proximity to the oocyte surface. ENaC-mediated Na\(^+\) currents, defined as currents sensitive to 5 μM benzamil, were increased significantly following the switch to fluid jet perfusion (Fig. 1, A–C; \(I_{\text{basal}}\) = 1.53 ± 0.19, n = 15, p < 0.05; +LSS versus before and after LSS). Channel activation was reversible, as currents fell to baseline levels when perfusion was changed from the vertical pipette back to the bath perfusion (Fig. 1, A–C). Increases in current induced by LSS were not associated with changes in reversal potential, suggesting that LSS did not alter the chemical driving force for Na\(^+\) transport (Fig. 1A). Increases in whole cell currents were not observed in non-injected oocytes in response to switching to fluid jet perfusion (Fig. 1D, upper trace). The inclusion of benzamil in the bath solution and vertical pipette abolished the response to LSS in ENaC-expressing oocytes (Fig. 1D, lower trace). Increases in whole cell Na\(^+\) currents were also observed if bath perfusion was maintained following initiation of fluid jet perfusion (\(I_{\text{basal}}\)–LSS = 1.28 ± 0.08, n = 33). We observed variability in the LSS response (\(I_{\text{LSS}}\)–LSS) across different batches of oocytes. For that reason, statistical comparisons were made with experiments performed with oocytes from the same batch.

A software-controlled perfusion system was utilized to initiate fluid jet perfusion in the setting of constant bath perfusion in order to characterize the kinetics of channel activation. To define the time course of activation by shear stress, whole cell currents were monitored at a clamp potential of −60 mV. Currents were measured with constant bath perfusion for 20 s prior to initiation of fluid jet perfusion. Oocytes expressing αβγ mENaC responded to shear stress with an increase in whole cell currents (Fig. 2A). The time required to achieve half-maximal activation (\(t_{1/2}\)) was obtained from fitting experimental data with a single exponential function. The \(t_{1/2}\) for activation in response to LSS was 5.3 ± 0.3 s (n = 8). Following channel activation, currents were fairly stable while LSS was maintained for period of 60 s. We did not observe adaptive behavior while LSS was maintained, as was described previously for other mechanosensitive channels (20). In some experiments, we observed current rundown that was present before and during stimulation by LSS. Oocytes expressing wild type ENaC were subjected to different amounts of shear stress to determine whether channels responded to increases in shear stress in a dose-dependent manner. Increasing rates of fluid jet perfusion (0 ml/min to 3.7 ml/min, corresponding to a LSS of...
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Increases in whole cell currents in response to shear stress 
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Mechanical stimuli may affect the trafficking of 
(Fig. 2B).

**Fig. 1. ENaC is activated by LSS.** A, current-voltage relationship of oocytes expressing aqby ENaC prior to fluid jet perfusion to generate LSS (closed circle), 1 min following initiation of fluid jet perfusion (open circle), 1 min following discontinuation of fluid jet perfusion (closed triangle), and following the application of 5 
benzamil in the bath (open triangle) (n = 15). B, representative tracings of shear stress-induced currents. Voltage steps (500 mV) from −140 to 60 mV in 20-mV increments were performed in oocytes expressing wild type ENaC as indicated. C, relative response to LSS of oocytes expressing wild type ENaC. Benzamil-sensitive currents recorded at −100 mV prior to initiation of LSS (before LSS), during stimulation by LSS (+LSS), and following recuperation (after LSS) were plotted as response relative to the mean of the currents recorded before stimulation (I_{0.60}) (n = 15, p < 0.05; +LSS versus before and after LSS, Kruskal-Wallis non parametric analysis of variance test). D, upper trace, whole cell current recorded from a non-injected oo-
cyte clamped at a holding potential of −90 
mV and subjected to stimulation by LSS as indicated by the horizontal bars (+LSS). The tracing is representative of four experiments. Lower trace, current re-
corded from an oocyte expressing wild type ENaC clamped at a holding potential of −60 mV. The black bar indicates the addition of benzamil (5 
M) to the bath solution to block currents mediated by ENaC. The black bar indicates the begin-
ning and duration of the vertical jet perfusion. Benzamil (5 
M) was also included in the jet perfusion pipette. Represen-
tative of seven experiments.

between −0 and 0.733 dynes/cm²) were associated with in-
creases in whole cell Na⁺ currents in a dose-dependent manner (Fig. 2B).

**LSS Regulates ENaC by Altering Gating Properties of the Channel**—Mechanical stimuli may affect the trafficking of membrane proteins as well as the gating properties of ion channels (8). To determine whether increases on whole cell Na⁺ currents in response to LSS occurred in association with increases in channel open probability, we expressed ENaCs with mutations within a gating domain localized to a region preceding the second membrane-spanning (pre-M2) domain of the channel that were previously shown to either increase or decrease channel open probability. αS580Cβγ channels have a low open probability. However, following covalent modification by the sulphydryl reactive reagent [2-(trimethylammonium)-ethyl]methanethiosulfonate bromide (MTSET), the open probability of this mutant approached a value of 1 (21). Oocytes expressing αS580Cβγ were subjected to stimulation by LSS. Increases in whole cell currents in response to shear stress (I_{+LSS}/I_{LSS} = 1.44 ± 0.12, n = 7) were similar in magnitude to that observed in oocytes expressing wild type ENaC (I_{+LSS}/

following treatment of oocytes expressing αS580Cβγ channels with MTSET (1 mM) to increase channel open probability, the modified channels were insensitive to the shear stress stimulus (Fig. 3, A and B, I_{+LSS}/I_{LSS} = 1.04 ± 0.08, n = 7, p < 0.05; −MTSET versus +MTSET). A loss of response to shear stress was also observed in oocytes expressing αS580Cβγ that were treated with MTSET (1 mM) prior to their initial exposure to shear stress (I_{+LSS}/I_{LSS} = 1.01 ± 0.04, n = 5). Previous studies identified a key site (degenerin site) in the pre-M2 domains of ENaC subunits, degenerins, and acid-sensing ion channels where the introduction of bulky residues or the introduction of a Cys residue and subsequent modification by MTS reagents results in channel activation (2, 22, 23). ENaCs with a Lys residue substitution at the degenerin site in the β-subunit (corresponding to mouse βS518K) were reported to have a high open probability (22). αβS518Kγ channels were insensitive to LSS (Fig. 3B; I_{+LSS}/I_{LSS} = 1.07 ± 0.06, n = 8). These data demonstrate that gain-of-function mutations or covalent mod-
ifications within a region that dramatically affect channel gating alters the channel’s response to mechanical stimulation.
To corroborate the proposition that increases in whole cell Na\(^+\) currents in response to LSS reflected increases in channel open probability rather than the insertion of channels from an intracellular pool into the plasma membrane, we expressed αS583Cβγ ENaC in oocytes. Covalent modification of this mutant by MTSET (1 mM) reduced whole cell benzamil-sensitive Na\(^+\) currents by >90% (\(I_{\text{LSS-MTSET}} = -5.13 \pm 0.60 \mu A\) and \(I_{\text{MTSET}} = -0.36 \pm 0.09 \mu A\), \(n = 6\)). If ENaC stimulation by LSS is due to insertion of channels from an intracellular pool, the increase in benzamil-sensitive Na\(^+\) currents in response to LSS in oocytes expressing this mutant before and following covalent modification by MTSET should be similar in magnitude. Oocytes expressing αS583Cβγ responded to LSS with an increase in benzamil-sensitive currents of 0.44 ± 0.04 µA (\(n = 6\)). In contrast, the absolute magnitude of the current response to LSS in oocytes modified by MTSET (1 mM) was dramatically reduced to 0.07 ± 0.10 µA (\(n = 6, p < 0.005\)) (see Table I). These data suggest that activation of ENaC by LSS is not due to the insertion of channels from an intracellular pool.

**Reduction in the Response to LSS following Repetitive Stimulation**—We examined whether ENaCs would respond to repetitive stimulation by LSS. Wild type channels responded to repetitive stimulation for periods of 60 s, alternating with 120-s periods of basal bath perfusion with increases in whole cell currents. However, the magnitude of the current increase in response to shear stress was reduced with repetitive stimulation (Fig. 4, A and D) in association with a variable decrease in baseline Na\(^+\) currents throughout the recording. The decrease in baseline Na\(^+\) currents may reflect channel down-regulation due to enhanced rates of Na\(^+\) entry with a constant holding potential of −60 mV. Increases in intracellular [Na\(^+\)] in oocytes expressing wild type ENaC has been shown previously to re-

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**Fig. 2. Kinetics and dose-dependent activation of ENaC by LSS.** A, activation of whole cell Na\(^+\) currents by LSS. ENaC-expressing oocytes were perfused with TEV solution, and a computer-controlled perfusion system was used to activate fluid jet perfusion via a vertical pipette as indicated by the horizontal bar (+LSS). Inset, increases in whole cell currents (bold solid line) in response to LSS were fit using an exponential function (gray line) as described under "Materials and Methods." B, ENaC activation in response to increasing rates of LSS. Whole cell currents were measured in ENaC-expressing oocytes perfused with TEV solution at a holding potential of −100 mV. Oocytes were subjected to fluid jet perfusion at varying rates to generate LSS for 1 min, and whole cell currents were recorded. The benzamil-insensitive component of the whole cell current was then determined by bath perfusion with a TEV solution containing benzamil (5 µM). The responses obtained for various rates of LSS relative to the maximal response to LSS (0.73 dynes/cm\(^2\)) for each batch of oocytes are shown. Experiments were performed with 14–32 oocytes from three separate batches. Each determination was performed with a single oocyte.

**Fig. 3. Constitutively active channels do not respond to LSS.** A, representative tracings of experiments performed with oocytes expressing αS580Cβγ. Currents were recorded under control conditions (−LSS) and following 1 min of stimulation by LSS (+LSS). Oocytes were subsequently perfused in the bath with TEV solution containing MTSET (1 mM) for 4 min. The thiol-reactive reagent was washed out by bath perfusion with TEV solution for 8 min. Currents were again recorded before and following 1 min of fluid jet perfusion. The benzamil-insensitive component of the whole cell current was then determined by bath perfusion with TEV solution containing benzamil (5 µM). B, relative responses to LSS of oocytes expressing αS580Cβγ and the degenerin mutant (αβS151Kγ). Currents recorded after 1 min of stimulation by LSS relative to currents recorded before stimulation are shown (\(I_{\text{LSS-MTSET}}\) versus \(I_{\text{LSS}}\)). Experiments using oocytes expressing αS580Cβγ were performed as described above. The relative response of oocytes expressing αS580Cβγ to shear stress was reduced with repetitive stimulation for periods of 60 s, alternating with 120-s periods of basal bath perfusion with increases in whole cell currents. However, the magnitude of the current increase in response to shear stress was reduced with repetitive stimulation (Fig. 4, A and D) in association with a variable decrease in baseline Na\(^+\) currents throughout the recording. The decrease in baseline Na\(^+\) currents may reflect channel down-regulation due to enhanced rates of Na\(^+\) entry with a constant holding potential of −60 mV. Increases in intracellular [Na\(^+\)] in oocytes expressing wild type ENaC has been shown previously to re-

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**Table I.** 

| Condition | Mean Current (µA) | SD | n |
|-----------|-----------------|----|---|
| Control   | 0.73            |    |   |
| +LSS      | 1.53            |    |   |
| +MTSET    | 0.36            |    |   |
| +MTSET + LSS | 0.07         |    |   |

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**Materials and Methods**

Wild type and degenerin (αβS151Kγ) ENaC-expressing oocytes were subjected to fluid jet perfusion at varying rates to generate LSS for 1 min, and whole cell currents were recorded. The benzamil-insensitive component of the whole cell current was then determined by bath perfusion with a TEV solution containing benzamil (5 µM). The responses obtained for various rates of LSS relative to the maximal response to LSS (0.73 dynes/cm\(^2\)) for each batch of oocytes are shown. Experiments were performed with 14–32 oocytes from three separate batches. Each determination was performed with a single oocyte.
duce amiloride-sensitive whole cell currents, a process referred to as feedback inhibition (or sodium-dependent down-regulation) that reflects a reduction in the number of channels in the membrane as well as a fall in channel open probability (24).

To examine this possibility, oocytes were maintained at a holding potential of −60 mV for either 20 or 560 s prior to stimulation by LSS. Prolonged (560 s) hyperpolarization of the membrane potential significantly reduced the channel’s relative response to LSS ($I_{\text{LSS}}/I_{\text{LSS}} = 1.56 \pm 0.08, n = 10$) when compared with a brief (20 s) hyperpolarization prior to LSS ($I_{\text{LSS}}/I_{\text{LSS}} = 2.29 \pm 0.27, n = 10, p < 0.05$; Mann-Whitney test). These data suggest that the relative response to LSS was dependent, at least in part, upon the length of time that whole cell currents were recorded prior to initiation of LSS. Feedback inhibition of ENaC is dependent on the presence of the C-terminal intracellular domains of the $\beta$ and $\gamma$ subunits and is mediated, in part, through interactions of ENaC with the ubiquitin-protein ligase Nedd4 (25).

Oocytes expressing Na$^+$ channels with truncations of the intracellular C-terminal domains of the $\alpha$, $\beta$, and $\gamma$ subunits responded to a single stimulation by shear stress with a relative increase in whole cell Na$^+$ currents that was similar in magnitude to that observed with wild type channels (see Fig. 4C). Furthermore, oocytes expressing channels with truncations of the intracellular C-terminal domains of the $\alpha$, $\beta$, and $\gamma$ subunits responded to repetitive stimulation by shear stress with relatively constant increases in whole cell Na$^+$ currents that did not decay over time (Fig. 4, B and D). These results suggest that intracellular $\alpha$, $\beta$, and $\gamma$ C termini contain elements that modify the channel’s response to repetitive stimulation by LSS. This may be related, in part, to feedback inhibition.

### DISCUSSION

Oocytes expressing $\alpha\beta\gamma$ mENaC exhibited reversible increases in whole cell Na$^+$ currents in response to LSS that were abolished by benzamil (5 $\mu$M). Whole cell Na$^+$ currents increased in response to increasing levels of shear stress in a

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**TABLE I**

|         | −LSS $I_{b_{\text{tot}}}^a$ | +LSS $I_{b_{\text{tot}}}^a$ | $\Delta I_{b_{\text{tot}}}^a$ |
|---------|-----------------------------|-----------------------------|-----------------------------|
| MTSET ($n = 6$)$^{\beta}$ | −5.13 ± 0.60                | −5.58 ± 0.61                | −0.45 ± 0.04                |
| +MTSET ($n = 6$)$^{\beta}$ | −0.36 ± 0.09                | −0.42 ± 0.09                | −0.06 ± 0.10                |

$^a$ Whole cell benzamil-sensitive Na$^+$ current.
$^\beta p < 0.005$; Mann-Whitney test.

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**FIG. 4. Adaptive response to repetitive mechanical stimulation.** A, representative tracing of an experiment performed in an oocyte expressing wild type ENaC and subjected to repetitive stimulation by LSS. The benzamil-insensitive component of whole cell current was subsequently determined by bath perfusion with TEV solution containing benzamil (5 $\mu$M). B, same protocol utilized in panel A was performed in an oocyte expressing $\alpha\delta\gamma$, $\beta\delta\gamma\tau$, and $\gamma\delta\gamma\tau$ (a$\tau$T$\tau$T) ENaC. C, relative responses to a single exposure of LSS of oocytes expressing $\alpha\beta\gamma$ or $\alpha\tau\beta\tau\gamma$T. Currents recorded at −100 mV after 1 min of stimulation by LSS relative to currents recorded before stimulation are shown ($I_{\text{LSS}}/I_{\text{LSS}}$) (n = 10−15, p = not significant). D, relative response of oocytes expressing wild type ENaC ($n = 16$) or $\alpha\tau\beta\tau\gamma$T ($n = 13$) to four repetitive periods of fluid jet perfusion and recuperation. The ratio of the whole cell current measured just prior to and 1 min following the initiation of fluid jet perfusion at a holding potential of −60 mV was determined for each stimulation. Data were normalized to the $I_{\text{LSS}}/I_{\text{LSS}}$ value obtained with the initial fluid jet perfusion. The relative response for oocytes expressing wild type channels (open circles; $n = 16$) at the second, third, and fourth stimulation were 68 ± 4, 53 ± 5, and 40 ± 5%, respectively. The relative response for oocytes expressing $\alpha\tau\beta\tau\gamma$T channels (closed squares; $n = 13$) were 98 ± 7, 82 ± 9, and 80 ± 12%, respectively. Significant differences in the relative response of oocytes expressing wild type ENaC were observed with the second, third, and fourth stimulation, compared with the response of oocytes expressing $\alpha\tau\beta\tau\gamma$T ($p < 0.01$; Mann-Whitney test).
dose-dependent manner. Oocytes expressing Na⁺ channels responded to LSS in a range of 0.013–0.733 dynes/cm² with increases in whole cell Na⁺ currents. Estimated tubular flow rates in rabbit collecting ducts are in the range of ~2 nL/min (26), and a recent study suggested that a rate flow of 5 nL/min established a LSS at the wall of the tubule of 0.52 dynes/cm² (27). Thus, the range of forces that were used in our study were similar in magnitude to forces that are exerted at the surface of collecting duct cells due to the laminar flow of tubular fluid at rates observed under normal physiologic conditions.

The properties of ENaC mechanosensitivity were further examined to determine potential mechanisms that permit the channel to respond to mechanical stimuli. Channels with specific mutations within a gating domain were used to determine whether ENaC activation in response to shear stress involved a change in channel gating. ENaCs with a high intrinsic open probability due to a mutation or covalent modification of an introduced Cys residue in the pre-M2 region were not activated by LSS, suggesting that shear stress increases whole cell Na⁺ currents by increasing channel open probability. Chemical modification of α583C/?γ channels by MTSET inhibits channels expressed at the plasma membrane. The subsequent response of MTSET-modified α583C/?γ channels to LSS was markedly blunted, suggesting that activation of whole cell Na⁺ currents by LSS is not primarily due to recruitment of Na⁺ channels from an intracellular pool.

Mechanosensitive channels expressed in mammalian cells respond to mechanical stimuli with latencies from a few millisecond to seconds (8). The t½ for activation of whole cell Na⁺ currents in response to LSS appears to be relatively slow (5.3 ± 0.3 s). This relatively slow response to mechanical stimuli reflects changes in the kinetics of ENaC gating that, under baseline conditions, exhibit a variable open probability with long open and closed times (13, 28). Interestingly, it was recently demonstrated that C. elegans responds to gentle touch stimulation with a time course of ~500 ms. The DEG/ENaC channel subunit MEC-4 and the stomatin homolog MEC-2 are required for this process. In this system, the MEC-4-dependent gentle touch mechanoreceptors responded primarily to motion rather than continuous pressure (29). Our data suggest that this ability to respond to motion is conserved in ENaC.

We observed that repetitive stimulation of ENaC-expressing oocytes by LSS was accompanied by a substantial reduction in the magnitude of the increase in whole cell Na⁺ currents. This was related, in part, to down-regulation of ENaC when oocytes were maintained at negative holding potential for long periods of time. Oocytes expressing ENaC subunits with carboxyl-terminal truncations showed a relatively constant response to repetitive stimulation by LSS, consistent with a proposed role of the carboxyl termini of ENaC subunits in the down-regulation of ENaC (24, 25). Our data suggest that ENaC intracellular C termini domains contain elements that affect the response to LSS.

Two molecular events have been proposed to describe changes in channel gating in response to mechanical stimuli, the bilayer model and the tethered model. The bilayer model proposes that tension develops in the membrane in response to a mechanical force that directly gates the channel, whereas the tethered model proposes that mechanical forces are transmitted directly to the channel through the cytoskeleton or extracellular tethers (8). Tethering of both the intracellular as well as the extracellular domains of the pore-forming subunits of mechanosensitive channels in C. elegans has been postulated to be required for mechanosensation (2, 6). Although our experiments suggest that ENaC is a mechanosensitive channel, mechanisms by which mechanical forces modulate ENaC gating have not been defined. Previous studies have suggested that ENaC is tethered to the cytoskeleton (30, 31). Although evidence does not exist for interactions between ENaC extracellular domains and the extracellular matrix or other extracellular proteins, approximately two-thirds of the mass of each Na⁺ channel subunit is present within the extracellular space. We postulate that ENaC extracellular domains may have a role in sensing mechanical forces such as LSS. Recent studies suggest that extracellular domains of ENaC may participate in the regulation of channel gating and Na⁺ self-inhibition (32–34). In addition, Coric et al. reported that a cluster of three residues in the extracellular domain of acid-sensing ion channel 1 determines desensitization kinetics (35). Shear stress may activate ENaC by affecting the structure of the extracellular domains, resulting in a change in channel gating. Alternatively, shear stress may directly affect the “gating” domain that precedes the selectivity filter and modulate channel activity.

In summary, ENaC is activated by LSS. Our results suggest that channel activation in response to shear stress is due to an increase in channel open probability rather than the insertion of channels from an intracellular pool into the plasma membrane. Channels are expressed in the apical membranes of specific epithelial structures such as renal collecting tubules, airway cells, and glandular ducts, where variations in rates of fluid flow and the associated changes in shear stress at the cell surface may modulate channel gating. Channel activation in response to LSS will result in enhanced rates of renal collecting duct Na⁺ transport and will likely contribute to the enhanced rates of renal K⁺ secretion that are observed in the setting of increased renal tubular flow rates (36).

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