SMALL MOLECULE ACTIVATOR OF THE HUMAN
EPITHELIAL SODIUM CHANNEL*

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Running Title: Identification of a Human ENaC Opener

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The epithelial sodium channel (ENaC), a heterotrimeric complex composed of α, β, and γ subunits, belongs to the ENaC/degenerin family of ion channels and forms the principal route for apical Na⁺ entry in many reabsorbing epithelia. Although high-affinity ENaC blockers, including amiloride and derivatives, have been described, potent and specific small molecule ENaC activators have not been reported. Here we describe compound S3969 that fully and reversibly activates human ENaC (hENaC) in an amiloride-sensitive and dose-dependent manner in heterologous cells. Mechanistically, S3969 increases hENaC open probability through interactions requiring the extracellular domain of the β subunit. hENaC activation by S3969 did not require cleavage by the furin protease, indicating that non-proteolyzed channels can be opened. Function of αβG37Sγ hENaC, a channel defective in gating that leads to the salt wasting disease pseudohypoaldosteronism type I, was rescued by S3969. Small molecule activation of hENaC may find application in alleviating human disease, including pseudohypoaldosteronism type I (PHA1) (6,7).

Conversely, potent and specific small molecule ENaC activators have not been previously described. Molecules reported to increase ENaC Na⁺ transport, such as glybenclamide, CPT-cAMP, capsazepine and icilin, all suffer from low efficacy, low specificity, and/or lack of effect on wild-type αβγ hENaC. Glybenclamide, an inhibitor of members of the ATP-binding cassette (ABC) superfamily including the cystic fibrosis transmembrane conductance regulator (CFTR) and the sulfonlurea receptor, increased amiloride-sensitive ENaC current by 50-100% (apparent concentration yielding 50% ENaC activation (EC₅₀) of 45 μM) in Xenopus oocytes and A6 kidney cells; glybenclamide activation of ENaC required the extracellular and transmembrane domains of the α subunit and doubled the open

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probability of individual channels (10,11). It is unclear if glybenclamide effects were indirect and attributable to primary regulation of ENaC activity. CPT-cAMP increased amiloride-sensitive current by ~300% (no EC_{50} determined) in *Xenopus* oocytes expressing chimeric ENaC channels composed of guinea pig α subunits in combination with rat βγ subunits; however, CPT-cAMP had no effect on wild-type, non-chimeric ENaC channels (12,13). Openers of δ ENaC, another sodium-selective pore-forming channel, have recently been reported. Capsazepine, a non-specific inhibitor of TRPV1, and icilin, an activator of TRPM8 temperature-sensitive non-selective cation channels, increased amiloride-sensitive current by 100-200% (apparent EC_{50} of 8 uM and 33 uM respectively) in *Xenopus* oocytes expressing δ or δβγ hENaC but not α or αδγ hENaC (14,15). In addition, agents that activate ENaC by removing Na+ self-inhibition, the rapid, transient increase in inward current followed by a decline to a lower steady state level after removal of amiloride or switch from a low to high extracellular Na+ solution, have been reported including Zn^{2+} ions, the mercurial agent p-chloro-mercuribenzoate, and trypsin-like as well as furin serine proteases (16-21).

This report describes the first compound, S3969, that fully and reversibly activates hENaC in heterologous cells. Mechanistically, S3969 requires the extracellular domain of the β subunit and increases hENaC open probability, thereby augmenting flux of Na^+ ions into cells. Accordingly, S3969 activated δβG37Sγ hENaC, a channel variant that exhibits reduced open probability and leads to the salt wasting disease PHA1. Promoting hENaC-dependent Na^+ and fluid transport across epithelia may find utility in modulating extracellular volume and electrolyte homeostasis in both normal and diseased states.

**EXPERIMENTAL PROCEDURES**

**Molecular Biology** – α, β, and γ hENaC were cloned from kidney cDNA (Origene, Rockville, MD) into pcDNA3 (Invitrogen, Carlsbad, CA) as described previously (22). δ hENaC was cloned from testis cDNA (BD Biosciences Clontech, Palo Alto, CA). Mouse α, β, and γ kidney ENaC (mENaC) clones were obtained from Thomas Kleyman (Univ. Pittsburgh) and subcloned into pcDNA3. βS520C hENaC (activated by MTSET), αR178A R181A R204A hENaC (furin variant), γR138A hENaC (furin variant), βG37S hENaC (PHA1 variant), and αR508X hENaC (PHA1 variant) were generated using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) following the manufacturer’s instructions. To ensure that no errors occurred during mutagenesis, all constructs were sequenced and subcloned into new pcDNA3 vector backbones that did not go through the QuikChange process.

β hENaC and β mENaC chimeras were constructed using overlapping PCR with Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA) to amplify individual fragments and PCR SuperMix High Fidelity (Invitrogen) to amplify overlapping products. Chimeras, summarized in Table 1, were sequenced to confirm desired junctions and the absence of other mutations introduced during amplification.

**In Vitro Transcription** – ENaC cRNA was generated from linearized plasmids using the mMESSAGE mMACHINE kit with T7 RNA polymerase according to the manufacturer’s instructions (Ambion, Austin, TX). cRNA quality was checked by denaturing agarose gel electrophoresis and spectrophotometric absorbance readings at 260 and 280 nm to ensure that full-length, non-degraded cRNA was generated.

**Frog Surgery, Oocyte Isolation, and Microinjection** - Female Xenopus laevis South African clawed frogs greater than or equal to 9 cm in length were obtained from NASCO (Fort Atkinson, WI). Frogs were anesthetized in 0.15% ethyl-3-aminobenzoate methanesulfonate (Sigma, St. Louis, MO) in distilled water and placed on ice. Using sterile surgical tools, sequential 1-2 cm incisions were made in the abdomen through both the outer skin layer and the inner peritoneal layer to reveal the ovaries. Excised ovarian lobes (containing 1000-2000 oocytes) were placed in OR-2 calcium-free media (82.5 mM NaCl, 2 mM KCl, 1 mM MgCl_2, 5 mM HEPES pH 7.5 with NaOH) and sequentially digested with 2 mg/ml collagenase type IA (Sigma), prepared...
immediately before use, for 45 min followed by 1 mg/ml collagenase type IA for 15 min on a rocking platform at room temperature. After enzymatic digestion, at which point the majority of oocytes are released from the ovarian lobes, oocytes were thoroughly rinsed in OR-2 without collagenase and transferred to a Petri dish containing Barth’s saline (88 mM NaCl, 2 mM KCl, 0.82 mM MgSO4, 0.33 mM Ca(NO3)2, 0.41 mM CaCl2, 2.4 mM NaHCO3, and 5 mM HEPES pH 7.5; Specialty Media, Phillipsburg, NJ) supplemented with 2.5 mM sodium pyruvate. Mature stage V or VI oocytes (~1 mm diameter) were selected for microinjection. Frog skin was closed using a C6 needle with a 3-0 black braid suture (Harvard Apparatus, Holliston, MA) and frogs were reused for subsequent oocyte isolations following a 2-3 month recovery period. Oocytes were microinjected in the animal pole with 10-15 nl containing 1 ng of each ENaC subunit cRNA. Following microinjection, oocytes were incubated in Barth’s solution supplemented with 2.5 mM sodium pyruvate at 18 °C overnight.

Two Electrode Voltage Clamping – ENaC function was measured using the two-electrode voltage clamp technique on an OpusXpress 6000A parallel oocyte voltage clamp system (Axon Instruments, Union City, CA) twenty-four hours post-microinjection unless noted otherwise. The OpusXpress system is an integrated workstation that allows electrophysiological recordings to be made from up to 8 oocytes simultaneously. This system has previously been used to examine the function of ion channels including nicotinic acetylcholine and serotonin 5HT3 receptors as well as CFTR (23-25). Oocyte impalement is automated and compound delivery is performed by a computer-controlled fluid handler; compounds are removed from 96-well plates using disposable pipet tips and applied to individual oocytes. Oocytes were placed in the OpusXpress system and perfused with ND-96 solution (96 mM NaCl, 2.5 mM KCl, 1 mM CaCl2, 1 mM MgCl2, and 5 mM HEPES pH 7.4 with NaOH). Oocytes were then impaled with voltage-sensing and current-passing electrodes back-filled with 3M KCl. Electrodes exhibited resistances between 2-10 Mohm for voltage-sensing electrodes and between 0.5-2 Mohm for current-passing electrodes. Following impalement, oocytes were voltage clamped to −60 mV and experimental recordings were initiated. Data were acquired at 50 Hz and low-pass filtered at 5 Hz.

Cell Culture and Transfection – HEK293 cells were maintained in high glucose Dulbecco’s modified Eagle’s medium with glutamine (DMEM; Invitrogen) containing 10% bovine calf serum (Hyclone, Logan, UT) and penicillin/streptomycin (Invitrogen) in 6% CO2-balanced air at 37 °C. CHO cells were maintained as above in Kaighn’s modified FK12 Nutrient Mixture (Invitrogen) containing 10% bovine calf serum and penicillin/streptomycin. Cells were subcultured by trypsinization (0.25% trypsin, 1 mM EDTA in Hanks’ balanced salt solution; Invitrogen) and seeded on No. 1 glass coverslips (Warner Instrument Corp., Hamden, CT) coated with growth-factor reduced Matrigel (BD Biosciences, Bedford, MA) for CHO cells. Transient transfection was carried out using the TransIT-293 and TransIT-CHO transfection reagents (Mirus Bio Corporation, Madison, WI) as per the manufacturer’s instructions. HEK-293 cells were transfected with 0.45 μg each of alpha, beta, and gamma hENaC in pcDNA3, 0.38 μg plasmid for green fluorescent protein and 5.2 ul TransIT-293 transfection reagent. CHO cells were transfected with 0.3 μg each of alpha, beta, and gamma hENaC in pcDNA3, 0.25 μg plasmid for green fluorescent protein, 0.85 μg pUC18 carrier DNA, 4 μl TransIT-CHO transfection reagent, and 1 μl CHO Mojo reagent. Green fluorescent protein served as a marker to identify cells expressing hENaC for electrophysiology experiments.

Whole-Cell Patch Clamp Electrophysiology - Whole cell currents were acquired from cells 24-72 hours post-transfection at room temperature (21-24 °C) using an Axopatch200B amplifier and DIGIDATA 1322A with pCLAMP9.2 software (MDS Analytical Technologies). Currents were sampled at 2 kHz and filtered at 1 kHz. Patch pipettes were pulled from borosilicate glass and had resistances of 3-6 Mohm. The pipette solution contained (in mM): 105 CsF, 35 NaCl, 10 EGTA, 2 Na-ATP and 10 HEPES (pH 7.4). The bath solution contained (in mM): 150 NaCl, 2 KCl, 1.5 CaCl2, 1 MgCl2 and
10 HEPES (pH 7.4). After establishment of the whole-cell configuration, currents were measured at a holding potential of -80 mV.

**Single Channel Patch Clamp Electrophysiology** – Single channel currents were measured at room temperature in oocytes using the outside-out patch clamp configuration. Oocytes were incubated for 5-10 min in ND-96 supplemented with 100 mM NaCl and the vitelline membrane was peeled away using fine forceps. Single channel currents were recorded using an Axopatch200B amplifier and Digidata 1322A as with whole-cell recordings. Currents were acquired at 4 kHz and low pass filtered at 1 kHz. Patch pipettes were pulled from borosilicate glass and fire-polished and had resistances of 8-12 Mohm. Patches were voltage clamped to a pipette potential of -80 mV. Bath solution contained 115 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl2, and 10 mM HEPES pH 7.5. Pipette solution contained 88 mM KCl, 9.6 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, 11 mM EGTA, 2 mM Na-ATP, and 5 mM HEPES pH 7.5. For data analysis and presentation, currents were subsequently filtered at 200 Hz in pCLAMP10.2. NP0 was determined over 1-2 min intervals from idealized traces using the single channel search feature. Similar values were obtained by fitting a Gaussian to an all-points current amplitude histogram. Open probability (P0) was estimated by dividing NP0 by the number of channels in the patch.

**Compounds** – Amiloride and trypsin (salt-free, lyophilized powder, minimum 10,000 BAEE units/mg protein, TPCK treated) were from Sigma. MTSET was from Toronto Research Chemicals (North York, Ontario) and Biotium (Hayward, CA). S3969 was diluted to appropriate concentrations in ND-96 from 100 mM stock solutions in DMSO. The final concentration of DMSO in experiments was < 0.1%; this level of vehicle had no effect on ENaC function. Statistics and Measurements - Data represent the mean +/- SEM. Unless otherwise noted, experiments were performed on two to four batches of independently injected oocytes harvested from different frogs. Statistical significance between different groups was determined using an unpaired, two-tailed Student’s t-test. Dose-response curves were plotted and both EC50 values and Hill coefficients were determined using GraphPad Prism v3.02 (GraphPad Software, San Diego, CA). Values for percent hENaC activation were calculated by re-dissolving in boiling water, treated with decolorizing carbon and filtered using filter paper. The solution was cooled to room temperature and was stored at 4°C overnight. The resulting pale yellow crystals were collected by filtration, washed with cold water (2 x 25 ml) and dried under vacuum to give a 69% yield of 4-methyl-1H-indol-3-yl carbamimidothioate hydroiodide. This product (1.0 g, 3.0 mmol) was mixed with 2 ml 1.5 N NaOH, heated to 90°C and stirred for 30 min. 2-Bromo-4-methylpentanoic acid (0.6g, 3.1 mmol) was added and the reaction mixture stirred for 2 hr. The solution was cooled to room temperature, acidified to pH 2 with 1 N HCl and extracted with ethyl acetate (3 x 10 ml). The combined organic layers were dried with sodium sulfate, filtered and concentrated under reduced pressure. Purification by flash chromatography (dichloromethane/ 5% MeOH) afforded 4-methyl-2-(4-methyl-1H-indol-3-y1thio)pentanoic acid in 75% yield as a colorless oil. This product (305.1 mg, 1.1 mmol) in dimethylformamide (4 ml) was combined with N-hydroxybenzotriazole (135.1 mg, 1.0 mmol), 1-ethyl-3-(3’-dimethylaminopropyl)carbodiimide hydrochloride, (211.0mg, 1.1 mmol), and 2-aminoethanol (61 ul, 1.0 mmol). The reaction mixture was stirred at room temperature for 16 hr, quenched with 1 ml of water and extracted with dichloromethane (3 x 10 ml) and water. The combined organic layers were collected, dried with sodium sulfate and concentrated under reduced pressure. Purification by preparative thin layer chromatography (dichloromethane/MeOH) afforded S3969 in 67% yield as an off white powder. The identities of intermediate reaction products were confirmed by 1H NMR and LCMS, and the identity of S3969 was confirmed by 1H NMR, 13C NMR, LCMS and elemental analysis.

**Synthesis of S3969** - A potassium iodide-iodine solution, prepared by mixing iodine (9.7 g, 38.2 mmol in 75 ml water) with potassium iodide (19.0 g, 114.5 mmol in 225 ml water), was added dropwise to a solution of 4-methylindole (5.0 g, 38.1 mmol) and thiourea (2.9 g, 38.1 mmol) in methanol (80 ml). The reaction mixture was stirred at room temperature for 2 hr and concentrated to dryness by rotary evaporation. The crude solid was redissolved in boiling water, treated with decolorizing carbon and filtered using filter paper. The solution was cooled to room temperature and was stored at 4°C overnight. The resulting pale yellow crystals were collected by filtration, washed with cold water (2 x 25 ml) and dried under vacuum to give a 69% yield of 4-methyl-1H-indol-3-yl carbamimidothioate hydroiodide. This product (1.0 g, 3.0 mmol) was mixed with 2 ml 1.5 N NaOH, heated to 90°C and stirred for 30 min. 2-Bromo-4-methylpentanoic acid (0.6g, 3.1 mmol) was added and the reaction mixture stirred for 2 hr. The solution was cooled to room temperature, acidified to pH 2 with 1 N HCl and extracted with ethyl acetate (3 x 10 ml). The combined organic layers were dried with sodium sulfate, filtered and concentrated under reduced pressure. Purification by flash chromatography (dichloromethane/ 5% MeOH) afforded 4-methyl-2-(4-methyl-1H-indol-3-y1thio)pentanoic acid in 75% yield as a colorless oil. This product (305.1 mg, 1.1 mmol) in dimethylformamide (4 ml) was combined with N-hydroxybenzotriazole (135.1 mg, 1.0 mmol), 1-ethyl-3-(3’-dimethylaminopropyl)carbodiimide hydrochloride, (211.0mg, 1.1 mmol), and 2-aminoethanol (61 ul, 1.0 mmol). The reaction mixture was stirred at room temperature for 16 hr, quenched with 1 ml of water and extracted with dichloromethane (3 x 10 ml) and water. The combined organic layers were collected, dried with sodium sulfate and concentrated under reduced pressure. Purification by preparative thin layer chromatography (dichloromethane/MeOH) afforded S3969 in 67% yield as an off white powder. The identities of intermediate reaction products were confirmed by 1H NMR and LCMS, and the identity of S3969 was confirmed by 1H NMR, 13C NMR, LCMS and elemental analysis.
dividing the magnitude of the inward current induced by S3969 by the magnitude of the inward current blocked by amiloride in the same oocyte and multiplying the ratio by 100%. The activity of S3969 was expressed as a percentage of the paired amiloride response due to variability in hENaC expression between different batches of oocytes. Amiloride was used at 1 uM for αβγ hENaC and 10 uM for δβγ hENaC in oocytes; these concentrations yield greater than 90% hENaC inhibition. Similar percent hENaC activation ratios were obtained in oocytes expressing amiloride-sensitive currents ranging from 0.1 to 1.0 uA that were typically observed in these studies. For whole cell patch clamp experiments, mammalian cells with amiloride-sensitive inward currents less than 1 nA were studied. Cells with amiloride-sensitive currents greater than 1 nA, suggestive of channels opened by proteolysis, responded poorly to S3969.

RESULTS

Identification of a Small Molecule Opener of αβγ hENaC – A high-throughput screen was conducted using a fluorescent membrane potential dye to identify novel compounds that open hENaC channels expressed in heterologous cells. When a compound opens hENaC, sodium flows into the cell resulting in cell depolarization and an increase in dye fluorescence. This report describes in detail one of the more potent compounds, termed S3969 (Fig. 1A). Chemistry optimization and structure activity relationships of S3969 functional groups will be described elsewhere. Treatment of oocytes expressing αβγ hENaC with 1 uM S3969 activated hENaC by 300.1 +/- 12.2% (n=85 determinations on separate oocyte batches using three independent synthetic preparations of S3969) and the time required for S3969 to achieve half-maximal hENaC activation was 33.1 +/- 1.6 sec (Fig. 1B). The maximal stimulatory effect of S3969 was reached ~3 min following compound application, and currents returned to baseline following compound washout (Fig. 1B), suggesting that interaction of S3969 with hENaC was non-covalent. Repeated application of S3969 did not result in hENaC desensitization; the ratio of hENaC activation from two successive S3969 applications was 95.0 +/- 3.0% (n=11). Amiloride inhibited current activation by S3969 demonstrating that S3969 effects were hENaC-dependent (Fig. 1C). S3969 failed to activate inward currents in sodium-free media, indicating that S3969 responses in sodium-replete media were attributable to sodium influx (Fig. 1D). In sodium-free media, small outward currents, attributable to sodium efflux from oocytes, were observed; the S3969-activated outward current in sodium-free solutions was 25.5 +/- 5.8% (n=11) of the inward amiloride-sensitive current in sodium-replete media (Fig. 1D). Dose-response experiments, performed to determine the potency of S3969, revealed that the threshold for S3969 hENaC activation was ~30 nM and maximal hENaC activation was achieved at ~30 uM (Fig. 1E). The apparent EC50 for S3969 activation of hENaC was 1.2 +/- 0.1 uM (n=46 determinations on separate oocyte preparations using three independent synthetic preparations of S3969) with a Hill coefficient of 1.0 +/- 0.1 (Fig. 1F). Current-voltage (I-V) curves from hENaC expressing oocytes were linear and S3969 increased amiloride-sensitive hENaC currents at both negative and positive holding potentials (Fig. 1G). By contrast, S3969 had no effect on currents at any holding potential in uninjected oocytes, illustrating that S3969-induced currents required hENaC expression (Fig. 1H). Treatment of HEK293 or CHO cells transiently expressing αβγ hENaC with 10 uM S3969, applied by bath perfusion, activated hENaC by 357.2 +/- 82.4% (n=24) and 148 +/- 50.7% (n=10) respectively (Fig. 1I-J), indicating that S3969 enhances hENaC in both amphibian and mammalian expression systems. The time required for S3969 to achieve half-maximal hENaC activation was 18.9 +/- 1.8 sec in HEK293 cells and 20.0 +/- 2.3 sec in CHO cells, qualitatively similar to the kinetics of hENaC activation by S3969 in oocytes. Amiloride inhibited current activation by S3969 (Fig. 1J) and S3969 did not affect currents in mock-transfected cells (data not shown), demonstrating that S3969 effects were hENaC-dependent. These results demonstrate that S3969 is a novel, potent, and reversible activator of hENaC.

S3969 Activates hENaC Following Removal of Amiloride– Experiments were next performed to begin to elucidate the mechanism of hENaC activation by S3969. Application of
Amiloride only (1 uM; Fig. 2A, treatment a) inhibited hENaC and application of S3969 only (1 uM; Fig. 2A, treatment b) activated hENaC. Co-application of 1 uM amiloride (a dose that yields greater than 90% hENaC inhibition) and 1 uM S3969 (a dose that yields ~50% maximal hENaC activation), inhibited hENaC (Fig. 2A, treatment c) similar to subsequent application of 1 uM amiloride alone (Fig. 2A, treatment d). Under these conditions, the effect of the hENaC blocker amiloride was dominant to the effect of the hENaC activator S3969. Following washout of amiloride and S3969 (Fig. 2A, immediately after treatment c), current rapidly increased to levels observed when oocytes were treated with S3969 alone (Fig. 2A, treatment b) before returning to near control levels. These results suggest that amiloride dissociates from hENaC faster than S3969, resulting in immediate, albeit transient, hENaC activation following removal of both compounds.

Amiloride is thought to occlude the pore of αENaC subunits in an open conformation; thus, amiloride functions as an open-state blocker (27). Experiments were performed to synchronize hENaC in the open state prior to stimulation with S3969. Oocytes were first treated with amiloride to prime hENaC in the open but occluded state (Fig. 2B, treatment b). Next, oocytes were treated with S3969 in the continued presence of amiloride (Fig. 2B, overlap of treatments b and c); no activation of hENaC was observed, consistent with data in Fig. 2A. Removal of amiloride, in the continued presence of S3969, resulted in an immediate and sustained activation of hENaC (Fig. 2B, treatment c), similar in magnitude to current activation with S3969 alone (Fig. 2B, treatment d). Quantitation of these data revealed no difference in hENaC activation when oocytes were treated with S3969 before or after amiloride priming (Fig. 2C). However, amiloride priming significantly decreased the apparent time required for S3969 to achieve half-maximal hENaC activation by a factor of 7 (from ~35 seconds to ~5 seconds; Fig. 2D). Taken together, these data indicate that S3969 activates ENaC following removal of amiloride.

**S3969 Increases hENaC Open Probability**

Macroscopic hENaC current is dependent on the number, single channel current, and open probability of functional hENaC channels in the plasma membrane. S3969 could activate hENaC by increasing one or more of these parameters. The immediate stimulatory effect of S3969 on hENaC function following amiloride priming (Fig. 2B) is temporally inconsistent with a mechanism whereby S3969 increases the number of hENaC channels in the plasma membrane by promoting vesicular trafficking of hENaC from an intracellular pool to the cell surface; agonist-stimulated trafficking of ion channels, including ENaC, to the plasma membrane in oocytes requires tens of minutes or more (28,29).

To determine if S3969 promotes hENaC function by increasing single channel current or open probability, experiments were performed with αβS520Cγ hENaC and the sulfhydryl reagent MTSET. Substitution of serine by cysteine at the degenerin site near the extracellular end of the second transmembrane domain of β hENaC, or at the equivalent site in rodent ENaC, generates a channel that can be covalently modified by MTSET; MTSET selectively reacts with cysteine 520 in the β subunit of conducting αβS520Cγ hENaC and locks channels in a constitutively open conformation (open probability 0.96-1.0) (30,31). Accordingly, S3969 should not further activate αβS520Cγ hENaC following MTSET treatment if S3969 functions to increase hENaC open probability; conversely, S3969 should activate αβS520Cγ hENaC following MTSET treatment if S3969 functions to increase hENaC single channel current. This rationale has been used to determine the mechanism of activation of rat ENaC by the serum- and glucocorticoid-inducible kinase SGK1 (32).

αβS520Cγ hENaC exhibited similar amiloride-sensitive currents and levels of S3969 activation compared to wild-type αβγ hENaC, demonstrating that the βS520C substitution did not interfere with hENaC activation by S3969. Amiloride (1 uM)-sensitive currents were 315 +/- 49 nA for wild-type αβγ hENaC and 285 +/- 32 nA for αβSS20Cγ hENaC (n=9; p>0.05). ENaC activation by S3969 (1 uM) was 369 +/- 38% for wild-type αβγ hENaC (n=7) and 352 +/- 32% for αβSS20Cγ hENaC (n=4; p>0.05). Treatment of oocytes expressing αβS520Cγ hENaC with MTSET induced an amiloride-sensitive inward current that persisted following MTSET washout (Fig. 3A), indicative of covalent hENaC.
identified as the endogenous oocyte Ca\(^{2+}\)-
activation of a transient inward current, formerly
Fig. 3
increase hENaC open probability. As shown in
following trypsin treatment if S3969 functions to
Accordingly, S3969 should not activate hENaC
stabilizes channels in an open conformation. S3969 can
maximize open probability of wild-type hENaC
through non-covalent interactions.

To independently determine if S3969 increases the open probability of wild-type hENaC channels, experiments were performed with the serine protease trypsin. Trypsin irreversibly increases macroscopic ENaC current by increasing the open probability of near electrically silent plasma membrane channels (18-20). These channels are incorporated in the plasma membrane but reside predominantly in the closed state. Similar to MTSET treatment of oocytes expressing αβS520Cγ hENaC, trypsin treatment of oocytes expressing wild-type ENaC stabilizes channels in an open conformation. Accordingly, S3969 should not activate hENaC following trypsin treatment if S3969 functions to increase hENaC open probability. As shown in Fig. 3C, trypsin treatment resulted in rapid activation of a transient inward current, formerly identified as the endogenous oocyte Ca\(^{2+}\)-activated Cl\(^{-}\) current (33), followed by slow activation of an amiloride-sensitive inward hENaC current, as described previously (19). Trypsin treatment increased macroscopic hENaC current more than 5-fold (Fig. 3D). S3969 failed to activate hENaC following trypsin application (Fig. 3E). Amiloride-sensitive currents and levels of hENaC activation were similar before and after co-treatment of oocytes with trypsin plus soybean trypsin inhibitor, indicating that trypsin effects were attributable to protease function. These data indicate that locking hENaC channels in a constitutively conducting state with trypsin blocks hENaC activation by S3969 and confirm that S3969 functions to increase hENaC open probability.

To validate that S3969 increases hENaC open probability, single channel patch clamp experiments were performed using the outside-out configuration in oocytes. Application of hENaC activator (30 uM) to the bath, contacting the extracellular patch surface, increased open probability (Fig. 3F-G) but did not affect single channel conductance. Open probability was 0.2 +/- 0.1 before and 0.6 +/- 0.1 after compound (p<0.05), while single channel conductance was 4.8 +/- 0.3 pS before and 5.1 +/- 0.2 pS after compound (p>0.05, n=3). Collectively, macroscopic and single channel recordings indicate that S3969 activates hENaC by increasing open probability and not by increasing single channel current or the number of channels at the plasma membrane.

S3969 hENaC Activation Requires α, β, and γ Subunit Coexpression – Studies were conducted to determine the subunit requirements for hENaC activation by S3969. To this end, all possible combinations of α, β, and γ hENaC were expressed and currents blocked by amiloride and activated by S3969 were measured. As shown in Fig. 4A and consistent with previous reports on human and rodent ENaC (34,35), α hENaC, αβ hENaC, or αγ hENaC generated significantly smaller amiloride-sensitive currents compared to αβγ hENaC, whereas β hENaC, γ hENaC, or βγ hENaC failed to generate any detectable amiloride-sensitive currents. The only subunit combination activated by S3969 was αβγ hENaC, demonstrating that expression of all three channel components is required for the stimulatory effect of S3969 on hENaC function (Fig. 4B).

Activation of δβγ hENaC but not αβγ mENaC by S6969 – Experiments were performed using additional hENaC as well as mENaC channels to identify which hENaC subunit(s) were essential for activation by S3969. To this end, the effects of S3969 on δβγ hENaC and αβγ mENaC function were investigated. Similar to α hENaC, δ hENaC can form functional amiloride-sensitive channels when expressed alone or in combination
with \( \beta \gamma \) hENaC; however \( \delta \beta \gamma \) hENaC is more than an order of magnitude less sensitive to amiloride compared to \( \alpha \beta \gamma \) hENaC (36,37). Expression of \( \delta \beta \gamma \) hENaC and \( \alpha \beta \gamma \) mENaC generated amiloride-sensitive currents; the IC\(_{50}\) for amiloride inhibition of \( \delta \beta \gamma \) hENaC was 2.7 +/- 0.3 \( \mu \)M (n=10), similar to previous reports (36,37), and much larger than the IC\(_{50}\) for amiloride inhibition of \( \alpha \beta \gamma \) hENaC (110 +/- 11 nM; n=16) or \( \alpha \beta \gamma \) mENaC (96 +/- 16 nM; n=3) (Fig. 5A).Activation of \( \delta \beta \gamma \) hENaC by S3969 was similar to \( \alpha \beta \gamma \) hENaC (Fig. 5B); the EC\(_{50}\) for S3969 activation of \( \delta \beta \gamma \) hENaC was 1.2 +/- 0.2 \( \mu \)M (n=11) compared to the EC\(_{50}\) for S3969 activation of \( \alpha \beta \gamma \) hENaC of 1.2 +/- 0.1 \( \mu \)M (n=46). Similar results were obtained with \( \alpha \beta \gamma \) hENaC, a splice variant of \( \alpha \) hENaC with 59 additional N-terminal amino acids (38); potencies of amiloride (IC\(_{50}\) 156 +/- 25 nM; n=3) and S3969 (EC\(_{50}\) 1.2 +/- 0.5 \( \mu \)M; n=3) on \( \alpha \beta \gamma \) hENaC were not significantly different from \( \delta \beta \gamma \) hENaC. In addition, the potencies of amiloride (IC\(_{50}\) 2.1 +/- 0.2 \( \mu \)M; n=3) and S3969 (EC\(_{50}\) 0.4 +/- 0.1 \( \mu \)M; n=3) on \( \delta \beta \gamma \) hENaC were comparable to \( \delta \beta \gamma \) hENaC; \( \delta \) hENaC is a splice variant of \( \delta \) hENaC with a N-terminal extension due to an alternative transcriptional start site and inclusion of an alternative exon (39,40). Conversely, S3969 did not activate \( \alpha \beta \gamma \) mENaC at concentrations yielding maximal activation of \( \alpha \beta \gamma \) hENaC or \( \delta \beta \gamma \) hENaC (Fig. 5B). Weak \( \alpha \beta \gamma \) mENaC activation was observed at high concentrations of S3969 (100-300 \( \mu \)M); however, dose-response curves did not reach saturation and an EC\(_{50}\) value could not be determined. Taken together, S3969 efficiently activated \( \alpha \beta \gamma \) hENaC and \( \delta \beta \gamma \) hENaC, including reported splice variants, but not \( \delta \beta \gamma \) mENaC.

The \( \beta \) Subunit is Critical for S3969 Activation of hENaC - Differential activation of hENaC and mENaC by S3969 prompted subunit mixing experiments to determine which specific channel subunit(s) were important for the S3969 response. To this end, all possible heterotrimeric combinations of mENaC and hENaC subunits were co-expressed and both amiloride-sensitive currents and activation by S3969 were measured. Subunit mixing experiments have been performed to examine ENaC function from different species (10,12,34). All human-mouse hybrid channels generated amiloride-sensitive currents, indicating the production of functional channels at the plasma membrane (Fig. 6A). S3969 activated heterotrimeric hybrids containing \( \beta \) hENaC but not \( \beta \) mENaC (Fig. 6B). Most importantly, \( \alpha \beta \delta \gamma \) channels (\( \alpha \) and \( \gamma \) subunits from human and \( \beta \) subunit from mouse) lost the ability to respond to S3969, whereas \( \alpha \beta \delta \gamma \) channels (\( \alpha \) and \( \gamma \) subunits from mouse and \( \beta \) subunit from human) gained the ability to respond to S3969 (Fig. 6B). Collectively, these results demonstrate that the human \( \beta \) subunit is critical for S3969 activation of hENaC.

S3969 Maps to the Extracellular Domain of \( \beta \) hENaC – ENaC subunits have two transmembrane domains, intracellular amino- and carboxy-termiini, and a large extracellular loop comprising ~75% of protein sequence. Alignment of human and mouse \( \beta \)ENaC protein sequences revealed that the two subunits exhibit 84% identity. Because most of the amino acid differences between human and mouse \( \beta \)ENaC localize to the large extracellular domain and because the effect of S3969 on hENaC function is rapidly reversible following compound washout (Fig. 1B), we hypothesized that S3969 required the extracellular domain of \( \beta \) hENaC to activate channel function. To test this hypothesis, both amiloride-sensitive currents and S3969 activation of heterotrimeric ENaC channels, consisting of \( \alpha \) and \( \gamma \) mENaC subunits with \( \beta \) subunit chimeras comprised of intracellular, transmembrane, and extracellular domains derived from \( \beta \) mENaC or \( \beta \) hENaC, were measured. ENaC subunit chimera experiments have been performed to examine the functional contribution of individual domains to channel biology (11,13,36). All chimeras generated amiloride-sensitive currents, indicating the production of functional channels (Fig. 6C). S3969 activated chimeras containing the extracellular loop of \( \beta \) hENaC (chimeras M-H and M-H-H) but not the extracellular loop of \( \beta \) mENaC (chimeras H-M and H-M-H) (Fig. 6D). A \( \beta \) hENaC splice variant lacking valine 348 in the extracellular loop, due to use of an alternative splice acceptor site, was identified in tongue tissue. Expression of hENaC lacking valine 348 in the \( \beta \) extracellular loop generated amiloride-sensitive channels that were not activated by S3969 (Fig. 6C, D). Therefore, S3969 opening of

\[ \text{Equation} \]
hENaC requires the β hENaC extracellular loop, especially valine 348 within this region, and is not critically dependent upon the β hENaC transmembrane and cytosolic domains.

**S3969 Opens ENaC Channels Not Cleaved by Furin Proteolysis** – Transport of sodium by ENaC requires proteolytic cleavage of alpha and gamma subunits in the extracellular loop distal to the first transmembrane domain by the serine endoprotease furin (41). Furin cleaves ENaC subunits, expressed in mammalian cells or oocytes, at the C-terminal side of conserved dibasic RXXR motifs (where R represents arginine and X represents any amino acid) (41,42). Thus, experiments were performed to determine if S3969 activation of hENaC requires channel proteolysis by furin. To this end, αR178A R181A R204AβγR138A hENaC, a channel in which all potential arginine residues targeted by furin proteolysis in alpha and gamma subunits were substituted with alanine, was generated. Homologous substitutions made in mENaC abolish furin proteolysis and dramatically decrease sodium transport in oocytes (41,43).

Expression of αR178A R181A R204AβγR138A hENaC generated small amiloride-sensitive currents compared to wild-type αβγ hENaC (Fig. 7A and 7B), consistent with inhibition of channel activation by furin proteolysis. Following stimulation with S3969, currents with αR178A R181A R204AβγR138A hENaC were equal in magnitude to wild-type αβγ hENaC (Fig. 7A and 7C). Since αR178A R181A R204AβγR138A hENaC exhibits small basal amiloride currents, S3969 induced larger fold activation changes compared to wild-type αβγ hENaC (Fig. 7D). Taken together, these data indicate that S3969 activation of hENaC does not require channel proteolysis by furin.

**S3969 Activates PHA1 ENaC Channels** - Loss of function hENaC mutations result in the salt-wasting genetic disease PHA1 (6,7). Clinical symptoms include hyponatremia, hyperkalemia, dehydration, elevated serum aldosterone, and mineralocorticoid unresponsiveness, and current treatments include sodium supplementation as well as dialysis to reduce elevated serum potassium (44). Experiments were performed to determine if S3969 activates two PHA1 hENaC channels previously characterized in the Xenopus oocyte expression system. αβG37Sγ hENaC has a glycine to serine substitution at amino acid 37 in the N-terminal intracellular domain and decreases open probability without affecting channel assembly or surface expression (6,7). αR508Xβγ hENaC has an arginine to stop codon substitution at amino acid 508 in the extracellular loop before the second transmembrane domain and decreases expression at the plasma membrane (7,45,46). Both PHA1 variants and wild-type ENaC exhibit the same affinity for amiloride (6,46).

Expression of αβG37Sγ hENaC and αR508Xβγ hENaC generated amiloride-sensitive currents that were ~20% of wild-type αβγ hENaC levels (Fig. 8A and 8B), consistent with reduced macroscopic currents reported previously (6,7,45,46). αβG37Sγ hENaC was strongly activated by S3969 (Fig. 8A). Following stimulation with S3969 (10 uM), currents with αβG37Sγ hENaC were greater than half of wild-type hENaC levels (Fig. 8C) and nearly two-fold wild-type hENaC amiloride-sensitive basal levels, indicating substantial correction of αβG37Sγ hENaC function. Since αβG37Sγ hENaC exhibits reduced open probability, S3969 induced larger fold activation changes compared to wild-type αβγ hENaC (Fig. 8D). The time required for S3969 to achieve half-maximal activation of αβG37Sγ hENaC (50.4 +/- 5.7 s; n=14) was nearly two-fold greater than wild-type hENaC (28.4 +/- 5.6 s; n =14; p<0.01). Dose-response experiments revealed the apparent EC50 for S3969 activation of αβG37Sγ hENaC was (1.2 +/- 0.4 uM; n=3), similar to wild-type αβγ hENaC (1.3 uM +/- 0.5; n=3) in the same batches of oocytes. In contrast, αR508Xβγ hENaC was only weakly activated by S3969 (Fig. 8A), although levels of activation were greater than βγ hENaC lacking the α subunit (Fig. 8C and 8D). Collectively, these data indicate that S3969 opens αβG37Sγ hENaC, a channel variant with defective gating, more effectively than αR508Xβγ hENaC, a channel variant with defective surface expression.

**DISCUSSION**

Small molecule ion channel modulators regulate the flux of cations and anions across biological membranes. Compounds that positively regulate ion channel function have been
identified and termed activators for CFTR Cl− channels (47,48) and L-type Ca2+ channels (49), openers for KCNQ (50,51) and maxi-K+ channels (52,53), potentiators/correctors for mutant ΔF508 CFTR Cl− channels (54,55), or enhancers for GABA A Cl− channels (56) and cardiac Na+ channels (57). These modulators generally function to increase the time channels reside in an open conformation, by stabilizing the open state, inhibiting inactivation, or increasing the frequency of channel opening events (49,57). Despite abundant literature identifying modulators for many ion channel families, potent and specific small molecule activators of ENaC have not been previously reported. This report describes the first compound, S3969, that fully and reversibly activates hENaC in heterologous cells.

Mechanistically, S3969 increases the open probability of hENaC channels through interactions requiring the extracellular domain of the β subunit. By increasing hENaC open probability, S3969 permits increased flux of Na+ ions across biological membranes into cells.

The maximal stimulatory effect of S3969 on hENaC function in oocytes required ~3 minutes (t1/2 ~30 sec) and currents returned to baseline following S3969 washout. Slow, yet reversible, hENaC activation suggested that S3969 interacted non-covalently with hENaC channels in the plasma membrane. Activation of hENaC by S3969 exhibits qualitatively similar kinetics in mammalian cells and oocytes, suggesting that the time course of hENaC activation is not unique to the oocyte expression system but an intrinsic property of S3969 interaction with the hENaC channel. Amiloride priming experiments, performed to synchronize hENaC in an open conformation with an occluded pore, revealed that S3969 activation of hENaC was rapid (t1/2 ~5 sec) following amiloride removal. The majority of hENaC channels in the oocyte plasma membrane are closed or in a quiescent, non-conducting state (18,19,58), consistent with reports that ENaC exhibits long closed times on the order of seconds (27,59,60). Closed or quiescent channels may be poorly accessible to S3969; following transitions from the closed to the open state, S3969 may interact with hENaC to stabilize an open conformation, thereby increasing open probability. Additional, single channel patch clamp studies are required to further elucidate the mechanism of S3969 activation of hENaC. In addition, S3969 may function to relieve hENaC from sodium self-inhibition, similar to Zn2+ ions, the mercurial agent p-chloro-mercuribenzoate, and trypsin-like as well as furin serine proteases (16-21).

An alternative interpretation of the slow kinetics of hENaC activation is that S3969 interacts with an intracellular and/or transmembrane domain of hENaC; the time required to activate hENaC would simply reflect the necessity for S3969 to diffuse across the oocyte plasma membrane or into a hydrophobic cavity within the transmembrane domains. We argue against this contention based on the following points. First, positively charged analogs of S3969 exhibit similar potencies and kinetics as S39692, which is uncharged at physiological pH. Charged derivatives should not readily diffuse into the oocyte cytoplasm and achieve relevant concentrations necessary to activate hENaC over the time course of our experiments. Second, activation of hENaC specifically required the extracellular domain of the β subunit. Replacement of the extracellular domain of βhENaC with the homologous region of βmENaC did not support activation of chimeric channels by S3969; similarly, the intracellular and transmembrane domains of β hENaC were not sufficient to recapitulate ENaC activation by S3969. Finally, activation of guinea pig α and rat βγ hybrid ENaC channels by CPT-cAMP was slow and required, in part, the extracellular domain of the α subunit (12,13), indicating that slow activation of ENaC is consistent with compound interaction with an extracellular region.

Rapid activation of macroscopic hENaC current following amiloride washout, the failure to activate constitutively open hENaC (αβS520Cγ hENaC) following MTSET treatment, and the inability to activate wild-type hENaC following trypsin treatment suggest a mechanism whereby S3969 increases hENaC open probability without significantly affecting the number of functional channels incorporated into the plasma membrane or the single channel current of individual channels. Single channel patch clamp experiments validated that the hENaC activator increased open probability without affecting
single channel current. Immediate S3969 activation of hENaC following amiloride priming is inconsistent with a mechanism whereby S3969 increases macroscopic hENaC current by stimulating trafficking of hENaC from an vesicular pool to the plasma membrane, a process requiring tens of minutes or more in the oocyte system (28,29).

Previous reports indicate that furin and trypsin-like serine proteases modulate the function of ENaC and ASIC channels in the ENaC/degenerin family (20,41,61,62). Similar to S3969, proteases increase macroscopic ENaC current by increasing ENaC open probability (18,19). However, unlike S3969, protease activation of ENaC is irreversible, in that ENaC currents do not return to baseline values after removal of trypsin due to covalent modification of ENaC channels (19,63). Because S3969 activation of hENaC is reversible and requires the β subunit, whereas known proteases target the α and γ subunits (41,63-65), and because S3969 equally activates wild-type hENaC and hENaC lacking all known furin proteolysis sites, we consider it unlikely that S3969 activates hENaC indirectly through primary modulation of protease function. Reduced activation of hENaC by S3969 in CHO cells compared to HEK293 cells or oocytes may be attributable to hENaC proteolysis by furin in CHO cells resulting in channels with larger initial open probabilities (41).

Although activation of hENaC by S3969 required the extracellular domain of the β subunit, S3969 did not activate channels composed of αβ hENaC subunits and S3969 only weakly activated αR508Xβγ hENaC channels. Indeed, all three wild-type αβγ hENaC subunits were necessary to elicit a robust S3969 response. These data suggest that the binding site(s) for S3969 on hENaC is only formed upon co-expression of all three channel subunits. In addition to the β extracellular loop, S3969 likely requires additional domains present in α and γ subunits to stabilize open hENaC channels. These domains may be sufficiently conserved between human and mouse channels such that no differential functional effect was observed in subunit mixing experiments. In fact, weak activation of hENaC channels composed of α, αβ, or αγ subunits was observed upon challenge with high concentrations of S3969 (~50% activation at 100 uM, which is slightly less than the activation observed for αβγ mENaC in Fig. 5B), suggesting that non-heterotrimeric channels containing the pore-forming α-subunit are low affinity receptors for S3969. We identified valine 348 as a critical amino acid within the β extracellular loop required for S3969 activation of hENaC. Valine 348 in β hENaC is predicted to localize to beta sheet β9 in the palm domain of ASIC1α, a member of the ENaC/degenerin family that was recently crystallized (66). This region may comprise part of a S3969 binding site or be necessary for transmitting conformational changes between extracellular to transmembrane domains during hENaC gating by S3969. The degenerin site at the extracellular end of the second transmembrane domain of β hENaC is likely not critical since substitution of serine 520 by cysteine did not affect activation of αβS520Cγ hENaC by S3969.

Molecules previously reported to increase Na⁺ transport through ENaC channels in the Xenopus oocyte expression system suffer from low efficacy, low specificity, and/or lack of effect on wild-type αβγ hENaC (10-15). In contrast, S3969 exhibits high efficacy (600-700% hENaC activation at 30 uM) as well as potency on wild-type hENaC (apparent EC₅₀ ~ 1 uM for αβγ hENaC and δβγ hENaC but not αβγ mENaC). Further medicinal chemistry efforts have led to the discovery of S3969 derivatives with potencies ~100 nM. Although detailed testing of S3969 on diverse ion channels was not performed, S3969 had no effect on endogenous oocyte channels, exogenous G-protein-activated inwardly rectifying K⁺ channels (GIRK1 and GIRK2), and exogenous cold and menthol-activated non-selective cation channels (TRPM8).

Our findings provide indirect support for the hypothesis that ENaC may function as a ligand-gated ion channel, similar to other members of the ENaC/degenerin family (67). For example, δβγ ENaC and ASICs are activated by protons and FaNaC is activated by the peptide FMRF-amide (1,68-70); in addition, ASICs are modulated by neuropeptides (71). It is conceivable that an endogenous peptide ligand exits that structurally resembles S3969 and modulates hENaC function in vivo. In support of this contention, S3969 has
several peptide-like characteristics including an indole ring, found in the amino acid tryptophan, as well as a peptide bond flanked by leucine- and serine-like side chains. Thus, S3969 may function as a peptidomimetic for an endogenous hENaC ligand.

Small molecule activation of ENaC may be applicable for blood pressure regulation, airway fluid reabsorption, rodent salt taste, and renal electrolyte homeostasis. Whereas inhibition of hENaC function with amiloride may decrease blood pressure in hypertensive individuals, including those expressing a T594M polymorphism in the α subunit of hENaC (72), activation of hENaC may prove useful in increasing blood pressure in individuals with hypotension by increasing hENaC-dependent renal Na⁺ reabsorption. Similarly, activation of hENaC in the apical membrane of distal airway epithelia could promote lung function in neonatal respiratory distress syndrome or pulmonary edema by increasing hENaC-dependent Na⁺ and lung fluid absorption (73,74). Activation of ENaC in rodent tongue papillae could enhance salt sensation by promoting Na⁺ transport into taste bud cells (75,76). Finally, activation of residual hENaC function in individuals with PHA1 could correct dehydration and salt wasting accompanying mineralocorticoid resistance (44). Indeed, S3969 rescued αβG37Sγ hENaC, a PHA1 variant with compromised channel gating (6), highlighting the potential to use hENaC channel openers in situations where improved Na⁺ flux across epithelial membranes is clinically desirable. In conclusion, promoting hENaC-dependent Na⁺ and fluid transport across epithelial membranes may find utility in modulating extracellular volume and electrolyte homeostasis in both normal and diseased states.

REFERENCES

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FOOTNOTES

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1 The abbreviations used are: ENaCs, epithelial sodium channels; hENaC, human ENaC; mENaC, mouse ENaC; ABC, ATP-binding cassette; SNP, single nucleotide polymorphism; ASIC, acid-sensing ion channel; CFTR, cystic fibrosis transmembrane conductance regulator; I-V curve, current-voltage curve; IC50, concentration yielding 50% inhibition; EC50, concentration yielding 50% activation; PHA1, pseudohypoaldosteronism type I.

2 Unpublished observations, M. Lu, F. Echeverri, and B. D. Moyer

3 Unpublished observations, A. Patron, W. Keung, R. Kimmich, and B. D. Moyer

FIGURE LEGENDS

Fig. 1. Activation of αβγ hENaC by S3969. A, Structure S3969 (N-(2-hydroxyethyl)-4-methyl-2-(4-methyl-1H-indol-3-ylthio)pentanamide; MW = 320.5 Da). B, S3969 (1 uM) reversibly activated an inward current in oocytes expressing αβγ hENaC. The magnitude of the S3969 current was 300.1 +/- 12.2% (n=85) the magnitude of the amiloride-sensitive current (Amil., 1 uM). C, Amiloride (1 uM) inhibited αβγ hENaC activation by S3969 (1 uM). D, S3969 (1 uM) activated inward currents in sodium-replete media (Na) but not sodium-free media (NMDG). E, Dose-response relationship for S3969 activation of hENaC. S3969 was applied at increasing concentrations from 30 nM to 30 uM. F, Dose-response curve for S3969 activation of hENaC (EC50 = 1.2 +/- 0.1 uM; Hill coefficient = 1.0 +/- 0.1; n=46). G, I-V curves plotting amiloride-sensitive currents for control (untreated) and S3969 (1 uM)-treated oocytes expressing αβγ hENaC. Currents were measured from –100 mV to +60 mV in 20 mV increments 3 min. following application of 1 uM S3969 (n=8). Reversal potentials, the points where I-V curves cross the x-axis, were between 0-10 mV, consistent with previous reports (17,38), and attributable to increased intracellular sodium concentrations following ENaC expression in oocytes. H, I-V curves plotting whole-cell currents for control (untreated) and S3969 (1 uM)-treated uninjected oocytes (n=8). Reversal potentials were highly negative, indicating currents were attributable to endogenous potassium and chloride channels (77). S3969 had no effect on measured currents in the absence of hENaC expression. I, S3969 (10 uM) reversibly activated an inward current in HEK293 cells expressing αβγ hENaC. The magnitude of the S3969 current was 357.2 +/- 82.4% (n=24) the magnitude of the amiloride-sensitive current (10 uM; 241.4 +/- 50.6 pA). J, Amiloride (10 uM) inhibited αβγ hENaC activation by S3969 (10 uM) in CHO cells. The magnitude of the S3969 current was 148 +/- 50.7% (n=10) the magnitude of the amiloride-sensitive current (430.0 +/- 87.2 pA).

Fig. 2. S3969 activates hENaC following removal of amiloride. A, Amiloride competition experiment with S3969. Application of amiloride only (1 uM; treatment a) inhibited hENaC function and application of S3969 only (1 uM; treatment b) activated hENaC function. Co-application of amiloride (1 uM) and S3969 (1 uM) inhibited hENaC function (treatment c), similar to subsequent treatment with amiloride (1 uM) alone (treatment d), but induced transient and rapid hENaC activation following amiloride and S3969 washout (immediately following treatment c). B, Amiloride priming experiment. Application of amiloride only (1 uM; treatment a) inhibited hENaC function and was used to verify hENaC expression in oocytes. Prolonged amiloride treatment (1 uM; treatment b) synchronized hENaC in the open, but occluded, conformation. Subsequent application of S3969 (1 uM; treatment c) induced an apparent rapid
and sustained hENaC activation following amiloride washout. Final application of S3969 (1 uM; treatment d), without amiloride priming, induced slow hENaC activation. C, Quantitation of % hENaC activation (magnitude of the inward current induced by S3969 divided by the magnitude of the amiloride-sensitive current, multiplied by 100%) for control (no amiloride priming; treatment d in panel B) and amiloride priming (treatments b and c in panel B) protocols (n=7). In the amiloride primed group, baseline currents for S3969 activation were measured before amiloride treatment a; this eliminates an additional 100% hENaC enhancement that would be obtained by measuring baseline currents for S3969 activation at treatment b, when all hENaC channels are non-conducting. If this correction is not performed, S3969 activates hENaC by 442.7 +/- 26.0% compared to 355.6 +/- 27.7 % with correction. D, The apparent time required to reach half-maximal hENaC activation (t½) is faster following amiloride priming (n=7). * p < 0.001 compared to control.

Fig. 3. S3969 increases hENaC open probability. A, MTSET (1 mM) irreversibly activated αβS520Cγ hENaC in an amiloride (1 uM)-dependent manner. B, Application of S3969 (1 uM), following treatment with MTSET (1 mM) to maximize αβS520Cγ hENaC open probability, did not induce further αβS520Cγ hENaC activation. Similar results were obtained with 10 uM S3969. C, Trypsin (2 ug/ml) irreversibly activated wild-type hENaC in an amiloride (1 uM)-dependent manner. S3969 (1 uM) activated ENaC prior to but not following trypsin treatment. D, Quantitation of amiloride (1 uM) current before (control) and after trypsin (2 ug/ml) treatment. E, Quantitation of % hENaC activation by S3969 (1 uM) before (control) and after trypsin treatment. * p < 0.01 compared to control. F-G, Representative outside-out patch clamp recordings before (Pre, F) and 2 min after (Post, G) hENaC activator (30 uM) application to the same patch containing two hENaC channels. C indicates the closed state, O1 indicates the open state for the first channel, and O2 indicates the open state for the second channel.

Fig. 4. Requirement of α, β, and γ subunits for S3969 activation of hENaC. Amiloride (1 uM)-sensitive currents (A) and S3969 (1 uM)-induced currents (B) in oocytes expressing all possible combinations of α, β, and γ hENaC. αβγ hENaC oocytes were injected with 1 ng cRNA per subunit, whereas all other groups were injected with 5 ng cRNA per subunit to increase macroscopic current levels. Amiloride-sensitive currents were only detectable in oocytes expressing α hENaC, alone or in combination with β and/or γ hENaC. By contrast, S3969-induced currents were only observed in oocytes expressing heterotrimeric αβγ channels. n = 6-7 oocytes/group. * p < 0.001 compared to αβγ hENaC.

Fig. 5. S3969 activates δβγ hENaC but not αβγ mENaC. A, Amiloride inhibition curves for αβγ hENaC (n=16), αβγ mENaC (n=3), and δβγ hENaC (n=10). Half-maximal inhibition of δβγ hENaC required greater than 10-fold higher concentrations of amiloride compared with αβγ hENaC and αβγ mENaC. B, Representative S3969 dose-response curves for αβγ hENaC (n=46), αβγ mENaC (n=4), and δβγ hENaC (n=11). S3969 activated δβγ hENaC with similar efficacy and potency as αβγ hENaC. By contrast, S3969 did not activate αβγ mENaC unless used at 100-300 uM, more than two log orders above concentrations required to induce half-maximal hENaC activation. Experiments with δβγ hENaC used 10 uM amiloride whereas experiments with αβγ hENaC and αβγ mENaC used 1 uM amiloride, concentrations yielding >90% hENaC inhibition, to calculate % hENaC activation values.

Fig. 6. The β subunit extracellular loop is critical for S3969 activation of hENaC. Amiloride (1 uM)-sensitive currents (A) and % ENaC activation by S3969 (10 uM) (B) in oocytes expressing all possible heterotrimeric combinations of αβγ hENaC and αβγ mENaC. H denotes human and M denotes mouse. All mouse-human hybrids generated amiloride-sensitive currents, demonstrating the production of functional ENaC channels; no significant differences existed between amiloride-sensitive currents when comparing αβγ hENaC to hybrid channels. ENaC activation by S3969 was strictly dependent on expression of β hENaC. S3969-induced currents were not observed in oocytes expressing hybrid...
channels containing β mENaC.  n = 8-13 oocytes/group.  * p < 0.001 compared to αβγ hENaC. Amiloride (1 uM)-sensitive currents (C) and % ENaC activation by S3969 (10 uM) (D) in oocytes expressing heterotrimeric ENaC channels, consisting of α and γ mENaC subunits with β subunit chimeras composed of intracellular, transmembrane, and extracellular domains derived from β mENaC (thin lines) or β hENaC (thick lines) as indicated. ΔV348 indicates deletion of valine 348 (small gray circle). All β subunit chimeras generated amiloride-sensitive currents, demonstrating the production of functional ENaC channels; no significant differences existed between amiloride-sensitive currents when comparing chimera H to other chimeras. ENaC activation by S3969 was strictly dependent on the extracellular loop of β hENaC. S3969-induced currents were not observed in oocytes expressing β subunit chimeras containing the mENaC extracellular loop. Similar results were obtained when β subunit chimeras were expressed with α and γ hENaC subunits2. n = 10-17 oocytes/group.  * p < 0.001 compared to β chimera H.

Fig. 7. S3969 opens ENaC channels not cleaved by furin proteolysis. (A) Representative traces illustrating amiloride (1 uM) and S3969 (10 uM) modulation of hENaC function in oocytes expressing wild-type αβγ (WT) and αR178A R181A R204AβγR138A (Furin variant) hENaC. Amiloride (1 uM)-sensitive currents (B), S3969 (10 uM)-sensitive currents (C), and % ENaC activation by S3969 (D) in oocytes expressing wild-type αβγ (WT) or αR178A R181A R204AβγR138A (Furin variant) hENaC or no hENaC channels (uninjected). S3969 activated currents were similar in wild-type αβγ and αR178A R181A R204AβγR138A hENaC, indicating predominant activation of channels not proteolyzed by furin. Following application of near saturating concentrations of S3969, currents do not completely return to control values following compound washout. n = 16-17 oocytes/group.  * p <0.001 compared to wild-type αβγ hENaC.

Fig. 8. S3969 activates PHA1 hENaC channels. (A) Representative traces illustrating amiloride (1 uM) and S3969 (10 uM) modulation of hENaC function in oocytes expressing wild-type αβγ (WT), αβG37Sγ (βG37S), and αR508Xβγ (αR508X) hENaC. Amiloride (1 uM)-sensitive currents (B), S3969 (10 uM)-sensitive currents (C), and % ENaC activation by S3969 (D) in oocytes expressing wild-type αβγ (WT), αβG37Sγ (βG37S), αR508Xβγ (αR508X), or βγ hENaC channels, or no hENaC channels (uninjected). S3969 strongly activated wild-type αβγ and αβG37Sγ hENaC, weakly activated αR508Xβγ hENaC, and failed to activate βγ hENaC or uninjected oocytes. Oocytes were tested 48-72 hours post-injection to allow sufficient time for PHA1 hENaC channel functional expression (46). The lower activation of wild-type αβγ hENaC by 10 uM S3969 compared to previous figures is likely due to a decreased driving force for sodium entry (increased intracellular sodium concentration) in oocytes expressing wild-type αβγ hENaC for 48-72 hours.  n = 10-15 oocytes/group.  * p <0.05 compared to wild-type αβγ hENaC.
Table 1. Summary of β ENaC chimeras.

<table>
<thead>
<tr>
<th>β Chimera</th>
<th>Human region</th>
<th>Human amino acids</th>
<th>Mouse region</th>
<th>Mouse amino acids</th>
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<td>1-71</td>
<td>ECL, TMD2, C-term</td>
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<td>1-71, 532-638</td>
</tr>
</tbody>
</table>

H indicates human and M indicates mouse. N-term is the amino terminus, TMD1 is the 1st transmembrane domain, ECL is the extracellular loop, TMD2 is the 2nd transmembrane domain, and C-term is the carboxy terminus. Human amino acids are numbered based on accession number NP_000327. Mouse amino acids are numbered based on accession number NP_035455.
Figure 2

A

Amil. S3969

B

Amil. S3969

C

% ENaC Activation

D

% ENaC Activation

Control Amil. Prime

% ENaC Activation

Control Amil. Prime
Figure 3

(A) Amil.  MTSET  Amil.

(B) Amil.  S3969  S3969

(C) Amil.  S3969  Trypsin  Amil.  S3969

(D) Amiloride current (nA)

(E) % ENaC Activation

(F) Pre

(G) Post
Figure 4

A

[Graph showing Amiloride current (nA) for different subunits.]

B

[Graph showing S3969 current (nA) for different subunits.]
Figure 5

A

% ENaC Inhibition

\[ \text{Amiloride log [M]} \]

B

% ENaC Activation

\[ \text{S3969 log [M]} \]
Figure 6

A

Amiloride current (nA)

<table>
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<th>alpha</th>
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B

% ENaC Activation

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</tr>
</tbody>
</table>

C

Amiloride current (nA)


D

% ENaC Activation

Figure 7

A

Amiloride current (nA)

% ENaC Activation

B

WT

Furin variant

Uninjected

C

S3969 current (nA)

D

% ENaC Activation

WT

Furin variant

Uninjected
Figure 8

A

WT

βG37S

αR508X

B

Amiloride current (nA)

WT βG37S αR508X βγ Uninjected

C

S3969 current (nA)

WT βG37S αR508X βγ Uninjected

D

% ENaC Activation

WT βG37S αR508X βγ Uninjected
Small molecule activator of the human epithelial sodium channel
Min Lu, Fernando Echeverri, Dalia Kalabat, Bianca Laita, David S. Dahan, Raymond D. Smith, Hong Xu, Lena Staszewski, Jeff Yamamoto, Jing Ling, Nancy Hwang, Rachel Kimmich, Peter Li, Erika Patron, Walter Keung, Andrew Patron and Bryan D. Moyer

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