A mutation in the β-subunit of ENaC identified in a patient with cystic fibrosis-like symptoms has a gain-of-function effect

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Cystic fibrosis (CF) is a common autosomal recessive hereditary disease that is usually caused by mutations in the CF transmembrane conductance regulator (CFTR) gene (46). The CFTR gene encodes a Cl− channel, which is found in the apical membrane of a wide range of epithelial cells (50). Defective CFTR results in reduced apical Cl− conductance, a typical hallmark of CF epithelia. Patients with CF present with diverse symptoms, e.g., chronic sinusitis, chronic pulmonary insufficiency, failure to thrive, or congenital bilateral absence of the vas deferens (13). Moreover, it has been reported that, in differentiated primary cultures of human CF and non-CF respiratory epithelia, the Na+ channel inhibitor amiloride produced similar reductions in transepithelial conductance and Na+ absorption, indicating that Na+ conductance in CF epithelia did not exceed that in non-CF epithelia (25). Thus at present it remains a matter of debate
whether enhanced ENaC function contributes to the pathophysiology of CF. It is conceivable that ENaC hyperactivity may play a role in only a subset of patients with CF.

Recently, a mutation in the β-subunit (BV348M) of ENaC has been identified in a patient with severe CF-like symptoms (recurrent respiratory infections, lung colonization by *Pseudomonas aeruginosa*, gastrointestinal symptoms, failure to thrive, diabetes mellitus, and protein energy malnutrition) (39). Moreover, the BV348 residue has been reported to be critical for the stimulatory effect of the novel ENaC activator S3969 (36). These findings suggest that this residue is functionally important and that the identified mutation is likely to alter channel properties. The aim of the present study was to investigate the effects of the BV348M mutation on ENaC function.

**MATERIALS AND METHODS**

**Chemicals and solutions.** Unless stated otherwise, chemicals were from Sigma (Taufkirchen, Germany). 2-(trimethylammonium)ethyl methanesulphonate bromide (MTSET) was obtained from Toronto Research Chemicals (Toronto, Ontario, Canada), collagenase type II (CLS II), fetal bovine serum, sodium pyruvate and nonessential amino acids were from Biochrom (Berlin, Germany); and Modified Eagle’s Medium was from PAA Laboratories (Cölbe, Germany). S3969 (36) was synthesized as described previously (24). The solutions used were: OR2 for isolation of oocytes (in mM: 82.5 NaCl, 2 KCl, 1 MgCl₂, 5 HEPES, pH 7.4 with NaOH), a low-Na⁺-containing solution for oocyte incubation (in mM: 87 NMDG-Cl, 9 NaCl, 2 KCl, 1.8 CaCl₂, 1 MgCl₂, 5 HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin, pH 7.4 with Tris), and ND96 as the bath solution for two-electrode voltage-clamp experiments (in mM: 96 NaCl, 2 KCl, 1.8 CaCl₂, 1 MgCl₂, 5 HEPES, pH 7.4 with Tris). Solutions used for outside-out patch-clamp procedures were: pipette solution (in mM: 90 K-glucuronate, 5 NaCl, 2 Mg-ATP, 2 EGTA, 10 HEPES, pH 7.28 with Tris), low-sodium NMDG-Cl bath solution (in mM: 95 NMDG-Cl, 1 NaCl, 4 KCl, 1 MgCl₂, 1 CaCl₂, 10 HEPES, pH 7.4 with Tris), and high-sodium bath solution (in mM: 95 NaCl, 4 KCl, 1 MgCl₂, 1 CaCl₂, 10 HEPES, pH 7.4 with Tris). Solutions used for whole-cell voltage clamp on HEK293 cells were: pipette solution (in mM: 130 CsCl, 2 Mg-ATP, 10 HEPES, EGTA-Na (tetrasodium salt), 2 MgCl₂, pH 7.2 with Tris), and bath solution (in mM: 160 NaCl, 1 CaCl₂, 2 MgCl₂, 10 HEPES, pH 7.4 with Tris).

**Plasmids.** Full-length cDNAs for all human ENaC subunits were cloned in pcDNA3.1 as previously described (1). Linearized plasmids were used as templates for cRNA synthesis using T7 RNA polymerase (mMessage mMachine; Ambion, Austin, TX). To minimize the risk of expression artifacts that may arise from differences in cRNA quality, cRNAs for wild-type (wt) and mutant ENaC were synthesized in parallel, and the experiments were performed using at least two different batches of cRNA. Point mutations were generated by site-directed mutagenesis (QuikChange Site-Directed Mutagenesis Kit; Stratagene, Amsterdam, Netherlands) and confirmed by sequence analysis.

**Isolation of oocytes and injection of cRNA.** Oocytes were obtained from adult female *Xenopus laevis* in accordance with German legislation, with approval by the animal welfare officer for the University of Erlangen-Nuremberg, and under the governance of the state veterinary health inspectorate. Animals were anesthetized in 0.2% MS222 (ethyl 3-aminobenzoate methanesulfonate salt). Ovarian lobes were obtained by partial ovariectomy, and oocytes were isolated by enzymatic digestion at 19°C for 3–4 h with 600–700 U/ml collagenase type II dissolved in OR2 solution. Isolated oocytes were stored in ND96 supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin to prevent bacterial overgrowth until injection. Defolliculated stage V–VI oocytes were injected (Nanoject II automatic injector; Drummond, Broomall, PA) with 0.5 ng cRNA per subunit of ENaC. The cRNAs were dissolved in RNase-free water, and the total volume injected was 46 nl. To prevent current rundown, sodium loading ENaC expressing oocytes, the injected oocytes were stored until use in a low-Na⁺-containing solution (supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin) at 19°C as previously described (21, 24, 44). HEK293 cell culture and transfection. HEK293 cells were cultured in Modified Eagle’s Medium supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 1% nonessential amino acids, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were transfected with plasmids coding for α-, γ- and β- or BV348M-ENaC 1 day after seeding at a level of ~60% confluence using X-treme Gene HP DNA transfection reagent (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s protocol. The transfection reagent was used in a ratio 1:4. pEGFP-C1 (Invitrogen, Darmstadt, Germany) was cotransfected to identify positively transfected cells in the patch-clamp experiments using fluorescence microscopy. After transfection, medium was supplemented with amiloride (10 µM) to avoid excessive sodium loading of the cells.

**Two-electrode voltage clamp.** Oocytes were routinely studied 1–2 days after injection using the two-electrode voltage-clamp technique as described previously (45, 58). The oocytes were placed in a small experimental chamber and constantly superfused (2–3 ml/min) at room temperature with ND96 supplemented with amiloride (2 µM). Bath solution exchanges were controlled by an ALA BPS-8 magnetic valve system in combination with a TIB14 interface (both HEKA, Lambrecht, Germany). Voltage-clamp experiments were performed using an OC-725C amplifier (Warner Instruments, Hamden, CT) interfaced via a LIH-1600 (HEKA) to a PC running PULSE 8.5a software (HEKA) for data acquisition and analysis. For continuous whole-cell current recordings, oocytes were routinely clamped at a holding potential of ~60 mV. Amiloride-sensitive whole-cell currents (Iamiloride) were determined by washing out amiloride with amiloride-free ND96 and by subtracting the whole-cell current measured in the absence of amiloride from that measured in the presence. Downward current deflections in current traces correspond to inward currents, i.e., movement of positive charge into the cell.

**Patch-clamp experiments on oocytes.** Single-channel recordings in conventional outside-out patches were essentially performed as described previously (15, 44). Oocytes were routinely studied 2–3 days after injection. Patch pipettes were pulled from borosilicate glass capillaries and had a tip diameter of ~1–1.5 µm after fire polishing. Pipettes were filled with potassium glutonate pipette solution. Seals were routinely formed in a low-Na⁺ NMDG-Cl bath solution. In this bath solution, the pipette resistance averaged ~7 MΩ. After seal formation, the bath solution was changed to a high-Na⁺ solution. Outside-out patches were routinely voltage clamped at ~70 mV, which is close to the calculated reversal potential of Cl⁻ (ECI = −77.2 mV) and K⁺ (E钾 = −79.4 mV) under our experimental conditions. Experiments were performed at room temperature (~23°C). The current level at which all channels were closed was determined in the presence of amiloride (2 µM). Downward current deflections correspond to cell membrane inward currents, i.e., movement of positive charge from the extracellular side to the cytoplasmic side. Single-channel current data were filtered at 1 kHz and digitized at 3 kHz before refiltering at 50 Hz to calculate single-channel current amplitude and channel activity. The current level at which all channels are closed (closed level) was determined in the presence of amiloride (2 µM). Binned amplitude histograms were used to determine the single-channel current amplitude (i) and to estimate channel activity as the product Nappo, where N is the number of channels and po is the single-channel open probability (14, 15, 33, 34). To analyze channel gating, we calculated the number of channel transitions per second per apparent number of channels (Transitions × s⁻¹ × Napp⁻¹) (34). Data analysis was performed using the program Patch for Windows written by Dr. Bernd Letz (HEKA Elektronik, Lambrecht/Pfalz, Germany).
**Whole-cell patch-clamp experiments in HEK293 cells.** Conventional whole-cell patch-clamp recordings from cultured HEK293 cells were performed at 37°C using an experimental protocol and setup essentially as described previously (41, 42). Green fluorescent protein fluorescence was used to facilitate selection of successfully transfected cells. Only recordings with a clear amiloride response were included in the data analysis (~20% of selected cells did not respond to amiloride). Pipettes were made from borosilicate glass and had a resistance of 3–4 MΩ. Seal resistance was 4–10 GΩ, and series resistance (Rs) was 5–8 MΩ. Membrane capacitance (Cm) and Rs were estimated using the automated capacitance compensation procedure of the EPC-9 amplifier. Rs was compensated by 70%; Cm ranged from 7 to 15 pF. Cells were voltage clamped at a holding potential of −60 mV. Current signals were filtered at 250 Hz and sampled at a rate of 1 kHz. For further analysis and presentation, they were routinely refiltered at 100 Hz. The amiloride-sensitive current (ΔIamil) was determined by subtracting the whole-cell current measured in the presence of amiloride from that measured in the absence of amiloride (2 μM). Current density was calculated as the ratio of ΔIamil to cell membrane capacitance.

Detection of βENaC at the cell surface. Biotinylation experiments were performed essentially as described (14, 22, 23) using 30 oocytes per group. All biotinylation steps were performed on ice. Oocytes were incubated in biotinylation buffer [in mM: 10 triethanolamine, 150 NaCl, 2 CaCl2, EZ-link sulfo-NHS-SSBiotin (1 mg/ml, pH 9.5; Pierce, Rockford, IL)] for 15 min with gentle agitation. The biotinylation reaction was stopped by washing the oocytes twice for 5 min with a quenching buffer (in mM: 192 glycine, 25 Tris, pH 7.5). Oocytes were lysed by passing them five times through a 27-gauge needle in lysis buffer (in mM: 500 NaCl, 5 EDTA, 50 Tris, pH 7.4) supplemented with a protease inhibitor cocktail (Complete Mini EDTA-free protease inhibitor cocktail tablets; Roche Diagnostics) according to manufacturer’s instructions. The lysates were centrifuged for 10 min at 1,500 g. After addition of 0.5% Triton X-100 and 0.5% Igepal CA-630, supernatants were incubated for 20 min on ice, and 100 μl of ImmunePure immobilized Neutravidin beads (Pierce) were added. After overnight incubation at 4°C with overhead rotation, the tubes were centrifuged for 3 min at 1,500 g. Supernatants were removed and used for the detection of intracellular βENaC. The remaining beads were washed three times with lysis buffer, and 100 μl of 2× SDS-PAGE sample buffer (Rotiload 1; Roth, Karlsruhe, Germany) were added. Samples were boiled for 5 min at 95°C and centrifuged for 3 min at 20,000 g before loading the supernatants on a 10% SDS-PAGE gel. Separated proteins were transferred onto a nitrocellulose membrane by semidy blotting and detected with a primary rabbit anti-human βENaC antibody (1:10,000) (22) and a horseradish peroxidase-labeled secondary goat anti-rabbit antibody (1:50,000; Santa Cruz Biotechnology, Heidelberg, Germany). Absence of intracellular proteins was determined by monitoring the β-actin signal. Densitometric analysis was performed using ImageJ 1.38x (National Institutes of Health, Bethesda, MD).

**ENaC modeling.** The structural effects of mutating V348 were investigated based on a previous model of ENaC (44) that was generated on the basis of the published crystal structure of ASIC1, which belongs to the ENaC/degenerin gene family (26). According to the conformation of the template, the present model is likely to represent a closed conformation of ENaC. Mutants were generated with the Swiss-PdbViewer (49). Structural analysis and visualization was performed using RasMol (48).

**Statistical methods.** Data are presented as means ± SE. N indicates the number of different batches of oocytes, n the number of individual oocytes studied. Statistical significance was assessed using the appropriate version of Student’s t-test or one-way ANOVA followed by Dunnett’s Multiple Comparison test with GraphPad Prism 4.03 for Windows (GraphPad Software, San Diego, CA).

**RESULTS**

The βV348M mutation stimulates ENaC-mediated whole-cell currents in Xenopus laevis oocytes. To study the effect of the βV348M mutation on ENaC function, we expressed wild-

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Fig. 1. βV348M mutation stimulates epithelial sodium channel (ENaC). Wild-type αβγENaC (wt) or mutant αβV348MγENaC (V348M) was expressed in Xenopus laevis oocytes, and amiloride-sensitive whole-cell current traces of a wild-type and a mutant ENaC-expressing oocyte at a holding potential of −60 mV. Amiloride (2 μM) was present in the bath as indicated by the black bars. A: representative whole-cell current traces of a wild-type and a mutant ENaC-expressing oocyte at a holding potential of −60 mV. Amiloride (2 μM) was present in the bath as indicated by the black bars. A: representative whole-cell current traces of a wild-type and a mutant ENaC-expressing oocyte at a holding potential of −60 mV. B: summary of similar experiments as shown in A performed in wild-type and mutant ENaC-expressing oocytes. C: summary of 3 time course measurements of wild-type and mutant ENaC-expressing oocytes. To correct for batch-to-batch variability, current values were normalized to wild-type control on day 1. Each data point represents mean ΔIamil measured in 53–62 oocytes. N indicates the number of different batches of oocytes; n or numbers in columns indicate the number of individual oocytes. *P < 0.05, **P < 0.01, ***P < 0.001, unpaired t-test.

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type αβγENaC or mutant αβV348MγENaC in *Xenopus laevis* oocytes and determined amiloride-sensitive whole-cell currents (ΔI_{ami}) with two-electrode voltage clamp. Figure 1A shows typical whole-cell current traces recorded from a wild-type and a mutant ENaC-expressing oocyte. In 34 different batches of oocytes, ΔI_{ami} averaged 4.0 ± 0.14 μA in αβγENaC-expressing control oocytes (n = 331) and 5.6 ± 0.22 μA in αβV348MγENaC-expressing oocytes (n = 332, P < 0.001) (Fig. 1B). Thus on average the βV348M mutation stimulated the ENaC-mediated whole-cell Na⁺ current by ~38%. In five batches of oocytes, we measured ΔI_{ami} on three consecutive days following cRNA injection (Fig. 1C). In wild-type and mutant ENaC-expressing oocytes, ΔI_{ami} increased from day 1 to day 3, which demonstrates that with longer incubation periods more ENaC protein is synthesized and delivered to the plasma membrane (27). Importantly, on days 2 and 3, ΔI_{ami} was significantly higher in mutant than in wild-type ENaC-expressing oocytes, confirming that the stimulatory effect of the βV348M mutation on ENaC function is a robust phenomenon. In conclusion, our results demonstrate that the βV348M mutation causes an increase in ENaC function.

**βV348M increases channel open probability.** The stimulatory effect of the βV348M mutation can result from an increased density of channels expressed at the plasma membrane, a higher channel open probability (P_o), or a higher single-channel conductance. To test whether the βV348M mutation increases the average channel P_o, we introduced a βS520C point mutation into the wild-type and mutant β-subunit (14, 22, 24, 36). Binding of the sulfhydryl reagent MTSET to this cysteine stabilizes the open state of the channel, thereby shifting channel P_o close to 1 (28, 52). Therefore, the ratio of baseline ΔI_{ami} to ΔI_{ami} after activation with MTSET provides an estimate of the average channel P_o before the application of MTSET. Application of MTSET (1 mM) on αβS520CγENaC-expressing oocytes stimulated ΔI_{ami} ~4.2-fold (Fig. 2), which indicates a P_o of ~0.24 before the application of MTSET. ΔI_{ami} of αβV348M-S520CγENaC-expressing oocytes was on average ~43% higher than that of αβS520CγENaC-expressing control oocytes, confirming a stimulatory effect of the βV348M mutation in this set of experiments. Application of MTSET to αβV348M-S520CγENaC-expressing oocytes stimulated ΔI_{ami} ~3.0-fold, which indicates an average P_o of ~0.33 before the application of MTSET. These findings support the concept that a higher P_o of mutant ENaC contributes to the gain-of-function effect of the mutation. Interestingly, ΔI_{ami} levels of αβS520CγENaC- and αβV348M-S520CγENaC-expressing oocytes after MTSET stimulation were comparable. Under the assumption that the single-channel conductance is not affected by the V348M mutation (see below), these findings indicate that an increase in channel surface expression does not contribute to the gain-of-function effect of the βV348M mutation.

To investigate whether the βV348M mutation alters the single-channel conductance of ENaC and to confirm that it increases P_o, we performed single-channel recordings using outside-out patches obtained from oocytes expressing wild-type αβγENaC or mutant αβV348MγENaC. Figure 3A shows typical single-channel current traces recorded at different holding potentials. The corresponding I/V plots are shown in Fig. 3B. The Goldman-Hodgkin-Katz fits of the data suggest that the wild-type and the mutant channel are sodium selective. In similar experiments, as shown in Fig. 3, the single-channel conductance of the mutant ENaC averaged 5.20 ± 0.04 pS (n = 7), which is only marginally lower than that of wild-type ENaC averaging 5.4 ± 0.05 pS (n = 6, P < 0.01). These single-channel conductance values are in good agreement with those previously reported for human ENaC (24, 44, 54). Thus we have no evidence that an increased single-channel conductance or an altered ion selectivity contribute to the gain-of-function effect of the βV348M mutation.

Importantly, our single-channel current recordings demonstrate that the βV348M mutation alters the gating kinetics of ENaC. The representative current traces shown in Fig. 3A suggest that the gating kinetics of mutant ENaC are slower than those of wild-type ENaC. To further analyze a possible effect of the mutation on channel gating, we performed additional continuous single-channel recordings at a holding potential of ~70 mV as illustrated in Fig. 4. At the beginning of these recordings, amiloride was present in the bath solution to determine the current level at which all channels are closed. Washout of amiloride resulted in the appearance of single-channel current activity. In the absence of amiloride, the average N_{P_o} of the mutant ENaC showed a nonsignificant trend to be higher than the average N_{P_o} of wild-type ENaC (Fig. 4C). The maximal number of apparent channel levels (N_{app}) observed in outside-out patches with mutant ENaC was similar to that observed in outside-out patches with wild-type ENaC (Fig. 4D). By calculating the ratio of N_{P_o} to N_{app}, we
estimated the single-channel \( P_o \) in several recordings similar to those shown in Fig. 4, A and B. On average the \( P_o \) of mutant ENaC (\( n = 11 \)) was \( \sim 61\% \) higher than that of wild-type ENaC (\( n = 11; P < 0.05; \) Fig. 4E). To further quantify the effect of the \( \beta V348M \) on channel gating, we counted the overall number of channel transitions per time as previously described (34). The average number of channel transitions per second and per \( N_{\text{app}} \) was largely reduced in outside-out patches with mutant ENaC compared with outside-out patches with wild-type ENaC (Fig. 4F). Thus, the \( \beta V348M \) mutation reduces the frequency of gating events, which is likely to contribute to the observed increase in \( P_o \). In summary, our single-channel data confirm that the \( \beta V348M \) mutation causes a gain-of-function effect by increasing channel \( P_o \).
Relative activation of $\Delta I_{\text{ami}}$ by chymotrypsin is reduced in mutant ENaC. ENaC can be activated by serine proteases, e.g., chymotrypsin (7, 8, 10, 14, 21, 31, 43, 44, 47). To test whether the $\beta V348M$ mutation alters the responsiveness of the channel to proteases, we measured the effect of chymotrypsin on $\Delta I_{\text{ami}}$ in wild-type $\alpha \beta \gamma$ ENaC- and in mutant $\alpha \beta V348M \gamma$ ENaC-expressing oocytes. Baseline $I_{\text{ami}}$ of mutant ENaC-expressing oocytes was on average ~36% larger than that of wild-type ENaC-expressing control oocytes, consistent with the results reported above. Application of chymotrypsin (2 $\mu$g/ml) on wild-type ENaC-expressing oocytes stimulated $\Delta I_{\text{ami}}$ by only ~3.0-fold. This is consistent with the reduced stimulatory effect of MTSET on oocytes expressing $\alpha \beta V348M \gamma$ ENaC. The finding that the relative activation of mutant ENaC by chymotrypsin is lower than that of wild-type ENaC indicates that the ENaC activator S3969 stimulates mutant ENaC less than wild-type ENaC. The ENaC activator S3969 stimulates mutant ENaC less than wild-type ENaC. Recently, S3969 has been identified as a novel small molecule activator of ENaC (36). At present, the precise mechanism by which S3969 activates ENaC is unknown. However, it has been reported that deletion of V348 in human $\beta$ENaC abolished the stimulatory effect of the activator. Therefore, we wanted to know whether the $\beta V348M$ activator S3969 is reduced. $A$: representative whole-cell current traces of an $\alpha \beta \gamma$-ENaC (wt)- and an $\alpha \beta V348M \gamma$ ENaC (V348M)-expressing oocyte at a holding potential of ~$-60$ mV. Amiloride (2 $\mu$M) and chymotrypsin (2 $\mu$g/ml) were present in the bath as indicated by the black and white bars, respectively. $B$: summary of similar experiments as shown in $A$ performed in oocytes from 5 different batches. Same data as shown in $B$ but normalized to the corresponding control value before S3969 application to demonstrate the relative stimulatory effect of S3969 on $\Delta I_{\text{ami}}$. $N$ indicates the number of different batches of oocytes; numbers in columns indicate the number of individual oocytes. ***$P < 0.001$, paired ($B$) or unpaired ($C$) $t$-test.

Fig. 5. Relative activation of $\Delta I_{\text{ami}}$ by chymotrypsin is reduced in mutant ENaC. $A$: representative whole-cell current traces of an $\alpha \beta \gamma$-ENaC (wt)- and an $\alpha \beta V348M \gamma$-ENaC (V348M)-expressing oocyte at a holding potential of ~$-60$ mV. Amiloride (2 $\mu$M) and chymotrypsin (2 $\mu$g/ml) were present in the bath as indicated by the black and white bars, respectively. $B$: summary of similar experiments as shown in $A$ performed in oocytes from 9 different batches. $C$: same data as shown in $B$ but normalized to the corresponding control value before S3969 application to demonstrate the relative stimulatory effect of chymotrypsin on $\Delta I_{\text{ami}}$. $N$ indicates the number of different batches of oocytes; numbers in columns indicate the number of individual oocytes. ***$P < 0.001$, paired ($B$) or unpaired ($C$) $t$-test.

Fig. 6. Stimulatory effect of S3969 on mutant ENaC is reduced. $A$: representative whole-cell current traces of an $\alpha \beta \gamma$-ENaC (wt)- and an $\alpha \beta V348M \gamma$ ENaC (V348M)-expressing oocyte at a holding potential of ~$-60$ mV. Amiloride (2 $\mu$M) and S3969 (10 $\mu$M) were present in the bath as indicated by the black and white bars, respectively. $B$: summary of similar experiments as shown in $A$ performed in oocytes from 5 different batches. Same data as shown in $B$ but normalized to the corresponding control value before S3969 application to demonstrate the relative stimulatory effect of S3969 on $\Delta I_{\text{ami}}$. $N$ indicates the number of different batches of oocytes; numbers in columns indicate the number of individual oocytes. ***$P < 0.001$, paired ($B$) or unpaired ($C$) $t$-test.

Fig. 7. Amino acid substitutions show diverse effects on ENaC function. $\Delta I_{\text{ami}}$ was measured in oocytes expressing different mutants as indicated below the columns. To pool data from different batches of oocytes, individual $\Delta I_{\text{ami}}$ values were normalized to the mean $\Delta I_{\text{ami}}$ of the corresponding wild-type ENaC (wt)-expressing control oocytes. $N$ indicates the number of different batches of oocytes. Numbers in or above columns indicate the total number of oocytes. *$P < 0.05$, **$P < 0.01$, ANOVA followed by Dunnett’s multiple-comparison test vs. wild-type control.
mutation reduces the stimulatory effect of S3969. In this set of experiments, $\Delta I_{\text{ami}}$ of mutant ENaC-expressing oocytes was on average $\sim 29\%$ higher than that of wild-type ENaC-expressing oocytes, consistent with a gain-of-function effect of the V348M mutation. Application of S3969 (10 $\mu$M) on wild-type ENaC-expressing oocytes activated $\Delta I_{\text{ami}} \sim 3.5$-fold (Fig. 6). In contrast, superfusion of mutant ENaC-expressing oocytes with S3969 stimulated $\Delta I_{\text{ami}} \sim 2.6$-fold. Thus the stimulatory effect of S3969 on mutant ENaC is smaller than that on wild-type ENaC. Overall the stimulatory effect of S3969 on both wild-type and mutant ENaC was lower than that of MTSET and chymotrypsin. This indicates that S3969, in contrast to MTSET and chymotrypsin, does not fully activate the channel, which is in agreement with the findings of Lu et al. (36). Nevertheless, our finding that the V348M mutation significantly reduces the stimulatory effect of S3969 is consistent with the interpretation that the mutation increases average channel $P_o$.

Different amino acid substitutions at residue 348 show diverse effects on ENaC function. In an additional series of experiments, we investigated whether varying size or charge of residue 348 in βENaC systematically alters ENaC function. We substituted the wild-type valine in position 348 with amino acids of different size or charge and expressed the individual mutants in oocytes. The results summarized in Fig. 7 demonstrate that, in addition to the V348M mutation, four other mutations (V348G, V348K, V348L, V348W) caused a gain-of-function effect with a significantly larger $\Delta I_{\text{ami}}$ than that for wild-type ENaC. Interestingly, both the small and nonpolar amino acid glycine (V348G) and the large and positively charged lysine (V348K) stimulated $I_{\text{ami}}$ more than the nonpolar methionine (V348M) with an intermediate size. The nonpolar leucine (V348L) and the nonpolar tryptophan (V348W) stimulated ENaC less than methionine (V348M). The negatively charged glutamic acid (V348E) had no effect on ENaC function, whereas the negatively charged aspartic acid (V348D) mediated a small but significant inhibitory effect. Interestingly, replacing valine by the polar glutamine (V348Q) or deleting the valine residue (V348del) nearly abolished ENaC function. To test whether these differences result from differences in protein expression, we detected intracellular and biotinylated membrane βENaC levels by Western blot analysis (Fig. 8). Intracellular βENaC protein expression levels...
of the two mutants with the strongest inhibitory effect (V348Q and V348del) were largely reduced (Fig. 8, A and B), and the corresponding membrane expression signals were hardly distinguishable from background signals (Fig. 8, A and C). This indicates that a decrease in protein and hence channel surface expression is responsible for the large loss-of-function effect of these two mutations. In contrast, the protein expression level of the other mutants was similar to that of wild-type βENaC both at the plasma membrane and intracellularly. Thus, in these other mutants, the differences in ΔI_{ami} are probably not the result of differences in channel protein expression but are likely to be caused by differences in channel P_{o}. To test this, we activated all mutants with chymotrypsin, with the exception of the V348Q and V348del mutants, which were not sufficiently expressed to be tested. As shown in Fig. 9A, application of chymotrypsin had a large stimulatory effect on ΔI_{ami} in all mutants tested. Under the assumption that chymotrypsin maximally activates ENaC, different average P_{o} values of the mutant channels should become apparent by differences in the relative activation of ΔI_{ami} by chymotrypsin. Figure 9B compares the normalized baseline ΔI_{ami} before chymotrypsin activation with the relative activation of ΔI_{ami} by chymotrypsin. This figure demonstrates that a higher baseline ΔI_{ami} is related to a lower relative activation by chymotrypsin. This supports the conclusion that the higher baseline currents observed for some of the mutant channels are caused by a higher average P_{o} before chymotrypsin activation. In summary, our data demonstrate that, in most cases, substitution of the βV348 residue by another amino acid affects channel function by altering average channel P_{o}. Only in some cases, with largely reduced ENaC currents (V348Q and V348del mutants), the loss-of-function effect was caused by reduced channel surface expression.

To better understand the effects of different amino acid substitutions at βV348, we used computational ENaC modeling according to the crystal structure of ASIC1 (26). Structural analysis of the trimeric ENaC model (Fig. 10A) reveals that V348 of the β-subunit is located within a globular domain (termed palm domain) that is located proximal to the membrane (Fig. 10B). Interestingly, our model indicates that V348 forms interactions not only within the palm domain but also with the residue M90, which is located in the linker between the palm and the β-ball domain (Figs. 10B and 11A). Molecular modeling reveals that the majority of the mutations investigated destabilize the structure of the closed ENaC for the following reasons: The lack of the side chain in the V348G mutant results in a loss of the contacts to M90 (Fig. 11B), thereby reducing the stability of the corresponding region. A mutation to lysine (V348K) is unfavorable because of steric clashes with M90 and electrostatic repulsion with K350 (Fig. 11C). Clashes of residue 348 with M90 are also observed for V348M (Fig. 11D), as well as V348L and V348W (data not shown). In summary, all mutants described above are either characterized by a loss of contact with M90 or by steric clashes with M90. Both effects are energetically unfavorable and are expected to destabilize the interaction between the palm and the β-ball domain (Fig. 10B). In contrast, a mutation to glutamate or aspartate allows the formation of a novel stabilizing salt-bridge with K350 (Fig. 11E). This may explain why mutating valine to glutamate or aspartate in this position has no stimulatory effect on the channel. Interestingly, the same type of interaction is also observed in the crystal structure of the homologous ASIC1 channel (Fig. 11F).

The βV348M mutation stimulates ENaC-mediated whole-cell currents in HEK293 cells. To rule out that the gain-of-function effect of the βV348M mutation is limited to the Xenopus laevis oocyte expression system, we transiently transfected HEK293 cells with wild-type αβγENaC or mutant αβV348MγENaC and measured amiloride-sensitive whole-cell currents using the patch-clamp technique. Figure 12A shows representative whole-cell current recordings from a cell expressing wild-type ENaC and a cell expressing mutant ENaC. ΔI_{ami} averaged 85 ± 15 pA (n = 15) in wild-type ENaC-expressing cells and 157 ± 26 pA (n = 12, P < 0.05) in mutant ENaC-expressing cells. To correct for differences in cell size, current density was calculated. Current density averaged 8.0 ± 1.3 pA/pF (n = 15) in wild-type ENaC-expressing cells and 14.8 ± 2.4 pA/pF (n = 12, P < 0.05) in mutant ENaC-expressing cells (Fig. 12B). These results indicate that the βV348M mutation also causes a gain-of-function effect in mammalian cells.

Fig. 9. Effect of chymotrypsin on different mutants. A: wild-type (wt) ENaC and different mutants were expressed in oocytes, and ΔI_{ami} was measured with two-electrode voltage-clamp technique. ENaC was activated with chymotrypsin as shown in Fig. 3. Data were pooled from 47 oocytes for wild-type and βV348M-ENaC and from 21–24 oocytes for the remaining mutants. To correct for batch-to-batch variability, data were normalized to mean ΔI_{ami} of wild-type ENaC-expressing oocytes before chymotrypsin activation. B: same data as in A. The relative chymotrypsin activation was calculated as the ratio of ΔI_{ami} after and before chymotrypsin activation and plotted against basal ΔI_{ami} before chymotrypsin activation.
DISCUSSION

In the present study, we investigated the effect of a mutation in βENaC (βV348M) on channel function. To the best of our knowledge, this is the first report on the functional effects of this mutation identified in a patient with severe CF-like symptoms (39). In the *Xenopus laevis* oocyte expression system, we found that the βV348M mutation stimulated ENaC whole-cell currents by ~40%. Using different experimental approaches, including single-channel recordings, we demonstrated that this gain-of-function effect is caused by an increased $P_o$ of the mutant channel. Moreover, we used transiently transfected HEK293 cells to confirm the gain-of-function effect of the βV348M mutation in a mammalian expression system. Our findings suggest that the gain-of-function effect of the βV348M mutation may contribute to CF pathophysiology by inappropriately increasing sodium and fluid absorption in the respiratory tract of affected patients.

Functional testing of the effect of the βV348M-ENaC mutation in the present study was performed in *Xenopus laevis* .
that MTSET increased the current densities after activation of ENaC including the recruitment of near-silent channels (36). This response of human ENaC is likely to reflect the situation in vivo at least under certain physiological conditions. Thus, the V348M mutation may well be relevant in native respiratory epithelia.

In the present study, chymotrypsin activated wild-type and Vβ348M-ENaC ~4.2-fold and ~3.0-fold, respectively. However, in previous studies on primary human nasal epithelia (17) or human bronchial epithelial cells (4, 11), trypsin failed to activate ENaC, which suggests that in these epithelia ENaC is already fully cleaved and active under resting conditions. In the oocyte expression system, the current levels of wild-type and Vβ348M-ENaC were similar after full stimulation with chymotrypsin. Thus, in respiratory epithelia with high protease activity and full proteolytic activation of all ENaCs present at the plasma membrane, the Vβ348M mutation may not result in a gain-of-function phenotype. However, it has been demonstrated that the effect of trypsin on ENaC in cell layers of respiratory epithelia depends on the experimental conditions used (40, 56). Prolonged equilibration periods in the Ussing chamber are thought to wash out endogenous protease inhibitors from the ASL, leading to disinhibition of proteases and hence to proteolytic ENaC activation. In contrast, shortly after changing the epithelial cell layers from the air-liquid interface to the Ussing chambers, trypsin has been shown to have a stimulatory effect on ENaC (5, 40). This latter situation is likely to reflect the situation in vivo at least under certain physiological conditions. Thus the gain-of-function effect of the V348M mutation may well be relevant in native respiratory epithelia.

The presence of V348 in βENaC was reported by Lu et al. (36) to be essential for the stimulatory effect of the novel small molecule ENaC activator S3969. This was concluded from the finding that deletion of βV348 abolished the stimulatory effect of S3969 on ENaC. We were unable to reproduce this finding because of an insufficient expression of the βV348del mutant at the oocyte membrane. We confirmed the stimulatory effect of S3969 on wild-type ENaC described by Lu et al. (36), but
the stimulatory effects of chymotrypsin and MTSET on ENaC were larger than that of S3969. This suggests that S3969 did not fully activate ENaC under our experimental conditions. A likely explanation for this finding is that the S3969 concentration used in the present study (10 μM) was not sufficient to achieve a maximal stimulation. Indeed, Lu et al. (36) reported a maximal stimulation of ENaC by S3969 at a threefold higher concentration (30 μM) (36) than that used in the present study. Importantly, βV348M-ENaC could also be stimulated by S3969. This demonstrates that the βV348M mutation has no impact on the stimulatory effect of S3969 on ENaC.

To further elucidate the molecular mechanism by which the βV348M mutation causes an increase in average channel P0, we tested the functional effect of different amino acid substitutions at the βV348 residue on ENaC function. Interestingly, substitution of the βV348 residue by several amino acids resulted in ENaC stimulation. However, this effect could not be correlated to the size or charge of the amino acids. Therefore, we used computational channel modeling to analyze the putative microdomain around βV348. The results of this modeling suggest a qualitative correlation between the structural effect of the mutants and the observed effect on ΔIuni. Mutants that destabilize the closed ENaC structure (V348 → G, K, M, L, W) generally result in increased ΔIuni, whereas stabilizing mutants do not affect (V348E) or even reduce ΔIuni (V348D). Therefore, it is tempting to speculate that mutants at position 348 that destabilize the closed ENaC conformation facilitate the closed to open transition, thereby increasing the open probability of the channel. This may also explain our experimental finding that the βV348M mutation reduced the number of channel transitions per second in single-channel recordings. A thorough structural interpretation of this effect, however, has to await the crystal structure of an open channel, which is currently not available.

Epithelia of conducting airways are covered by a mucus layer, which not only prevents desiccation but also scavenges inhaled particles. The mucus layer floats on a liquid or gel-like layer, the so-called periciliary layer (PCL), and is transported by cilia to the pharynx, where it is removed from the airways by coughing or swallowing, a process termed mucociliary clearance. Together, the mucus layer and the PCL are often referred to as the ASL layer (6). A well-defined microenvironment is essential for proper cilia function. In cultured human airway epithelial cells from healthy donors, the PCL was found to have the same height as that of outstretched cilia (~7 μm), whereas the height of the PCL was largely reduced (~3 μm) in cultured airway epithelial cells from patients with CF (38). Flattening of the PCL leads to steric hindrance of the cilia and thus impairs mucociliary clearance (32). Precise regulation of ASL volume is therefore an important prerequisite for proper lung defense. Interestingly, PCL height was similar in excised tracheal epithelia from newborn non-CF and CFTR−/− pigs, which had not yet developed airway inflammation (9). Thus it is still unclear whether flattening of the PCL in patients with CF occurs before the onset of inflammation or whether inflammation occurs first and affects transepithelial electrolyte transport, which then reduces PCL height. The gain-of-function effect of the βV348M mutation could favor disease in both situations. On the one hand, increased Na+ absorption via overactive ENaC could flatten the PCL and thus reduce mucociliary clearance. On the other hand, the βV348M mutation could increase the vulnerability of airway epithelia to inflammatory processes by reducing the threshold for development of CF symptoms and thus influence the manifestation and intensity of the disease. In this context, it is of interest that the patient reported by Mutesa et al. (39) was compound heterozygous for the βV348M mutation and a F693L-CFTR mutation. The F693L-CFTR mutation was described as a polymorphism that, compared with wild-type CFTR, did not affect channel maturation in COS1 cells or chloride transport ability in oocytes (57). Therefore, it seems unlikely that the F693L-CFTR mutation alone is sufficient to cause the disease. However, in combination with the βV348M-ENaC gain-of-function mutation, it may contribute to the development and/or manifestation of CF disease.

In summary, we have shown that the βV348M mutation found in a patient with severe CF-like symptoms (39) stimulates ENaC activity by an increase in average channel P0. The underlying mechanism for this increase is probably a destabilization of the closed channel state by the mutation. Our finding that the βV348M mutation has a gain-of-function effect may provide an explanation for the symptoms of the affected patient according to the pathophysiological concept that elevated Na+ transport reduces mucociliary clearance in respiratory epithelia and favors CF-like pulmonary symptoms. However, additional factors may contribute to the patient’s phenotype, and further studies are needed to elucidate the role of ENaC in the pathophysiology of CF.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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