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A Gain-of-Function Mutation in Gating of *Corynebacterium glutamicum* NCgl1221 Causes Constitutive Glutamate Secretion

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The A-to-V mutation at position 111 (A111V) in the mechanosensitive channel NCgl1221 (MscCG) causes constitutive glutamate secretion in *Corynebacterium glutamicum*. Patch clamp experiments revealed that NCgl1221 (A111V) had a significantly smaller gating threshold than the wild-type counterpart and displayed strong hysteresis, suggesting that the gain-of-function mutation in the gating of NCgl1221 leads to the oversecretion of glutamate.

Corynebacterium glutamicum is used worldwide for the industrial fermentative production of glutamate. Under biotin-limiting conditions, this organism secretes a large amount of glutamate (11). Reagents that result in a change in the cell surface structure, such as fatty acid ester surfactants and penicillin, induce glutamate secretion even in the presence of biotin (5). Recent studies have revealed that NCgl1221 (MscCG), a homolog of the mechanosensitive channel of small conductance (MscS) of *Escherichia coli*, functions as a glutamate exporter. Mutations in NCgl1221 lead to constitutive glutamate secretion, and disruption of the gene abolishes its secretion (8). NCgl1221 is localized at the plasma membrane (14) and has mechanosensitive channel activity when expressed in *E. coli* spheroplasts (2) and *Bacillus subtilis* provacuoles (4). These findings suggest that the mechanosensitive gating of NCgl1221 caused by membrane distortion triggers glutamate secretion.

In this study, to elucidate the molecular basis of the relationship between NCgl1221 gating and glutamate secretion, we investigated electrophysiological properties of the NCgl1221 (A111V) mutant protein, having an A-to-V mutation at position 111, which causes constitutive glutamate secretion (8). On the basis of amino acid sequence alignment between NCgl1221 and *E. coli* MscS, Ala^{111} in NCgl1221 was shown to be a residue corresponding to Ala^{106} or Ala^{110} in *E. coli* MscS. These alanine residues of MscS reside in the third transmembrane domain (TM3; residues 95 to 126), which forms an ion-conducting pore (1), and the mutations alter the gating threshold (3). Thus, we hypothesized that the A111V mutation in NCgl1221 alters the gating property of the channel.

The difference in gating between wild-type NCgl1221 and NCgl1221 (A111V) was examined by patch-clamping *E. coli* spheroplasts expressing these channels. The NCgl1221 gene was amplified by PCR from pVK9-NCgl1221 (8) and cloned into the expression vector pB10b (9) with the In-Fusion cloning kit (TaKaRa). The A111V mutation was created with a mutagenesis kit (Toyobo). *E. coli* spheroplasts were prepared as described previously (7). Strains PB113 (Δ*mscS* Δ*mscK*) (6) and MJF612 (Δ*mscM* Δ*mscS* Δ*mscK* Δ*mscL*) (10) harboring pB10b, pB10b-NCgl1221, or pB10b-NCgl1221 (A111V) were incubated to the exponential growth phase. NCgl1221 expression was induced by 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at 37°C for 30 min. Single-channel currents were recorded with inside-out excised patches. The pipette solution contained 200 mM KCl, 90 mM $MgCl_2$, 10 mM $CaCl_2$, and 5 mM HEPES-KOH (pH 7.2). The bath solution consisted of the pipette solution supplemented with 300 mM sucrose to stabilize the spheroplasts. Currents were amplified using an Axopatch 200B amplifier (Molecular Devices, California) and filtered at 2 kHz. Current recordings were digitized at 5 kHz using a Digidata 1322A interface with pCLAMP9 software (Molecular Devices). Negative pressure was applied to the patch membrane as mechanical stimulation using a high-speed pressure clamp (HSPC) apparatus (ALA Scientific Instruments).

When PB113 was transfected with an empty vector and an increasingly negative pressure was applied to the patch membrane by suction at constant ramp rate (Fig. 1A), endogenous *E. coli* MscL with ~80 pA single-channel currents were elicited at approximately 200 mmHg (Fig. 1B). When transfected with a vector harboring wild-type NCgl1221, on the other hand, NCgl1221 started to open at approximately 60 mmHg (Fig. 1C, OP_{CG}), and endogenous *E. coli* MscL was subsequently elicited at approximately 170 mmHg (OP_L). The magnitude of single-channel currents of NCgl1221 was 5 pA. NCgl1221 was distinguished from MscL by the smaller single-channel current and lower activation threshold. When the negative pressure was reduced at the same ramp rate, *E. coli* MscL and wild-type NCgl1221 closed at pressures (CP_L and CP_{CG}) similar to those for the opening. In contrast, NCgl1221 (A111V) opened at a negative pressure lower than that for the wild type (Fig. 1C): NCgl1221 (A111V) opened at approximately 20 mmHg (OP_{CG}) and *E. coli* MscL was subsequently elicited at approximately 130 mmHg (OP_L). Interestingly, NCgl1221 (A111V) closed barely a few seconds after the complete release of the negative pressure (CP_{CG}), while *E. coli* MscL closed at the same pressure (CP_L) as OP_L (Fig. 1D).

To evaluate the changes in the gating threshold of NCgl1221, the ratio of the pressure required for the gating of *E. coli* MscL (P_L) to NCgl1221 (P_{CG}) was calculated using P_L as an internal standard. This ratio rather than the pressure was used

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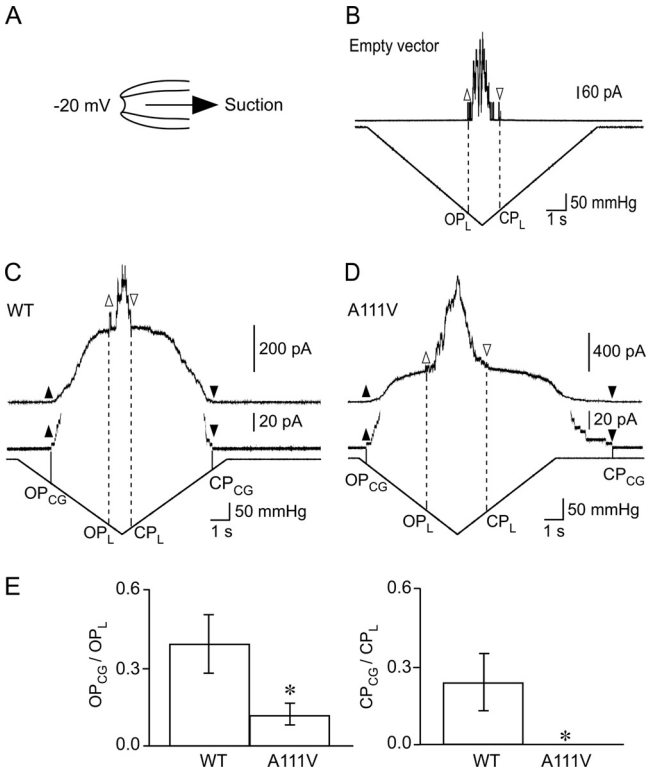

FIG 1 Electrophysiological analysis on gating of wild-type NCgl1221 and NCgl1221 (A111V). (A) Configuration of mechanosensitive current recording in *E. coli* spheroplasts. Negative pressure to the patch membrane was applied by suction with HSPC. All experiments were performed at holding potential of −20 mV. (B) Mechanosensitive currents in strain PB113 (*mscS mscK*) harboring an empty vector. Upper traces and lower traces show the current and the negative pressure applied to the patch membrane through the pipette, respectively. Mechanosensitive current recording in PB113 expressing wild-type NCgl1221 (WT) (C) and NCgl1221 (A111V) (D). Middle traces show vertical enlargements of the upper traces. Upward and downward arrowheads represent the start and end of channel activity, respectively, of NCgl1221 (black) and *E. coli* MscL (white). (E) Quantitative analysis of the threshold for gating in wild-type NCgl1221 and NCgl1221 (A111V). The ratio (OP_{CG}/OP_L) of the pressure required for the opening of *E. coli* MscL (OP_L) to NCgl1221 (OP_{CG}) is shown in the left panel, and the ratio (CP_{CG}/CP_L) of the pressure required for the closing of *E. coli* MscL (CP_L) to NCgl1221 (CP_{CG}) is shown in the right panel. *, $P < 0.01$, compared to wild-type NCgl1221 (Student's *t* test). Error bars represent standard deviations of the data of wild-type NCgl1221 ($n = 8$) and NCgl1221 (A111V) ($n = 5$).

because mechanosensitive channels are activated by membrane tension (T), which is defined by the radius (r) of membrane curvature and magnitude of pressure (P) according to Laplace's law ($T = Pr/2$) (12), but the radius varies slightly from patch to patch. The use of the ratio allows for compensating the effect of the radius because the radius is expected to be the same when examined on identical patches. OP_{CG}/OP_L for the opening of NCgl1221 (A111V) (0.12 ± 0.04, $n = 5$) was significantly smaller than that of wild-type NCgl1221 (0.39 ± 0.11, $n = 8$) (Fig. 1E), suggesting that the A111V mutation makes NCgl1221 easy to open. While CP_{CG}/CP_L for the closing of wild-type NCgl1221 (0.24 ± 0.11, $n = 8$) did not differ statistically from OP_{CG}/OP_L for the opening, NCgl1221 (A111V) did not close during the presence of negative pressure in all experiments. The difference in the thresholds for opening

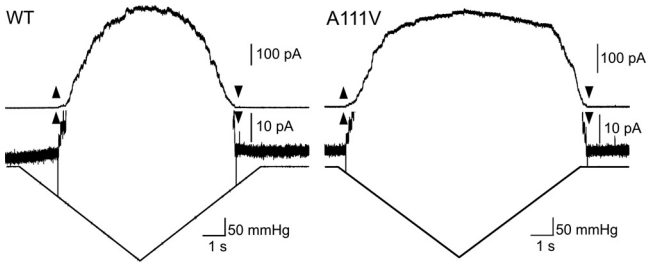

FIG 2 Mechanosensitive channel activity of wild-type NCgl1221 (left) and NCgl1221 (A111V) (right) in *E. coli* strain MJF612 (Δ*mscM* Δ*mscS* Δ*mscK* Δ*mscL*). The membrane current and its vertical enlargement and pressure are shown from the top to the bottom. Upward and downward arrowheads represent the start and end of the channel activity of NCgl1221, respectively. All experiments were performed at a holding potential of −20 mV.

and closing shows that the gating of NCgl1221 (A111V) has strong hysteresis, which is not evident in wild-type NCgl1221.

To exclude possible influence of MscM and MscL, we examined NCgl1221 in strain MJF612 (Δ*mscM* Δ*mscS* Δ*mscK* Δ*mscL*), lacking all four mechanosensitive channels cloned so far (10). NCgl1221 (A111V) expressed in MJF612 also opened at low pressure and displayed strong hysteresis, whereas wild-type NCgl1221 did not (Fig. 2). This result suggests that the change of gating kinetics in NCgl1221 (A111V) is not influenced by endogenous *E. coli* mechanosensitive channels.

E. coli MscS is a homoheptamer, each subunit of which has three transmembrane domains and a cytoplasmic domain (1). Replacement of conserved alanines in the pore region with glycine (A106G and A110G) decreases the gating threshold and results in a gain-of-function phenotype (3, 13). These alanines (knobs) are proposed to line up against the conserved glycines (holes) of an adjacent subunit and stabilize the closed state (3). Despite the steric importance, amino acid sequence alignment of TM3 between *E. coli* MscS and NCgl1221 shows that the conserved alanines and glycines present in *E. coli* MscS are poorly conserved in NCgl1221 (Fig. 3), suggesting that the low threshold of NCgl1221 may be due to the loose alignment of TM3.

NCgl1221 is a mechanosensitive channel gated by membrane tension and functions not only for glutamate secretion but also for osmotic safety valves (2, 4). Thus, we propose that fluctuations in membrane tension caused by endogenous osmotic pressure activate NCgl1221 (A111V), which has a gating threshold significantly lower than that of wild-type NCgl1221. The strong hysteresis of the gating of NCgl1221 (A111V) possibly keeps this channel open, leading to constitutive glutamate secretion. Thus, the present study suggests that constitutive glutamate secretion by NCgl1221 (A111V) is tightly associated with the altered gating property of NCgl1221.

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MscS      95 SVIAVLGAAGLAVGLALQGSLSNLAAGVLLVM 126
NCgl1221  95 GAAIPATIASAAIGLGAQSIVADFLAGFFILT 126
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FIG 3 Amino acid sequence alignment of the transmembrane segment TM3 between *E. coli* MscS and NCgl1221. The positional regularities of conserved alanines (black) and glycines (gray) present in *E. coli* MscS are highlighted. Asterisks represent identical residues.

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