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, Ala111 in NCgl1221 was shown to be a residue corresponding to Ala106 or Ala110 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 (∆mscS ∆mscK) (6) and MJF612 (∆mscM ∆mscS ∆mscK ∆mssL) (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 MgCl2, 10 mM CaCl2, 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 (OP1). 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 (CP1 and CP1CG) 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 170 mmHg (OP1). Interestingly, NCgl1221 (A111V) closed barely a few seconds after the complete release of the negative pressure (CP1CG), while *E. coli* MscL closed at the same pressure (CP1) as OP1 (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 (P1) to NCgl1221 (P1CG) was calculated using P1 as an internal standard. This ratio rather than the pressure was used.
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) ($0.12 \pm 0.04$, $n = 5$) was significantly smaller than that of wild-type NCgl1221 ($0.39 \pm 0.11$, $n = 8$) (Fig. 1E), suggesting that the A111V mutation makes NCgl1221 easy to open. While $CP_{CG}/CP_L$ for the closing of wild-type NCgl1221 ($0.24 \pm 0.11$, $n = 8$) 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 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 ($\Delta mscM \Delta mscS \Delta mscK \Delta mscL$), 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 alamines in the pore region with glycine (A106G and A110G) decreases the gating threshold and kinetics in NCgl1221 (A111V) is not influenced by endogenous E. coli mechanosensitive channels.
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