Involvement of pore helix in voltage-dependent inactivation of TRPM5 channel

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ABSTRACT

The transient receptor potential melastatin 5 (TRPM5) channel is a monovalent-permeable cation channel that is activated by intracellular Ca\textsuperscript{2+}. Expression of TRPM5 has been shown in taste cells, pancreas, brainstem and olfactory epithelium, and this channel is thought to be involved in controlling membrane potentials. In whole-cell patch-clamp recordings, TRPM5 exhibited voltage-dependent inactivation at negative membrane potentials and time constant of voltage-dependent inactivation of TRPM5 did not depend on the intracellular Ca\textsuperscript{2+} concentrations between 100 and 500 nM. Alanine substitution at Y913 and I916 in the pore helix of TRPM5 increased time constant of voltage-dependent inactivation. Meanwhile, voltage-dependent inactivation was reduced in TRPM5 mutants having glycine substitution at L901, Y913, Q915 and I916 in the pore helix. From these results, we conclude that the pore helix in the outer pore loop might play a role in voltage-dependent inactivation of TRPM5.

1. Introduction

Transient receptor potential melastatin 5 (TRPM5) is a cation channel that is permeable to monovalent cations, but not divalent cations. Although most TRP channels have multiple ligands, there are few reports concerning TRPM5 ligands. Only one de...

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inner pore by the N-terminal region. In contrast, C-type inactivation is thought to involve a subtle conformational change around the selectivity filter. TRP channels also exhibit voltage dependence and mutant analyses suggested that the pore helix is involved in voltage-dependent inactivation in TRPA1 and TRPP3 [13,14]. Although TRPM5-mediated currents also exhibit voltage dependence, its inactivation mechanisms remain unclear.

In this study, we demonstrate that voltage-dependent inactivation of TRPM5 is independent of [Ca\textsuperscript{2+}] and that the pore helix in the outer pore loop could be involved in the voltage-dependent inactivation.

2. Results

First, we confirmed the voltage-dependency of TRPM5-mediated currents using a whole-cell patch-clamp method in HEK293T cells expressing mouse TRPM5 (mTRPM5). Application of step-pulses in the presence of 0 or 30 nM Ca\textsuperscript{2+} did not evoke any current in HEK293T cells expressing mTRPM5. On the other hand, application of step-pulses in the presence of intracellular 100, 300 or 500 nM Ca\textsuperscript{2+} produced currents that had a slowly-activated large outward components and a rapidly-declined inward components in HEK293T cells expressing mTRPM5, while currents in the presence of intracellular 100 nM Ca\textsuperscript{2+} looked smaller than those seen in the presence of intracellular 300 and 500 nM Ca\textsuperscript{2+} (Figure 1A). Furthermore, the kinetics of the activating current induced by 100 nM Ca\textsuperscript{2+} at positive membrane potentials appeared to be slower than those by 300 and 300 nM Ca\textsuperscript{2+}, suggesting that this channel kinetics depend on [Ca\textsuperscript{2+}], or its activity. The peak currents at -160 mV in the presence of 300 and 500 nM Ca\textsuperscript{2+} were significantly larger than those in the presence of 0, 30 and 100 nM Ca\textsuperscript{2+} (Figure 1B). We next analyzed the inactivation kinetics of mTRPM5 currents at -160 mV. The current ratio, which provides an indication of the inactivation ability, was similar among 100, 300 and 500 nM Ca\textsuperscript{2+} (Figure 1C), although the peak current amplitude with 100 nM Ca\textsuperscript{2+} was smaller than those in the presence of 300 nM and 500 nM Ca\textsuperscript{2+}. The inactivating time constants were not

![Figure 1. Relationship between intracellular Ca\textsuperscript{2+} concentration and voltage dependency in the activation of TRPM5. (A) Representative traces of whole-cell currents stimulated by step-pulses in the presence of intracellular 0, 30, 100, 300 or 500 nM Ca\textsuperscript{2+} in HEK293T cells expressing mouse TRPM5 (mTRPM5). Box indicates the current expanded from 100 nM Ca\textsuperscript{2+}-induced current at negative membrane potentials. The inset shows the step-pulse protocol. (B) Dose-response profiles of current densities in HEK293T cells expressing mTRPM5 at -160 mV. (C) The ratio of peak to steady-state (at 200 ms) current in HEK293T cells expressing mTRPM5 activated by intracellular 100, 300 or 500 nM Ca\textsuperscript{2+}. (D) Inactivation time constant of whole-cell currents at -80, -120 or -160 mV in HEK293T cell expressing mTRPM5 activated by intracellular 100, 300 or 500 nM Ca\textsuperscript{2+}. Each column represents the mean ± S.E.M. for 6–12 cells. Statistical significance was assessed using ANOVA followed by the two-tailed multiple t-test with Bonferroni correction. **; P < 0.01 vs. 0 or 30 nM Ca\textsuperscript{2+}.](image-url)
largely different among -80, -120 and -160 mV, suggesting that the inactivating time constants do not depend on membrane potentials (Figure 1D), and the representative fitting curves were shown in Figure 6A). Furthermore, the inactivating time constants were also not different among 100, 300 and 500 nM Ca\(^{2+}\) (Figure 1D).

We hypothesized that the pore helix could be involved in the voltage-dependent inactivation of TRPM5. To test this hypothesis, we next constructed and analyzed mTRPM5 mutants. As shown in Figure 2A and B, 16 amino acids are thought to comprise the pore helix of TRPM5 channel based on alignment analysis of the amino acid sequence between TRPM4 and TRPM5. Therefore, we constructed single mutants in which alanine was substituted for each of the 16 amino acids in the pore helix (L901A, E902A, I904A, F905A, V908A, Y913A, Q915A and I916A). We excluded W903A, R906A, R907A, I909A, V911A, P912A and L914A mutants from analysis, because channels having these substitutions showed no current activation in response to step-pulses even in the presence of intracellular 500 nM Ca\(^{2+}\) (Figure 3A). We confirmed the protein expression of TRPM5 mutants in HEK293T cells by immunocytochemistry using an anti-TRPM5 antibody. While TRPM5 L901A and L909A appeared to be localized to the intracellular organelles, fluorescent signals of TRPM5 wild-type (WT) and the other TRPM5 mutants was observed throughout the HEK293 cells (Figure 4), suggesting that the protein of TRPM5 mutants were expressed in HEK293 cells. We then examined the currents of L901A, E902A, I904A, F905A, V908A, Y913A, Q915A and I916A mutants in the presence of 500 nM Ca\(^{2+}\), and found that inactivation of Y913A and I916A mutants at negative membrane potentials appeared to be weaker than that of TRPM5 WT (Figure 3A). Although the current ratios of TRPM5 mutants at -80, -120 and -160 mV did not differ from that for TRPM5 WT, the inactivation time constants (\(\tau_{\text{inact}}\)) at -120 and -160 mV for Y913A and I916A were significantly larger than that for TRPM5 WT (Figure 3A). In addition, substitution of amino acids was thought to affect the structure of the selective filter. Structural information of hTRPM4 and mTRPM4 was referenced from cryo-EM structural dynamics simulations. We confirmed that substitution of glutamine at 915 to glycine reduced the stability of the \(\alpha\)-helix structure at G918 and Q919 compared to TRPM5 WT, and increased the probability of the \(\alpha\)-helix structure at Q918 and Q919 compared to TRPM5 WT, while substitution to alanine did not affect to the structure of the selective filter from P917 to Q919 (Figure 7).

3. Discussion

In this study, we showed that TRPM5 channels exhibited voltage-dependent inactivation at negative membrane potentials that was independent of [Ca\(^{2+}\)]\(_i\) (Figure 1). In addition, substitution of amino acids within the pore helix of TRPM5 channel impaired the voltage-dependent inactivation.

The peak amplitude of activating currents of TRPM5 depends on [Ca\(^{2+}\)]\(_i\) above 100 nM at both positive and negative membrane potentials, which is similar to the report by another study [15]. On the other hand, inactivation of TRPM5 was independent of [Ca\(^{2+}\)]\(_i\) (Figure 1). Changes in [Ca\(^{2+}\)]\(_i\) to higher than 40 \(\mu\)M induce not only activation but also desensitization of TRPM5 in the excised patch membrane [4]. The time constant of desensitization were between 0.6 and 4 s, which are much larger than that for voltage-dependent inactivation (i.e., several dozens of ms), suggesting that mechanisms for Ca\(^{2+}\)-dependent desensitization and voltage-dependent inactivation are different. There are two major mechanisms for voltage dependence of TRP channels: 1) voltage-dependent pore blockage by divalent cations and 2) intrinsic

Figure 2. Putative amino acid sequence of the pore helix of mouse TRPM5. (A) Schematic diagram of mouse TRPM5 (mTRPM5). H: pore helix; F: selective filter; T: transmembrane domain. (B) Sequence alignment of the pore regions in human TRPM4 (hTRPM4), mTRPM4 and mTRPM5. Highlighted areas denote the pore helix region and areas outlined with a dotted line denote the selective filter. Structural information of hTRPM4 and mTRPM4 was referenced from cryo-EM structural analysis by Windler et al. [11] and Guo et al. [10].
Figure 3. The effect of alanine substitutions in the pore helix region on voltage-dependent inactivation of mouse TRPM5. (A) Representative traces of whole-cell currents stimulated by step-pulses with intracellular 500 nM Ca\(^{2+}\) in HEK293T cells expressing mTRPM5/L901A, E902A, W903A, I904A, F905A, R906A, R907A, V908A, L909A, Y910A, R911A, P912A, Y913A, L914A, Q915A and I916A mutants. (B) The ratio of peak to steady-state (at 200 ms) current in HEK293T cells expressing mTRPM5 wild-type (WT) and L901A, E902A, I904A, F905A, V908A, Y913A, Q915A or I916A mutants activated by 500 nM Ca\(^{2+}\) at -80, -120 and -160 mV. (C) Inactivation time constant of whole-cell currents in HEK293T cells expressing mTRPM5 WT and L901A, E902A, I904A, F905A, V908A, Y913A, Q915A or I916A mutants activated by intracellular 500 nM Ca\(^{2+}\) at -80, -120 and -160 mV. Each column represents the mean ± S.E.M. for 6–18 cells. Statistical significance was assessed using Dunnett’s test. *, P < 0.05 vs. WT.
Another possible mechanism is transition of the pore from an open state to an inactivated state that involves tilting of the lower portions of the TM6 helices toward the center of the pore, as is seen for the effect of calmodulin on TRPV6 [17]. TRPM4, which is closely related to TRPM5, also exhibits the voltage-dependent inactivation at negative membrane potentials, similar to that of TRPM5. Voltage dependence of TRPM4 does not appear to be affected by divalent cations [18]. In addition, voltage-dependent inactivation of TRPM4 can be observed in inside-out and outside-out configurations [18]. These reports and our findings may indicate that voltage-dependent inactivation of TRPM5 is not caused by the interaction with intracellular molecules such as Ca$^{2+}$ and calmodulin.

C-type inactivation is seen for voltage-gated K$^+$ channels, and structural analyses of the KcsA K$^+$ channel suggested that such inactivation results from a conformational change in the selective filter. X-ray crystal structure analysis of the KcsA channel [19] showed partial collapse of the selective filter prevents K$^+$ conduction. Functionally, mutation of glutamate at 71 in the pore helix, which is an adjacent subunit to the selectivity filter, abolished the inactivation ability of the KcsA channel [20]. Although glutamate, an amino acid with electrically charged side chain, is not conserved in voltage-gated K$^+$ channels, the structural rearrangement of hydrogen bond network between selective filter and pore helix rather than a protonation of E71 is reported to be important for the inactivation of KcsA channel [21]. C-type inactivation is conserved among voltage-gated K$^+$ channels, as studies with Shaker (KCNA3) channel demonstrated that substitution of amino acids within the pore helix impairs the inactivation [20]. In this study, we found that voltage-dependent inactivation of mTRPM5 at negative membrane potentials was impaired by substitution of alanine at 915 to glycine (Figures 3 and 5), indicating that C-type inactivation could be involved in voltage-dependent inactivation of TRPM5 channels. It is also possible that pore collapse underlie the voltage-dependent inactivation of TRPM5 channels. We and another group previously reported that zinc ion and proton inactivate TRPM5 irreversibly [3,22]. While TM5-TM6 region was involved in these irreversible inactivation, electrical interaction with charged amino acids (such as glutamate and aspartate) was necessary for its inactivation, suggesting that the different mechanisms underlie between voltage-dependent inactivation and irreversible inactivation by zinc ion and proton in TRPM5.

Interestingly, voltage-dependent inactivation of TRPM5 was impaired by substitution of glutamine at 915 to glycine but not to alanine (Figures 3 and 5) and substitution of glutamine at 915 to glycine can reduce the structural stability of the selective filter (Figure 7), suggesting that the pore helix structure could modulate the gating of selective filter for voltage-dependent inactivation. Voltage-dependent inactivation of TRPM5 was also impaired by substitution of leucine at 901 to glycine but not to alanine. Although we cannot make definitive conclusions, one possible explanation is that substitution of leucine at 901 to glycine affects the stability of the selective filter. We also found that Y913 and I916 are also involved in voltage-dependent inactivation (Figures 3 and 5). The side chains of Y971 and I974 in TRPM4, which correspond to Y913 and I916 in TRPM5, are at conformationally opposite sides of the selective filter and near TM5 and TM6 [10]. Although structural analysis of TRPM5 with atomic resolution, such as cryo-EM analysis, is necessary, it is possible that the Y913 and I916 could interact with TM5 and TM6, and that modulate voltage-dependent inactivation of TRPM5. Our results suggest that the latter part of the pore helix may play an important role in voltage-dependent inactivation. Further analysis will help elucidate the precise mechanisms of voltage-dependent inactivation of TRPM5.

Given that the amino acid sequences of the pore helix are highly conserved among TRPM channels (Figure 2B and [10]), similar mechanisms could also be associated with the voltage-dependent inactivation of
TRPM4. Comparison of the amino acid sequence of the pore regions of TRPM5 and TRPM4 demonstrate that the amino acid sequences of both the pore helix and the selective filter are highly conserved (Figure 2B), suggesting that the same mechanisms may underlie the voltage-dependent inactivation of TRPM4. Similar inactivation was also reported for other TRP channels, such as TRPM2 with same pore helix sequences, which exhibits inactivation by pore collapse caused by conformational changes in selective filter [23]. In TRPA1 channels, L906 in the pore helix is considered to be essential for voltage-dependent gating [13]. In case of TRPP3 (also named PKD2L1), N533 in the pore loop region is essential for its voltage-dependent inactivation [24]. Thus, conformational dynamics of the pore region during voltage-dependent inactivation could be conserved among TRP channels.

4. Methods

4.1. Cell culture

Human embryonic kidney-derived 293T (HEK293T) cells were maintained in DMEM (WAKO Pure Chemical Industries, Ltd., Osaka,
Japan) containing 10% FBS (Thermo Fisher Scientific Inc, Massachusetts, USA), 100 units/mL penicillin (Thermo Fisher Scientific Inc), 100 μg/mL streptomycin (Thermo Fisher Scientific Inc), and 2 mM L-glutamine (GlutaMAX, Thermo Fisher Scientific Inc) at 37 °C and 5% CO2. For patch-clamp recordings, 1 μg of plasmid DNA containing mouse TRPM5 in pcDNA3.1 and 0.1 μg pGreen Lantern 1 plasmid in OPTI-MEM medium (Thermo Fisher Scientific Inc) were transfected to HEK293T cells using Lipofectamine Plus Reagent (Thermo Fisher Scientific Inc). After incubating for 3–4 h, cells were reseeded on coverslips and further incubated at 37 °C in 5% CO2. Whole-cell patch-clamp recordings were performed one day after transfection.

4.2. Construction of TRPM5 mutants

The mutants of mouse TRPM5 (mTRPM5) were made using a modified QuickChange Site-Directed Mutagenesis method (Stratagene Corp., California, USA), as we reported previously [25]. In detail, PCR was performed using mTRPM5 expression vectors as templates, two synthetic oligonucleotide primers containing specific mutations (Table 1), and PrimeSTAR Max DNA Polymerase (Takara Bio Inc., Shiga, Japan). The PCR products were digested with DpnI at 37 °C for 1 h and transformed into DH5α competent cells. The entire sequence including the desired substitution in the mutants was confirmed.

![Figure 6](image_url). Representative fitting curve for determination of inactivation time constants. (A) Representative traces of whole-cell currents (black), fit with exponential functions (orange) stimulated by step-pulses in the presence of intracellular 100 or 500 nM Ca2+ in HEK293T cells expressing mouse TRPM5 wild-type (WT). (B) Representative traces of whole-cell currents (black), fit with exponential functions (orange) stimulated by step-pulses in the presence of intracellular 500 nM Ca2+ in HEK293T cells expressing mouse TRPM5 L901A and Y913A. (C) Representative traces of whole-cell currents (black), fit with exponential functions (orange) stimulated by step-pulses in the presence of intracellular 500 nM Ca2+ in HEK293T cells expressing mouse TRPM5 E902G and I916G.

![Figure 7](image_url). Results of molecular dynamics simulation confirming the stability of the structures of mouse TRPM5 wild-type, Q915A and Q915G. (A) Percent helical occupancy for each amino acid residue from L894 to R929 of mouse TRPM5 wild-type (WT), Q915A and Q915G. (B) Snapshots of the molecular dynamics simulation of mouse TRPM5 Q915G at 0 ns (left) and at 100 ns (right). Amino acid residues at 915 are shown in a stick representation.
Table 1. Primers used for construction of TRPM5 mutants.

| Mutation | Sense Primer (5′→3′) | Antisense primer (5′→3′) |
|----------|-----------------------|--------------------------|
| L916A    | CATGATGGCGCTGGGGAGTTTGT    | GAAATTCACCTCCGAGGCATGTG |
| E902A    | GATGCGCCGCTGGGGAGTTTGT    | GCCGCTTATCCGGGAAAGACTG   |
| W903A    | GCGTTGAGGCGAGTTTTCGCGG   | ACGGGCTATCCGGGAAAGACTG   |
| I904A    | CGTTGAGGCGAGTTTTCGCGG   | CACGGCCTATCCGGGAAAGACTG   |
| F905A    | TTGGAGTGGATTGGCCGCCGTG    | TTAGAGATCGTGGATGCGCTT    |
| I904A    | CGTTGAGGCGAGTTTTCGCGG   | CACGGCCTATCCGGGAAAGACTG   |
| E902A    | GATGGCCGTTTGGGGTGGATTTTC | GTATAGCACAGCGCGGAAAATCCA |
| L901A    | CATGATGGCCGTGGGGAGTGGATTT | GTATAGCACAGCGCGGAAAATCCA |

4.3. Electrophysiology

HEK293T cells on coverslips were mounted in an open chamber (Warner Instruments LLC, Connecticut, USA) and superfused with a bath solution containing 140 mM NaCl, 5 mM KCl, 2 mM CaCl2, 2 mM MgCl2, 10 mM glucose and 10 mM HEPES (pH 7.4 with NaOH). The pipette solution was adjusted to a Ca2+ concentration of 0, 30, 100, 300 or 500 nM (calculated by Calibuto; http://www.kuleuven.be/fysio/trp/cabu) and was used for all experiments. The pipette solution contained 120 mM K-aspartate, 10 mM KCl, 1 mM MgCl2, 5 mM EGTA, 10 mM HEPES (pH 7.4 with KOH), and CaCl2 was added as follow: 0 mM CaCl2 for 0 nM Ca2+, 1.603 mM CaCl2 for 30 nM Ca2+, 3.032 mM CaCl2 for 100 nM Ca2+, 4.105 mM CaCl2 for 300 nM Ca2+, or 4.428 mM CaCl2 for 500 nM Ca2+. Data from whole-cell voltage-clamp recordings were sampled at 10 kHz and filtered at 5 kHz for analysis (Axopatch200B amplifier with pCLAMP10 software, Axon Instruments, California, USA). The membrane potential was clamped at 0 mV and voltage step-pulses from -160 to +160 mV (200 ms) were applied every second. All experiments were performed at room temperature (RT).

The inactivating time constant was estimated by fitting a single exponential curve between the peak current and the stimulus offset (200 ms):

\[ I = I_0 \times \exp(-t/\tau_{\text{inact}}) + I_o \]

Representative curve fittings were shown in Figure 6.

The current ratio was calculated as follow:

The current ratio = the current amplitude at 200 ms (steady-state)/the peak current amplitude.

Analysis of currents was performed using pCLAMP10 software.

4.4. Immunocytochemistry

HEK293 cells were cultured on collagen I and poly-L-lysine-coated 4-well slides (Lab-Tek II chamber slide, Thermo Fisher Scientific Inc) and incubated with Lipofectamine 3000 reagent (Thermo Fisher Scientific Inc) with 0.5 µg of each plasmid per well. Two days later, the cells were treated with 4% paraformaldehyde (15 min, 37 °C) and then 0.1% TritonX-100 (5 min, RT), followed by incubation with 10% goat serum. Then, the cells were incubated with anti-TRMP5 (1:200, Cat No. 18027-1-AP, Proteintech, Illinois, USA) for 2 h at RT, followed by a 1-hour incubation with anti-rabbit IgG-Alexa Fluor 488 (1:800, Abcam, Cambridge, UK) and TO-PRO-3 iodide (1:800, Thermo Fisher Scientific Inc) at RT. Fluorescence was observed using a confocal microscope (LSM510; Carl Zeiss Micromaging GmbH, Jena, Germany). ZEN 2009 (Carl Zeiss) was used for image processing.

4.5. Molecular dynamics simulations

Molecular dynamics (MD) simulations were carried out to investigate the structures of the pore helix regions of TRMP5, Q915A and Q915G. Residues 894–929 were employed as the pore helix region in our MD simulations. Because the tertiary structure of TRMP5 has not yet been determined, the tertiary structure of TRMP4 (PDB ID: 6BLU) was utilized for modeling the pore helix regions of TRMP5 and its mutants. We obtained the initial structures by replacing the sidechains in TRMP4 with those in TRMP5 (or the mutants). The N- and C- termini were capped by ace- and N-methyl group, respectively. Each system consisted of one chain of the respective pore helix regions, one sodium ion, and water molecules. The number of water molecules was 3015 for the WT and Q915A systems and 3016 for the Q915G system. These molecules were prepared in a cubic simulation box with a side length of 46.608 Å. Four different initial velocities were prepared for the statistical analysis. MD simulations were performed by the Generalized-Ensemble Molecular Biophysics (GEMB) program [26], which was developed by one of the authors (H. Okumura). This program has been applied for several protein and peptide systems [27,28]. We applied the AMBER parm14SB force field [29] for the peptides and counter ions, and the TIP3P rigid body model [30] for the water by adopting the symplectic [31] quaternion scheme [32,33]. The electrostatic potential was calculated using the particle mesh Ewald (PME) method [34]. The cut-off distance was 12 Å for the Lennard-Jones (LJ) potential. To maintain the N- and C-terminal
positions, Cα atoms of the 894L and 929R residues were restrained with a harmonic potential, as in ref [27]. Reversible multiple time-step MD techniques were also applied. The time step was taken to be δt = 0.5 fs for the bonding interactions of the peptide atoms, δt = 2.0 fs for the Lennard-Jones (LJ) interactions and the real part of the PME calculation of the peptide atoms, and those between the peptide atoms and solvent molecules, and δt = 4.0 fs for the LJ interaction and the real part of PME calculation between the solvent molecules and the reciprocal part of the PME calculation of all atoms. Because the symplectic rigid body algorithm was used for the water molecules, δt can be taken to be as long as 4.0 fs [33]. MD simulations in the canonical ensemble were performed for 100 ns at 310 K. The temperature was controlled using the Nose–Hoover thermostat [35,36,37]. The first 20 ns simulation was regarded as the equilibration, and the following 80 ns simulation was used for the analysis. Error bars in physical quantities were calculated for the four MD simulations from the different initial conditions by the Bootstrap method with 10⁶ bootstrap cycles [38].

4.6. Statistical analysis

Data are expressed as means ± standard error of the mean (S.E.M.). Statistical analysis was performed by the unpaired two-tailed Student’s t-test for comparison between two groups. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by a two-tailed multiple t-test with Bonferroni correction for comparison of multiple groups, or Dunnett’s test for comparison with the control group using the Origin® software (Origin Lab Corp., Massachusetts, USA). P values less than 0.05 were considered significant.

Declarations

Author contribution statement

Kunitoshi Uchida: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Tomo Kita: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Mitsutoki Hatta: Contributed reagents, materials, analysis tools or data.

Jun Yamazaki, Satoru G. Itoh, Hisashi Okumura: Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Author contribution statement

Kunitoshi Uchida: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Tomo Kita: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Mitsutoki Hatta: Contributed reagents, materials, analysis tools or data.

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