A Dimer Is the Minimal Proton-Conducting Unit of the Influenza A Virus M2 Channel

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http://dx.doi.org/10.1016/j.jmb.2014.05.002
Edited by I. Shimada

Abstract

When influenza A virus infects host cells, its integral matrix protein M2 forms a proton-selective channel in the viral envelope. Although X-ray crystallography and NMR studies using fragment peptides have suggested that M2 stably forms a tetrameric channel irrespective of pH, the oligomeric states of the full-length protein in the living cells have not yet been assessed directly. In the present study, we utilized recently developed stoichiometric analytical methods based on fluorescence resonance energy transfer using coiled-coil labeling technique and spectral imaging, and we examined the relationship between the oligomeric states of full-length M2 and its channel activities in living cells. In contrast to previous models, M2 formed proton-conducting dimers at neutral pH and these dimers were converted to tetramers at acidic pH. The antiviral drug amantadine hydrochloride inhibited both tetramerization and channel activity. The removal of cholesterol resulted in a significant decrease in the activity of the dimer. These results indicate that the minimum functional unit of the M2 protein is a dimer, which forms a complex with cholesterol for its function.

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Introduction

The M2 protein of influenza A virus has been proposed to form an acid-activated, proton-selective ion channel that exhibits multiple functions upon viral infection [1]. The proton permeation through M2 channels in low-pH endosomes after receptor-mediated endocytosis has been shown to acidify the viral interior and facilitate the dissociation of viral nucleoproteins from the matrix protein M1, an essential process for subsequent entry of the virus genome into the nucleus of the host cell [2]. M2 has also been suggested to prevent premature conformational changes in newly synthesized hemagglutinin in the trans-Golgi network membrane (pH ~ 6.0) [3] by equilibrating the lumenal pH [4]. Therefore, M2 is an important target for anti-influenza drugs [5].

The M2 protein is a single-transmembrane (TM) protein composed of an unstructured N-terminal region (residues 1–24), TM region (residues 25–46), and C-terminal region (residues 47–97) including the amphipathic helix (AH) (residues 51–59) (Supplemental Fig. S1). It is widely thought that the TM region of M2 is the core of association into a four-helix bundle that conducts protons [6] and binds the antiviral drug amantadine hydrochloride (Am) [7,8]. Recent X-ray crystallography [9], NMR [10], and ultraviolet resonance Raman spectroscopy [11] studies using fragment peptides containing TM regions incorporated into detergent micelles or liposomes proposed a molecular model in which M2 stably formed a tetrameric channel irrespective of pH, and intermolecular interactions between protonated His37 and Trp41 at acidic pH were involved in channel activation, assuming an open or closed conformation in the M2 tetramer. However, these artificial environments do not necessarily provide a native structure in cell membranes, and TM peptides may behave differently from the full-length protein. Although the M2 channel has been extensively investigated, the oligomeric states of the full-length M2 protein in living cells have not yet been assessed directly.

In addition to the oligomeric states, the mechanisms by which adamantane-based antiviral drugs bind to M2 also remain to be elucidated [9,10]. Although Am has been proposed to bind to the pore...
olf the tetramer [9], the existence of a different Am-binding site has also been suggested in lipid-facing surface of the TM helices by surface plasmon resonance and rotational-echo double-resonance NMR methods [12,13].

In the present study, we examined the oligomeric states of the full-length M2 proteins [A/Udorn/72 virus (H3N2)] on the plasma membrane of living CHO-K1 cells, the membrane lipid constituents of which [14] were similar to those of the viral envelope membrane [15], using fluorescence resonance energy transfer (FRET) among the M2 proteins labeled with fluorophores by the coiled-coil method [16]. This method utilizes a tight heterodimer formation between the E3 tag (EIAALEK)₃ (net charge: −3) fused to the N-terminus of the target protein and the synthetic fluorescent K4 probe, fluorophore-(KIAALKE)₄ (net charge: +4). In addition to electrostatic interaction between E and K, hydrophobic interaction between L and I drives the heterodimer formation. Due to the parallel association between the E3 and K4 colis [17], the fluorophore is located near the N-terminus of the tag when K4 is bound (Supplemental Fig. S2). Our techniques are suitable to investigate dynamic transition of oligomerization in living cells because we have already validated the method using several standard membrane proteins [18]. In contrast to previous models, FRET analyses indicated that full-length M2 formed dimers at neutral pH and these dimers were reversibly converted to tetramers at acidic pH. Moreover, not only the tetramer but also the dimer exhibited proton channel activities. Am inhibited tetramerization and channel activity by inactivating dimers. To elucidate the mechanism of channel activity in the dimeric state, we performed alanine scanning of the TM region and removal of cholesterol. The H37A mutation nullified channel activity, which indicated that His37 was crucial for conducting protons in the dimer. Significant reductions in the activity of the dimer were also observed by mutating Ala for Ile at the position of 35 or by treatments with cholesterol-removal agents. Taken together, we proposed a functional dimer model in which the minimum functional unit of M2 exhibiting channel activity was not the tetramer, but the dimer, and cholesterol was essential for the function of the dimeric channel.

**Results and Discussion**

**M2 forms a tetramer at acidic pH, whereas it exists as a dimer at neutral pH**

We investigated the oligomerization of E3-tagged full-length M2 proteins (E3-M2) in living cells by FRET using the coiled-coil labeling technique [16] and spectral imaging. E3 tagging was confirmed not to affect the channel activity of M2 (see Fig. 5a and b). E3-M2 was labeled with mixtures of the donor Alexa568-K4 and the acceptor Alexa647-K4 at various donor mole fractions (Xₐ) at 50 nM. Fluorescence spectra for the cell membranes were deconvoluted into the donor and acceptor contributions, as depicted in Fig. 1d. The apparent FRET efficiency E_app was calculated and compared with theoretical values as described in Materials and Methods to determine the oligomeric states. Consistent with proposed models [9–11], our FRET method revealed that E3-M2 formed a tetramer at pH 4.9 (Fig. 1a). Surprisingly, however, E3-M2 existed as a dimer at pH over 5.5 (Fig. 1a and b). Moreover, interconversion between the dimer and tetramer was reversible when external pH (6.0) was lowered to pH 4.9 and then raised to 6.0 (Fig. 1c and d). These results indicate that the oligomeric states of M2 are dependent on pH. Note that the dissociation constants of the donor and acceptor probes were important factors for stoichiometric analysis, and their high affinities to the E3 tag (Kₐ ~ 5 nM) did not vary over the pH range 4.9–7.3 (labeling efficiencies: ~90%) (Supplemental Fig. S3). Furthermore, based on their normal morphology, exposure to acidic pH did not affect the mock cells (transfected with the empty vector pcDNA3) (data not shown). The H37A and W41A mutants lost the ability to form tetramers at pH 4.9 (Fig. 2), which demonstrated that the driving force of tetramerization could be attributed to the cation–π interaction between protonated His37 and Trp41 at acidic pH [11]. We considered that the abovementioned FRET results reflect to the interconversion between the dimer and tetramer instead of a significant separation above 82 Å (Förster distance of this donor–acceptor pair) among the N-termini in a tetramer for the following two reasons. (i) The estimated average distance between the N-termini of E3-M2 [61 Å, giving an E (true FRET efficiency) value of ~90%] was much shorter than the Förster distance (82 Å) (Supplemental Fig. S2). (ii) The N-terminal truncated E3-M2(25–97) mutant exhibited a dimeric FRET signal at pH 6.0 similar to full-length E3-M2 (Fig. 3), which suggested that FRET signals were unaffected by the presence of the N-terminus.

The dimer–tetramer equilibrium has been suggested by immunoprecipitation and SDS-PAGE [19,20], and the active form was concluded to be a tetramer by simultaneously expressing mixtures of the WT (wild type) and Am-insensitive V27S mutant proteins on the plasma membranes of oocytes [19]. The DeGrado group studied the oligomerization of M2 proteins in vitro. In 1999 and 2000, they used synthetic polypeptides of M2 in detergent micelles...
and analytical ultracentrifugation method and proposed a reversible monomer–tetramer (slight octamer) equilibrium model. They pointed out that a too high protein density in the micelles facilitated tetramerization of M2, although their results that the tetramerization was more favorable at basic pH at a peptide-to-detergent mole ratio of 1/100 were opposite to our findings [8,21]. In 2003, they applied disulfide cross-linking to quantitatively measure the thermodynamics of M2 protein association in detergent micelles and revealed the monomer–dimer–tetramer equilibrium under reversible redox conditions in a thiol-disulfide buffer [22]. In comparison with these previous studies, the full-length M2 in living cell was found to assume different oligomeric states with different pH dependence, suggesting the importance of appropriate membrane environments and extramembrane regions to study the oligomerization of M2.

**The dimer–tetramer equilibrium of M2 channel is not dependent on the protein density**

To examine if the oligomeric state of the M2 protein is dependent on the protein density, we determined $E_{\text{app}}$ values at $X_D = 0.74$ as different M2 expression levels (Supplemental Fig. S4). The $E_{\text{app}}$ values did not increase significantly even if the protein density increased more than twice, suggesting that a pH drop is needed to induce tetramerization. However, it should be noted that the protein-to-lipid ratios [23] in the cellular experiments ($<1/8000$) were far below...
those typically used in biophysical experiments (>1/100). Thus, tetramerization may be facilitated even at neutral pH at such extremely high protein densities.

The AH in the C-terminus of M2 is essential for dimer formation

To determine the region required for dimer formation, we examined the oligomeric states of several M2 mutants. SDS-PAGE analysis [24] indicated that the formation of at least one of the two possible disulfide bonds between monomers at Cys17 or Cys19 in the N-termini stabilized the M2 tetramer, although it was not essential for oligomeric assembly. In our study, both E3-C17A·C19A-M2 and N-terminal truncated E3-M2(25–97) mutants did not dissociate into monomers at neutral pH (Fig. 3). Furthermore, alanine scanning of the TM region (residues 25–46) showed that the region was also not responsible for dimerization (Fig. 6a). We then focused on AH (residues 51–59) in the C-terminal juxtamembrane region. The AH-defective mutant, E3-M2(22–46), exhibited a monomeric FRET signal (Fig. 3). This result clearly demonstrates that the interaction between AHs drives dimerization, as observed for the M2 fragment peptides in detergent in the previous report [10] in which M2(18–60) including both the TM region and 15 residues of C-terminal region forms a stable tetramer in the micelles whereas the TM-only peptide, M2(18–46), does not assemble to a tetramer as indicated by SDS-PAGE and chemical cross-linking.

Am inhibits M2 tetramerization by preventing the protonation of His37 and the consequent cation–π interaction between His37 and Trp41

We further investigated the effect of Am on the oligomeric states of E3-M2. First, we confirmed that a pretreatment with 100 μM of Am completely blocked the channel activity of E3-M2 at pH 4.9 [25,26] (Supplemental Fig. S5). Tetramerization was inhibited when E3-M2 was preincubated with 100 μM Am for 15 min at pH 7.3 and then exposed to acidic pH (4.7–6.0) (Fig. 1b). Moreover, a decrease in intracellular pH was not observed in the presence of Am as described below (see Fig. 5a and b), indicating that Am-bound M2 channels could not transport protons into the inside of cells. Thus, Am inhibits the protonation of His37 and the consequent cation–π interaction between the His37 and Trp41 at acidic pH, which is important for tetramerization (Fig. 2). In accordance with this, Okada et al. reported that changes in Raman spectra of the M2 peptide upon acidification in the presence of Am were much smaller than those in the absence of Am [11].
The tetramer and the dimer exhibit channel activity

We examined the relationship between the oligomeric states of M2 and channel activity on living cells using the cytosolic pH-indicator dye SNARF-4 F 5-(and-6)-carboxylic acid, acetoxymethyl ester, acetate (SNARF-4 F AM acetate). The pH-dependent spectral shift in SNARF-4 F allows quantitative measurement of H+ concentrations in terms of the ratio (R_{665:535}) of fluorescence intensities measured at two different wavelengths (635 and 665 nm) (Supplemental Fig. S6). Firstly, we constructed a calibration curve for [H+] measurements inside the cells using ionophores (nigericin and valinomycin [27]) to equilibrate the intracellular pH of dye-loaded cells to the extracellular pH and measuring the R_{665:535} at several pH points (Supplemental Fig. S6). M2-induced increases in intracellular [H+] were analyzed using the single exponential equation [Eq. (4) in Materials and Methods], which gives an apparent channel activity independent of the [H+] gradient across the plasma membrane. We determined the channel activities of the M2 mutants at different extracellular pH values and then estimated the expression level of M2 on each cell per protein (not channel) (8.0 × 10^{-20} A (0.5 proton s^{-1}) at pH 6 (10× larger at pH 5), in comparison with the amplitude of the currents measured for Xenopus oocytes (0.01–1.0 × 10^{-6} A) [1,19,20]. However, this small current is sufficient to the acidification of viral interior because only three protons are present in a virus with a diameter of 100 nm at pH 5. In our study, the net proton transport by M2 was underestimated due to the intracellular buffering capacity (β = 2.0–5.0 × 10^{-2} M/pH at pH 7.2–7.4) [29–31]. Moreover, the generation of an inside-positive diffusion potential by the proton influx can suppress the further conduction of protons through the M2 channel, particularly at lower pH, where the proton influx is large, because intracellular pH did not approach pH 4.9 (Fig. 5a). In consistent with this interpretation, the addition of the K+ ionophore valinomycin resulted in a steep increase in intracellular [H+] by effluxing intracellular K+ ions and thus dissipating the diffusion potential (Supplemental Fig. 9). At a higher pH of 6.0, the final intracellular [H+] approached the outside [H+] (Fig. 5a), indicating that the electrogenic effect was not significant at least in pH 7.4–6.0, probably because the Na^+-K^+ pump and/or the K^+ leak channel compensated the diffusion potential. We roughly estimated the channel activity corrected for the diffusion potential at pH 4.9 by dividing the very initial slope in the region intracellular [H+] ≤ 1 × 10^{-6} M, where the diffusion potential effect was negligible, by the [H+] gradient across the plasma membranes at t = 0. The corrected channel activity at pH 4.9 was estimated to be 4.2 ± 0.7 × 10^{-9} (s^{-1} protein^{-1}) (mean ± SEM, n = 8 cells; SEM, standard error of the mean), which was even lower than the uncorrected value [3.0 ± 0.4 × 10^{-8} (s^{-1} protein^{-1})]. On the other hand, there was no difference between the corrected and uncorrected values at pH 6.0 [1.2 ± 0.2 × 10^{-7} (s^{-1} protein^{-1})] (mean ± SEM, n = 8 cells), indicating that this rough estimation is reliable. These results demonstrate that the channel activity at pH 6.0 is higher than that at pH 4.9, ruling out the possibility that a minor fraction of tetramers at pH 6.0 that were undetectable with our FRET method mainly contributed to channel activity. Furthermore, the cholesterol dependence of channel activity supports this conclusion (vide infra).

As observed for E3-M2(22–46) mutant (Fig. 5c), Ma et al. also failed to detect any significant membrane current for M2(21–51) expressed on oocytes [32], whereas Hu et al. reported that E3-M2(22–46) mutant itself was active in liposomes at a very high protein-to-lipid ratio (1:100) [33].

His37 is crucial for the channel activity of the dimer, and cholesterol is essential for proton conduction

To elucidate the mechanism of channel activity by the dimer at pH 6.0, we performed alanine-scanning analysis of 20 residues in the TM region. As previously noted, although substitution of any residues in the TM
region did not influence the oligomeric state, the H37A mutation nullified channel activity (approximately 35-fold lower than that with WT) (Fig. 6a). The temporal change in intracellular \[\text{H}^+\] of the H37A mutant was indistinguishable from that of the mock (Fig. 6b), which indicated that His37 was crucial for channel activity by the dimer. Pinto’s group reported that replacements of His37 resulted in ionic current that was not dependent on pH, suggesting that the protonation of the side chain of this amino acid was responsible for the activation by low pH\(^1\,\text{34}\) and also that these mutants might conduct ions other than protons under an electric potential difference. In contrast, the channel activity in our study indicates

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Fig. 4. A flow chart of apparent channel activity. (a) Cells were incubated with PBS(+) (pH 7.3) containing 10 \(\mu\)M of cytosolic SNARF-4 F AM acetate to load the dye into the cells. After incubation for 20 min, cells were excited at 514 nm to obtain a spectrum at \(t = 0\). Typical cells are shown in the right figure. (b) Time-lapse imaging (excitation: 514 nm, 20-s intervals for 600 s) started as soon as neutral pH buffer was exchanged with acidic pH buffer. The neighbor figure is the representative curve for the increase in intracellular \([\text{H}^+]\) at extracellular pH 6.0. As shown in the inserted figures, the pseudocolor for the dyes inside cells changed from light to dark green. The curve was fitted with Eq. (4), \([\text{H}^+]_\text{in}(t) = [\text{H}^+]_\text{out}(0) + ([\text{H}^+]_\text{in}(0) - [\text{H}^+]_\text{out}(0))e^{-kt}\), to calculate the rate constant, \(k\) (s\(^{-1}\)). In Am-blocking experiments, the buffer in steps (a) and (b) contained 100 \(\mu\)M of Am. (c) Cells were stained with the primary and secondary antibodies in PBS(+) (pH 7.3) for 20 min, respectively. Exemplary spectra are represented. The observed spectrum (excitation: 637 nm, black) obtained from the membrane region was deconvoluted into spectra for the antibody (red) and SNARF-4 F (orange) with the least-squares method. The calculated curve (blue) closely fit the observed one. All spectral images are shown in pseudocolor, which are composite of 19 channels in the respective ranges (565–745 nm, resolution: 10 nm)\(^{17}\).
the amount of only protons passed through the M2 channel because we used the pH-indicator dye SNARF-4 F.

Interestingly, the I35A mutation also significantly decreased channel activity (approximately 10-fold decrease) (Fig. 6a and b). The β face of cholesterol has been proposed to interact with Ile and Val side chains in the TM helices of membrane proteins [35]. Therefore, we examined the effect of cholesterol depletion with methyl-β-cyclodextrin (MβCD) and mevastatin (also known as “compactin”) on channel activity. Regarding E3-M2 and untagged M2, the treatment of cells with the two removal regents with different mechanisms of action significantly reduced channel activity at pH 6.0. In contrast, channel function was not affected by cholesterol removal at pH 4.9 (Fig. 6c). FRET signals at pH 6.0 and 4.9 were unchanged in the presence of the agents (Fig. 6d), which indicates that the elimination of cholesterol interrupted the channel activity of the dimer only without disturbing the oligomeric states. These results suggest that the M2 dimer interacts with cholesterol to form a proton-conductive pathway, whereas the tetramer does not require cholesterol for its function.

Despite similar activities, dimeric and tetrameric channels appear to have different H+-conducting mechanisms because only the former was cholesterol sensitive (Fig. 6c) and the latter had a conducting pore in the center [9,10]. The mechanism of the tetrameric channel has been extensively investigated [26,32,33,36]. Regarding the dimeric channel, the conventional model in which the channel assumed an open or closed state by His37 (a pH sensor) and Trp41 (a gate) [10,11] was inconsistent with the observation that a decrease was not observed in activity for the W41A mutant (Fig. 6a). It is clear that His37 is the core of channel activity because the H37A mutation nullified activity (Fig. 6a and b). Mould et al. concluded from D2O replacement experiments that H+ ions did not pass through the M2 channel as hydronium ions, but they interacted with titratable groups [26]. The interaction of Ile35 with cholesterol [35] appears to be important for proton transport because cholesterol removal significantly reduced channel activity.
without disturbing the oligomeric states (Fig. 6c and d). The hydroxyl group of cholesterol may help protons to access the His residue. Alternatively, cholesterol may stabilize the specific conformation of the dimeric helices necessary for proton conduction. A detailed mechanism is currently being investigated using molecular dynamic simulations. These findings lead an interesting question: what is the physiological significance of the tetramer if the dimer has channel activity? The answer may be that the two oligomeric states for proton permeation are double insurance against cholesterol-rich and cholesterol-poor membrane environments because the lipid composition of the envelope was previously shown to be host specific [37,38].

The minimal functional unit of the M2 channel is a dimer

While the vast majority of groups consider that M2 exists as a stable tetramer using fragment peptides incorporated into artificial environments, dynamic equilibrium between the oligomeric states of the full-length M2 protein in biomembranes has never been examined. In this study, we revealed that dimerization of M2 was driven by the interaction between AH in the C-termini of monomers, and the tetramerization of dimers was promoted by low pH in a reversible manner (Fig. 7). The midpoint of the dimer–tetramer transition was pH ~ 5.1 (Fig. 1b), which was close to the reported third pK_a value of 4.9 ± 0.3 [36]. In the dimeric state, one His residue was considered to be protonated at pH 6.0, deduced from four pK_a values for tetramer [36]. The protonation of the third His residue in the tetrad induces tetramerization via a cation–π interaction between His37 and Trp41 (Figs. 2 and 7) [11]. The minimal functional unit of the M2 channel is a dimer because both dimeric and tetrameric forms of M2 protein exhibited channel activity, whereas monomeric form was inactive. Cholesterol was essential for dimeric channel activity. Am inhibited both tetramerization and channel activity.
Our findings propose a new approach for elucidation of resistance mechanism for adamantane-based drugs and discovery of a novel drug with different mode of action.

**Materials and Methods**

All experiments were performed at room temperature (~25 °C). The pH of PBS(+) [phosphate-buffered saline (PBS) added with 0.33 mM MgCl₂ and 0.90 mM CaCl₂] was controlled to the desired value with 1 mol/L hydrochloric acid (Nacalai Tesque) and was stable for at least 3 h.

**Vector construct**

For transient expression, cDNA encoding E3-M2 was inserted into pcDNA3 (Invitrogen) as follows. Oligo DNA encoding M2 of influenza virus A [A_Udorn_1972 (H3N2)] (GenScript), which had a NheI site at the 5' terminus and an Apal site at the 3'-terminus, was digested with Nhel and Apal. The DNA of M2 was inserted into the vector DNA (E3/pcDNA3 vector) to obtain an E3-M2/pcDNA3 vector. Point mutations were carried out with QuickChange II Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer’s instructions. Truncated mutants including E3-untagged M2 were constructed by amplifying the template E3-M2/pcDNA3 vector with designed primers (Invitrogen) containing a NheI or an Apal recognition site by PCR. The product was digested with either Nhel or Apal, and then ligated with Ligation High Version 2 (Toyobo) to construct the vector encoding desired truncated M2.

**Cell culture**

CHO-K1 cells were cultured in Ham’s F12 medium supplemented with 10% fetal bovine serum (FBS), L-glutamine, and antibiotics at 37 °C in a 5% CO₂ incubator. Penicillin at 50 units/mL and streptomycin at 50 μg/mL (Nacalai Tesque) were used for the passage of cells.

**Transfection**

Cells were seeded at 1.0 × 10⁶ in 2 mL antibiotic-free F12/10% FBS on a 35-mm glass bottom dish (advanced TC treated; Greiner Bio-One). The medium was changed to 2 mL serum-free medium and cells were incubated with a transfection mixture composed of 2.0 μg plasmid DNA, 4.0 μL Lipofectamine LTX (Invitrogen), and 400 μL Opti-MEM (Invitrogen) per dish at 30 h after seeding. The medium

**Fig. 7.** An oligomeric state model of M2 proteins without and with Am. Monomeric M2 proteins dimerize by an interaction between AHs in C-terminal juxtamembrane regions. Dimerization facilitates the formation of disulfide bonds between Cys17 and Cys19 in the N-termini to rigidly stabilize the structure. The dimer–tetramer state equilibrates in a pH-dependent manner; at acidic pH, the cation–π interaction between the protonated His37 and Trp41 derives the dimers to form a tetramer, whereas the tetramer dissociates to dimers at neutral pH. At neutral pH, Am binds the dimer to induce an inactive state, resulting in the inhibition of tetramerization. Note that the true Am to M2 stoichiometry is not known.
was changed to a fresh medium containing 10% FBS 5 h after transfection. CHO-K1 cells were imaged 18 h after transfection. The transfection mixture was composed of 4.0 μg plasmid DNA, 4.0 μL Lipofectamine LTX, 4.0 μL PLUS reagent (Invitrogen), and 400 μL Opti-MEM per dish in order to transfect E3-untagged M2. In the present study, we used cells transiently expressing M2 below 4.0 × 10^5 proteins/cell.

**Determination of the minimal effective concentration of Am**

1-Adamantaneamine (Nacalai Tesque) was dissolved in diluted hydrochloric acid to prepare the Am stock. To determine the minimal effective concentration of Am, we performed experiments at 0, 10, 100, 500, 800, and 1600 μM. Cells were incubated with PBS(+) (pH 7.3) containing 10 μM of SNARF-4 F AM acetate and each concentration of Am for 30 min and were then exposed to the pH 4.9 buffer for 30 min. We determined the minimal concentration that gave an intracellular pH indistinguishable from that of the mock (Supplemental Fig. 5).

**Analysis of E_{app} values**

The binding assay for K4 probes has been described elsewhere [18]. Mixtures of the Alexa568-K4 and Alexa647-K4 probes at various donor mole fractions (X_D) were diluted with pH-controlled PBS(+) to produce 50 nM K4 probe solutions (1 mL). After one-time washout with probe-free PBS(+) (pH 7.3), cells were incubated in pH-controlled solutions for 30 min and were then sequentially excited at 561 nm and 637 nm. Spectra in the range 565–745 nm (resolution: 10 nm) for the cell membranes were acquired using a confocal microscope equipped with a spectral detector (Nikon C1). The analysis procedure for the apparent FRET efficiency (E_{app}) has been previously described [18]. Briefly, we quantified the sensitized emission of the acceptor (FRET signal) to determine the E_{app} value. With the use of the reference spectra of the donor (excitation: 561 nm) and acceptor (excitation: 637 nm), the spectrum (excitation: 561 nm) was deconvoluted into the donor (fitting parameter: f_1) and acceptor (f_2) fractions by the least-squares method. The spectrum (excitation: 637 nm) was fitted with the reference spectrum of the acceptor to acquire the parameter f_2 and was multiplied by the constant R_{561/637} (the ratio of the intensities of the acceptor excited at 561 and 637 nm) to estimate the contribution of directly excited acceptor fluorescence (f_2 × R_{561/637}). The E_{app} value was determined by the following equation:

\[
E_{app} = \frac{f_A (\lambda_{647 \text{ ex}})}{f_D (\lambda_{647 \text{ ex}})} \times \frac{F_{AD} - F_A}{F_A} \\
= 0.39 \times \frac{f_2}{f_3 \times R_{561/637}}^{-1}
\]

where ε_A (\lambda_{647 \text{ ex}}) and ε_D (\lambda_{647 \text{ ex}}) represent the molar extinction coefficients of the acceptor and donor at 561 nm, respectively, and F_{AD} and F_A indicate the acceptor emissions (arbitrary unit) excited at 561 nm in the presence and absence of the donor, respectively.

When examining the effect of Am preincubation, we pretreated cells with 100 μM of Am in PBS(+) (pH 7.3) for 20 min and then labeled them with the K4 probes at X_D = 0.74 (50 nM) in the pH-controlled PBS(+) (pH 4.7, 4.9, 5.1, 5.5, and 6.0) for 30 min, independently.

**Theoretical curves**

The theoretical curve for FRET analysis is given as

\[
E_{app} = E \times \frac{X_D}{1 - X_D} \times \left[1 - \left(X_D (1 - X_U) + X_U\right)^N\right]
\]

where X_D, X_U, and N indicate the donor molar fraction, unlabeled receptor fraction, and association number, respectively. In the present study, the X_U value was estimated to be 10% according to a labeling efficiency of ~90% for K4 probes at 50 nM. E represents the true FRET efficiency in the oligomers and was determined by the distance and mutual orientations of donor and acceptor.

**Calibration of pK_a for SNARF-4 F**

The pH-indicator dye SNARF-4 F AM acetate was purchased from Molecular Probes. The ionophores nigericin and valinomycin were obtained from Wako.

To accurately measure intracellular pH, we examined whether SNARF-4 F loaded into cells exhibited the same pK_a value as in dilute solutions. The dye (50 μg) in a vial was dissolved in 10 μL of anhydrous dimethylsulfoxide (DMSO). The DMSO solution was diluted 1:854 into loading PBS(+) (pH 7.3) to prepare a 10 μM dye solution (DMSO < 0.12%). To calibrate of pK_a, we loaded mock cells (transfected with the empty vector pcDNA3) with 1 mL of the dye solution for 30 min, and then substituted them to 1 mL of pH-controlled 20 mM Hepes buffer containing 1 mM of MgCl_2, 150 mM of KCl, 10 μg/mL of nigericin, and 5 μg/mL of valinomycin to equilibrate pH inside and outside the cells for 5 min. The dye was excited at 514 nm, and the fluorescence spectra in the range 555–745 nm (resolution: 10 nm) were acquired at the endpoints of the titration, respectively. The obtained pK_a value was determined by measuring fluorescence intensities measured at 635 and 665 nm for each spectrum (R_{665/635}) were plotted versus pH (Supplemental Fig. S6) and fitted with the below equation:

\[
R_{665/635} = \frac{\text{Top}}{1 + 10^{(pH-pK_a)}} + \text{Bottom}
\]

where Top and Bottom indicate the basic and acidic endpoints of the titration, respectively. The obtained pK_a value of 6.42 ± 0.10 for SNARF-4 F was consistent with the reported value in the buffer (6.52 ± 0.01, excitation: 514 nm) [39], which demonstrated that the dye was not disturbed by intracellular constituents and reflected the precise intracellular pH.

**Measurement of the channel activity per M2 protein**

SNARF-4 F solution [10 μM in PBS(+) (pH 7.3)], prepared as described above, was loaded into cells for 20 min. Before substitution to acidic pH PBS(+), the spectra of SNARF-4 F inside cells were acquired for the measurement of intracellular pH at t = 0. After buffer replacement, the spectra of the same cells were
successfully gained at 20-s intervals for 600 s. In Am-blocking experiments, the buffer before and after the exchange contained 100 μM of Am. After measurement of the temporal pH changes, 1 μg/mL of the primary anti-M2 antibodies (14C2, Abcam) and 5 μg/mL of the secondary antibodies labeled with Alexa Fluor 680 (Invitrogen) in PBS(+) (pH 7.3) were sequentially added to cells and incubated for 20 min, respectively, to identify the expression level of M2 proteins on each cell (Fig. 4). The incubation time was 5 min when using Alexa568-K4 (50 nM).

Intracellular [H\(^+\)]\(\text{in}\) was calculated using Eq. (3) from the \(R_{\text{bioasys}}\) value at each time point and plotted as a function of time. Assuming the passive diffusion of H\(^+\) through the M2 channel into cells according to the [H\(^+\)]\(\text{in}\) gradient across the cell membranes, we fitted the function with Eq. (4) derived from Fick’s first law to calculate the apparent proton permeability, \(k\) (s\(^{-1}\)). The flux of H\(^+\) \(J\) (mol m\(^{-2}\) s\(^{-1}\)) can be written as

\[
J = \frac{1}{4\pi R^2} \frac{d[H^+]_{\text{in}}(t)}{dt} - \frac{4}{3} \pi R^3 \frac{d[H^+]_{\text{out}}(t)}{dt} = -D \frac{[H^+]_{\text{in}}(t) - [H^+]_{\text{out}}(t)}{\Delta t}
\]

where \(R\) denotes the radius of the cell (m), \([H^+]_{\text{in}}(t)\) and \([H^+]_{\text{out}}(t)\) are the concentrations of intracellular and extracellular H\(^+\) (M) at time \(t\), and \(D\) and \(\Delta t\) denote the diffusion coefficient of H\(^+\) (m\(^2\) s\(^{-1}\)) and the thickness of the membrane (m). Since \([H^+]_{\text{out}}(t)\) is almost constant, the abovementioned differential equation can be easily solved as

\[
[H^+]_{\text{in}}(t) = [H^+]_{\text{ax}}(0) + \left( [H^+]_{\text{in}}(0) - [H^+]_{\text{out}}(0) \right) e^{-\frac{t}{\Delta t}}
\]

where \(k\) is 3D/\(\text{Rd}\) (s\(^{-1}\)).

Expression was mainly identified with the antibodies, whereas that of E3-M2(22–46) was exceptionally determined with Alexa568-K4 because the mutant lacked the recognizable region of the anti-M2 antibody and the spectrum for Alexa647-K4 could not be deconvoluted from that of SNARF-4 F. Cells treated with antibodies or Alexa568-K4 were excited at 637 or 561 nm, respectively. The incubation time was 5 min when using Alexa568-K4 (50 nM).

Calibration curves to estimate M2 expression levels

The E3-β2AR stably expressed on CHO cells [E3-β2 AR-CHO (stable)] was used as a secondary standard because no stoichiometric M2 ligand is known. Its expression level was previously identified using the radioisotopic ligand \(^{3}H\)CGP12177 [40]. E3-M2 and E3-β2 AR-CHO (stable) were labeled with Alexa568-K4 (50 nM) for 5 min at pH 7.3. The first spectral image (excitation: 561 nm) was obtained. Regarding E3-β2AR-CHO (stable), we plotted the fluorescence intensity of the plasma membrane regions at 605 nm as a function of its expression level. E3-M2 was then treated with 1 μg/mL of the primary anti-M2 antibodies (14C2) (Abcam) and 5 μg/mL of the secondary antibodies labeled with Alexa Fluor 680 (Invitrogen) at pH 7.3 for 20 min, respectively, and the second image (excitation: 637 nm) for the identical cells was acquired. Regarding E3-M2, we plotted the peak intensity of the first spectrum as a function of that at 705 nm of the second. Data were fitted using regression lines through the origins. These calibration curves enabled us to estimate the M2 expression level with the antibody via the fluorescence intensity of Alexa568-K4.

Dissipation of proton influx-induced diffusion potential with valinomycin at pH 4.9

Valinomycin was dissolved in EtOH to prepare a stock solution at a concentration of 1 mg/mL. The intracellular [H\(^+\)]\(\text{in}\) measurement was performed in 1 mL PBS(+ buffer (pH 4.9) as described above. At \(t = 660\) s, cells were treated with valinomycin by addition of a 10 μg/mL solution (1.0% EtOH) in 1 mL PBS(+) buffer (pH 4.9) [a final concentration of 5 μg/mL (0.5% EtOH)]. As controls, cells expressing E3-M2 and mock cells were treated with additional 1 mL PBS(+) buffer (pH 4.9) containing 1.0% EtOH and 10 μg/mL of valinomycin (1.0% EtOH) at \(t = 660\) s, respectively.

Cholesterol removal

MβCD (Sigma Aldrich) and mevastatin (LKT Laboratories, Inc.) were dissolved in PBS(+) (pH 7.3) and DMSO (Nacalai Tesque), respectively. In the channel activity assay, cells were treated with 10 mM MβCD for 2 h after loading the dyes, whereas 1 μM mevastatin (DMSO < 0.1%) was added to the medium when cells were seeded on the dishes and were then incubated with the regent until immediately prior to loading the dyes except for a period of the transfection time. When investigating the influence of cholesterol removal on the oligomeric states, after treatment with mevastatin as described above, we removed the culture medium and then incubated cells with the K4 probes at \(X_D = 0.74\) (50 nM) in the PBS(+) (pH 6.0 or 4.9) for 30 min.

Confocal microscopy

All imaging experiments were performed using a confocal microscope (Nikon C1) under a water-immersed 60× objective (Plan Apo VC) with 514 nm, 561 nm, and 637 nm lasers. Spectral images were obtained with a spectrum detector.

Acknowledgments

This work was financially supported in part by Japan Society for the Promotion of Science KAKENHI Grant.
Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jmb.2014.05.002.

Received 26 December 2013; Received in revised form 30 April 2014; Accepted 1 May 2014
Available online 9 May 2014

Keywords:
M2 proton channel; coiled-coil labeling; dimer–tetramer equilibrium; FRET; spectral imaging

Abbreviations used:
AH, amphipathic helix; FRET, fluorescence resonance energy transfer; TM, transmembrane; MβCD, methyl-β-cyclodextrin; PBS, phosphate-buffered saline; FBS, fetal bovine serum.

References


