Kv4.2 and accessory dipeptidyl peptidase-like protein 10 (DPP10) subunit preferentially form a 4:2 (Kv4.2:DPP10) channel complex

Running title: Stoichiometry of Kv4/DPP complex

Masahiro Kitazawa‡, §, Yoshihiro Kubo‡, §, Koichi Nakajo‡, §, 1

‡ Division of Biophysics and Neurobiology, Department of Molecular Physiology, National Institute for Physiological Sciences, Okazaki, Aichi 444-8585, Japan

§ Department of Physiological Sciences, SOKENDAI (The Graduate University for Advanced Studies), Hayama, Kanagawa 240-0155, Japan

1To whom correspondence should be addressed: Division of Biophysics and Neurobiology, Department of Molecular Physiology, National Institute for Physiological Sciences, Okazaki, Aichi 444-8585, Japan, Tel: +81-564-55-7831; Fax: +81-564-55-7834; E-mail: ykubo@nips.ac.jp or Department of Physiology, Osaka Medical College, Takatsuki, Osaka 569-8686, Japan, Tel: +81-72-684-7283; Fax: +81-72-684-6521; E-mail: knakajo@art.osaka-med.ac.jp

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Background: Kv4/DPP forms a complex of unknown stoichiometry.

Results: Current properties and Kv4.2/DPP10 stoichiometry are altered depending on their relative expression levels.

Conclusion: The stoichiometry of Kv4.2/DPP10 is variable with a preference for the 4:2 configuration.

Significance: Kv4/DPP stoichiometry may provide important information for treatment of some psychiatric diseases.

ABSTRACT

Kv4 is a member of the voltage gated K⁺ channel family and forms a complex with various accessory subunits. Dipeptidyl aminopeptidase-like protein (DPP) is one of the auxiliary subunits for the Kv4 channel. While DPP has been well characterized and known to increase the current amplitude and accelerate the inactivation and the recovery from inactivation of Kv4 current, it remains to be determined how many DPPs bind to one Kv4 channel. To examine whether or not the expression level of DPP changes the
biophysical properties of Kv4, we expressed Kv4.2 and DPP10 in different ratios in *Xenopus* oocytes and analyzed the currents under two-electrode voltage clamp. The current amplitude and the speed of recovery from inactivation of Kv4.2 changed depending on the co-expression level of DPP10. This raised the possibility that the stoichiometry of the Kv4.2/DPP10 complex is variable and affects the biophysical properties of Kv4.2. We next determined the stoichiometry of DPP10 alone by subunit counting using single-molecule imaging. Approximately 70% of DPP10 formed dimers in the plasma membrane and the rest existed as monomers in the absence of Kv4.2. We next determined the stoichiometry of the Kv4.2/DPP10 complex: Kv4.2-mCherry and mEGFP-DPP10 were co-expressed in different ratios and the stoichiometries of Kv4.2/DPP10 complexes were evaluated by the subunit counting method. The stoichiometry of the Kv4.2/DPP10 complex was variable depending on the relative expression level of each subunit with a preference for 4:2 stoichiometry. This preference may come from the bulky dimeric structure of the extracellular domain of DPP10.

**INTRODUCTION**

Kv4, a member of the voltage-gated K$^+$ channel (Kv) family, is expressed in neurons and cardiac myocytes. In neurons, Kv4 contributes to the somatodendritic subthreshold A-type current ($I_{SA}$) which is involved in the control of repetitive firing and the attenuation of the back-propagation of action potentials (1-5). In cardiac ventricular myocytes, Kv4 generates the transient outward K$^+$ current ($I_{to}$) which plays an important role in the early phase of cardiac action potential repolarization (6-14). Kv4 forms multimolecular complexes with various subunits in physiological conditions (14-20). Dipeptidyl aminopeptidase-like protein (DPP) is one of the accessory subunits for Kv4 and was originally identified as a 115 kDa protein (21-24). DPPs belong to the prolyl oligopeptidase subfamily of the serine protease family and contain a well-known catalytic triad (Ser, His, Asp). However, DPP6 (also known as DPPX) and DPP10 (DPPY) lack the catalytic serine (it is substituted to aspartic acid in DPP6 or glycine in DPP10) and hence catalytic activity (18,25-27). The common molecular structure of the DPP family comprises a short N-terminal region, a single transmembrane region and a large extracellular C-terminus region including a glycosylation domain, a cysteine-rich domain and an aminopeptidyl peptidase (-like) domain (21).

DPP6 is expressed in many regions in the brain including hippocampal CA1 neurons, where it controls the A-current (Kv4) expression level and even dendritic morphogenesis by interacting with fibronectin in the extracellular matrix.
DPP6 has been implicated in neuropsychiatric pathologies including autism spectrum disorders (30,31), mental retardation (32) and amyotrophic lateral sclerosis (33,34). DPP10 is also broadly expressed in the brain including hippocampus, neocortex, cerebellum and main olfactory bulb, where Kv4/KChIP/DPP form a ternary complex (35). DPP10 is also implicated in neuropsychiatric diseases such as autism (30,36) and Alzheimer’s disease (37). Therefore, the Kv4/KChIP/DPP complex could be a good target for therapeutic approaches to these diseases although a better understanding of the complex composition and formation would be required.

DPP increases the current amplitude of Kv4 both by increasing the single channel conductance and by facilitating trafficking of Kv4 protein from the endoplasmic reticulum (ER) to the plasma membrane (38-41). DPP also accelerates both the inactivation and the recovery from inactivation of Kv4 although the underlying mechanism has not been fully determined (42-46). The region from the N-terminus to the start of the transmembrane domain of DPP10 plays an important role in binding to the S1-S2 segments of Kv4 in complex formation (47). However, it remains uncertain how many DPP subunits can bind to a tetrameric Kv4 channel. Although there is no available crystal structure for the Kv4/DPP complex, a crystal structure of the extracellular domains of DPP6/10 has been determined and it is suggested that DPP6 and DPP10 exist as a dimer in the plasma membrane (26,48). This implies the possibility that Kv4 and DPP preferentially form a 4:2 (Kv4:DPP) channel. On the other hand, a previous experiment using tandem repeat constructs suggests that the Kv4.2 channel requires four DPP6 subunits to be fully modulated (49).

We previously reported that the stoichiometry of Kv4 and another accessory subunit KChIP is variable and concluded that up to 4 KChIP molecules can bind to a single Kv4 tetramer (4:4 stoichiometry) (50). The stoichiometry of Kv4/KChIP is dependent on the relative expression levels of KChIP and Kv4 with no preferred stoichiometry (50).

In the present study, we aimed to determine the stoichiometry of Kv4 and DPP10. We first examined how the expression level of DPP10 affects the electrophysiological properties of Kv4.2 and observed that the current amplitude and the recovery from inactivation changed depending on the expression level of DPP10. Next, we confirmed the dimeric structure of DPP10 alone in the plasma membrane by subunit counting using single-molecule imaging under total internal reflection fluorescence (TIRF) microscopy. Interestingly, approximately 70% of DPP10 on the plasma membrane existed as dimers and the rest existed as monomers. Finally, we determined the stoichiometry of the
Kv4.2/DPP10 complex. The stoichiometry of the Kv4.2/DPP10 complex was again variable and dependent on the expression level of DPP10 as is the case for the Kv4/KChIP complex (50). However, in contrast, Kv4.2/DPP10 showed a clear preference for the 4:2 stoichiometry which is probably due to the large dimeric extracellular domain.

EXPERIMENTAL PROCEDURES

Molecular biology

Human Kv4.2 (KIAA1044; NM_012281) and Human DPP10d (KIAA1492; NM_001004360) (44,45) were provided by Kazusa DNA Research Institute and were subcloned into the pGEMHE expression vector. mEGFP and mCherry with a flexible linker were kindly provided by Dr. M. Ulbrich (Univ. of Freiburg) (51,52). mEGFP tagged Kv4.2 (Kv4.2-mEGFP) was constructed as previously described (50). For mEGFP-DPP10, a XhoI site was added to the N-terminus of DPP10 and a cleavage was made at the unique BstXI site in DPP10 (‘‘XhoI-DPP10-BstXI’’). mEGFP with a flexible GGS linker on the C-terminus was cleaved at the unique XhoI site in the linker, and cleavage was made at the unique KpnI site in the pGEMHE vector (‘‘KpnI-mEGFP-linker-XhoI’’). The two fragments were ligated at the XhoI sites and inserted into the KpnI and BstXI sites in the DPP10-pGEMHE construct. A C-terminal truncated version of mEGFP-DPP10 (mEGFP-DPP10 D-Extra) was made by PCR as previously described (41). To construct Kv4.2-mCherry, a BglII site was introduced into the N-terminus of mCherry with a flexible GGS linker (BglII-mCherry). mEGFP in Kv4.2-mEGFP was then replaced with mCherry by using the BglII site. All constructs were confirmed by DNA sequencing. cRNA was transcribed from the linearized plasmid cDNA using T7 mMessage mMachine kit (Ambion).

Preparation of Xenopus oocytes

Oocytes were surgically obtained from Xenopus laevis anesthetized in water containing 0.15% tricaine for 30 minutes. Xenopus frogs were allowed to recover from anesthesia before returning to the tank. To remove the follicular cell layer, oocytes were treated with collagenase for 6 - 7 h at room temperature. Defolliculated oocytes of similar size at stage V or VI were microinjected with 50 nl of cRNA solution and incubated for 1-3 days at 17°C in frog Ringer solution containing (in mM): 88 NaCl, 1 KCl, 2.4 NaHCO3, 0.3 Ca(NO3)2, 0.41 CaCl2, and 0.82 MgSO4, pH 7.6, with 0.1% penicillin-streptomycin solution (Sigma-Aldrich). All experiments were approved by the Animal Care Committee of the National Institute for Physiological Sciences, and performed following the institutional guidelines.

Two-Electrode Voltage Clamp

One to three days after cRNA
injection, ionic currents were recorded under two-electrode voltage clamp with an OC-725C amplifier (Warner Instruments) at room temperature. The bath chamber was perfused with ND-96 containing (in mM): 96 NaCl, 2 KCl, 1.8 CaCl2, 1 MgCl2 and 5 HEPES, pH 7.2. The microelectrodes were drawn from borosilicate glass capillaries (World Precision Instruments) to a resistance of 0.2 - 0.5 MΩ and filled with 3 M K-acetate and 10 mM KCl (pH 7.2). Holding potential was -80 mV or -100 mV. Voltage clamp protocols and data acquisition were performed using a digital converter (Digidata 1440) and pCLAMP 10.3 software (Molecular Devices).

**Subunit counting by single-molecule imaging**

Twelve to twenty h after cRNA injection, to remove the extracellular matrix, the oocytes were treated with 1 U/ml neuraminidase (Sigma-Aldrich) and 1 mg/ml hyaluronidase (Sigma-Aldrich) for 15 minutes at 12°C (52). To remove the vitelline membrane, oocytes were put into 2x frog Ringer solution which makes them osmotically shrink. The vitelline membrane was then manually removed with forceps. The oocytes were put on a high refractive index coverslip (n = 1.78) (Olympus) and observed through an objective lens (Olympus 100 ×, N.A. 1.65) mounted on an inverted microscope (Olympus, IX71). Movies of 400 frames (20 Hz, 20 s) were recorded with an iXon3 EMCCD camera (Andor) and SOLIS software (Andor). ND-96 was used as the extracellular solution. mEGFP was excited with a Cyan 488-nm solid laser (Spectra-Physics) and mCherry was excited with an Orange 588-nm solid laser (Coherent). The density of ion channels was kept low enough (approximately 1 spot/µm²) to minimize the probability of two spots overlapping within a diffraction-limited spot (52,53). A 25.6 × 25.6-µm² area was illuminated and the fluorescence intensity of each spot was measured off-line from the recorded movie. Stable spots throughout the recording were selected manually to analyze the bleaching steps and overlap of green and red spots.

**Data analysis for electrophysiology**

pCLAMP 10.3 (Molecular Devices) and Igor Pro software (WaveMetrics, Inc) were utilized for the ionic current analyses. All current traces were analyzed without leak subtraction.

The kinetics of the recovery from inactivation was fitted with a single exponential function:

\[ I = I_{\text{peak}} (1 - e^{-t/\tau_{\text{rec}}}) \]

Where \( I_{\text{peak}} \) is the average of peak current amplitudes at prepulse and \( \tau_{\text{rec}} \) is the recovery time constant at -100 mV.

**Data analysis for subunit counting**

The number of subunits in the optical spots was analyzed based on the distribution
histogram of bleaching steps. Obtained histograms were fitted with a binomial distribution $P(X = a)$,

$$P(X = a) = \binom{n}{a} p^a (1-p)^{n-a}$$

where $A$ is the total number of spots, $\binom{n}{a}$ is the number of combinations of choosing $a$ from among $n$, $n$ is the number of bound subunits, $a$ is the number of observed bleaching steps and $p$ is the fluorescence probability of mEGFP.

**Statistical Analyses**

All data are presented as the mean ± S.E. and $n$ represents the number of samples. Statistical differences were evaluated by Tukey-Kramer test. Values of $p < 0.05$ were considered significant. *** indicates $p < 0.001$.

**RESULTS**

**DPP10 changes Kv4.2 properties in an expression level dependent manner**

DPP10 is an accessory subunit for Kv4 and is known to increase the current amplitude of Kv4.2 and accelerates the inactivation and the recovery from inactivation of Kv4.2 (38,42,54). In our previous report, we demonstrated that the biophysical properties of Kv4.2 change with increasing expression level of KChIP4 (50). We first investigated whether the biophysical properties of Kv4.2 were also variable and dependent on the expression level of DPP10 as in the case of KChIP4.

2.5 ng of Kv4.2 cRNA was injected with various amounts of DPP10 cRNA (0, 0.025, 0.25, and 2.5 ng) into Xenopus oocytes and the currents were recorded under two electrode voltage clamp on one to three days after the injection. Kv4.2 current was evoked by depolarizing pulses from -100 to +50 mV (Fig. 1A). The current amplitudes were not significantly changed by up to 0.25 ng of DPP10 cRNA, and were significantly changed by between 0.25 ng (3.2 ± 0.4 µA, $n = 19$) and 2.5 ng (8.6 ± 1.2 µA, $n = 10$; $p < 0.001$) (Fig. 1B). On the other hand, the normalized I-V relationships were not significantly different among groups (Fig. 1C).

Another prominent change induced by DPP10 co-expression was in the kinetics of the recovery from inactivation. To evaluate the recovery from inactivation, a +40 mV depolarizing prepulse was applied for 500 ms to fully inactivate the current and a second +40mV test pulse was subsequently applied after hyperpolarizing at -100mV for 10 - 490 ms to measure the recovery (Fig. 2A). The timecourse of the recovery from inactivation was best fitted with a single exponential function (Fig. 2B). The time constant was decreased from 145.9 ± 2.7 ms (Kv4.2 (2.5 ng) with DPP10 (0.025 ng), $n = 7$) to 69.3 ± 3.2 ms (Kv4.2 (2.5 ng) with DPP10 (2.5 ng), $n = 8$; $p < 0.001$), suggesting that the recovery from inactivation of Kv4.2 accelerated when more than 0.25 ng of DPP10 cRNA was injected with Kv4.2 cRNA (Fig. 2C).
Taking these results together, the current amplitude and the recovery from inactivation were changed depending on the expression level of DPP10. This raised the possibility that the stoichiometry of the Kv4.2/DPP10 complex might be variable and dependent on the relative expression level of each subunit as we previously observed for the Kv4.2/KChIP4 complex (50).

Approximately 70% of DPP10 on the membrane exists as dimers

Determination of stoichiometry had not been an easy task with a classical biochemical method. However, the recent development of single molecule imaging enabled us to count the number of molecules directly (52,53,55). The idea of the method is that one can count the number of subunits in a single membrane protein complex (e.g. ion channel tetramer) by counting irreversible bleaching steps from a fluorescence-tagged protein (e.g. GFP-tagged subunit): one step means a single bleaching event of GFP, indicating the existence of single subunit. It is an especially powerful method for examining stoichiometry because each individual macromolecule complex can be evaluated (counted) (50,51,56,57). Before determining the stoichiometry of the Kv4.2/DPP10 complex, we first determined the stoichiometry of DPP10 alone.

DPP6 is a member of the dipeptidyl aminopeptidase-like protein family and is also known as an accessory subunit for Kv4 (29,58,59). The crystal structure of the extracellular domain shows that DPP6 forms a dimeric structure (26). The recently published crystal structure of the extracellular domain of DPP10 shows a similar dimeric structure (48). To examine whether the full-length DPP10 actually exists as a dimer in the membrane we constructed mEGFP tagged DPP10 (mEGFP-DPP10) in which mEGFP was fused to the intracellular N-terminus of DPP10 (Fig. 3A). mEGFP-DPP10 alone was expressed in Xenopus oocytes and the fluorescent spots were observed under TIRF illumination (Fig. 3B; Supplemental Movie 1). We counted the bleaching steps from 185 spots in total and the numbers of spots for each bleaching step were plotted as a histogram (Fig. 3C, D). Most of the spots showed one or two bleaching steps with a few exceptions of three/four bleaching steps (probably due to an occasional overlapping of two fluorescent spots(53)), suggesting that DPP10 exists as dimers as expected from the DPP6/10 structures, and possibly also as monomers (Fig. 3C, D). We then estimated the proportion of monomers, if present, and dimers based on a binomial distribution, with a variable fluorescence probability $p$ (Table. 1). According to some previous reports, the probability that mEGFP is fluorescent is around 0.7-0.8 (50-52). If that is also the case here, 65-81% of the spots on the membrane would be dimers and the rest
would exist as monomers (Table 1).

**4:2 stoichiometry is preferred in the Kv4.2/DPP10 complex**

Next we determined the stoichiometry of Kv4.2 and DPP10. To identify mEGFP-DPP10 co-assembled with Kv4.2, Kv4.2 was tagged with the red fluorescent protein mCherry (Kv4.2-mCherry) (Fig. 4A). We first confirmed that Kv4.2-mCherry (2.5 ng) and mEGFP-DPP10 were functional. Kv4.2-mCherry (2.5 ng) with mEGFP-DPP10 (2.5 ng) showed fast inactivating currents similar to those of wild-type Kv4.2 (2.5 ng) with wild-type DPP10 (2.5 ng) (Fig. 4B). To confirm the co-assembly of Kv4.2-mCherry and mEGFP-DPP10, mCherry was first excited with a 588 nm laser (Fig. 4C, left) and mEGFP was subsequently excited with a 488 nm laser (Fig. 4C, middle; Supplemental Movie 2). The mCherry and mEGFP frames from the obtained movie were superimposed (Fig. 4C, right). The yellow spots indicate co-assembly of Kv4.2-mCherry and mEGFP-DPP10. To examine the idea of variable stoichiometry, Kv4.2-mCherry and mEGFP-DPP10 were co-expressed in Xenopus oocytes with various cRNA ratios (100:1 (1 ng:0.01 ng), 10:1 (1 ng:0.1 ng), 1:1 (0.1 ng:0.1 ng)), and the bleaching steps from each mEGFP fluorescent spot were counted (Fig. 4D). Throughout the three different expression levels, the two bleaching step spots were consistently predominant although the three and four bleaching step spots were gradually increased with increasing DPP10 expression (Fig. 4E). The result suggests that the stoichiometry of the Kv4.2/DPP10 complex is variable depending on the expression level of DPP10, and has a preference to form the 4:2 (Kv4.2:DPP10) complex.

**Co-expression of KChIP4 does not significantly change the stoichiometry of the Kv4.2/DPP10 complex**

It is known that Kv4, DPP and KChIP form a ternary complex in neurons (35,60,61). To examine if the co-expression of KChIP4 affects the stoichiometry of the Kv4.2/DPP10 complex, Kv4.2-mCherry, mEGFP-DPP10 and KChIP4 were all co-expressed (Fig. 5A). Before analyzing the stoichiometry of the Kv4.2/DPP10 complex, we first confirmed that the co-expression of KChIP4 modulated the ionic current of the Kv4.2-mCherry/mEGFP-DPP10 complex as expected. The inactivation of the Kv4.2-mCherry/mEGFP-DPP10 complex in the presence of KChIP4 (Fig. 5B, bottom) was apparently slowed compared to the case in the absence of KChIP4 (Fig. 4B, bottom), and showed similar kinetics to that of wild-type Kv4.2, DPP10 and KChIP4 (Fig. 5B, top). The stoichiometry of the Kv4.2/DPP10 complex with various ratios of Kv4.2, DPP10 and KChIP4 (Kv4.2-mCherry:mEGFP-DPP10:KChIP4 = 100:1:100 (5 ng:0.05 ng:5 ng), 10:1:10 (5 ng:0.5 ng:5 ng) and 1:1:1 (1 ng:1 ng:1 ng))
was examined by subunit counting. In all three cases, the two bleaching step spots were predominant and the three and four bleaching steps were increased along with increasing mEGFP-DPP10 expression (Fig. 5C). Compared with the stoichiometry of the Kv4.2/DPP10 complex in the absence of KChIP4 (Fig. 4E), no notable stoichiometry changes were observed in the presence of KChIP4. As we previously showed that the stoichiometry of Kv4.2/KChIP4 is independent of DPP10 (50), the stoichiometry of the Kv4.2/DPP10 complex is independent of KChIP4 expression as well.

The extracellular domain of DPP10 may prevent the binding of third and fourth DPP10 subunits to the 4:2 channel

The extracellular domain of DPP6/10 forms a dimeric structure according to the crystal structure (26,48) and our subunit counting experiment (Fig. 3). Because the extracellular domain is large (residues 50-789) and bulky, we predicted that a deletion of the extracellular domain might change the preference of the 4:2 stoichiometry in the Kv4.2/DPP10 complex. Lin et al. recently made the deletion mutant of DPP6 (DPP6-D-Extra), which lacks the entire C-terminal extracellular domain (41). Interestingly, DPP6-D-Extra was very well expressed on the cell surface of HEK293 cells and accelerated the recovery from inactivation, yet it failed to increase the current amplitude (41). To examine whether the extracellular domain has an influence on the stoichiometry of Kv4.2/DPP10, we made a similar C-terminal deletion mutant DPP10-D-Extra, which lacks the entire extracellular domain (residues 50-789) (Fig. 6A). First, we injected cRNA of mEGFP-DPP10-D-Extra (2 ng) alone and observed the expression in Xenopus oocytes. This was well expressed on the cell surface in agreement with Lin et al. Most of the fluorescent spots were mobile (supplementary movie 3), in sharp contrast to mEGFP-DPP10 with the extracellular domain, which mostly stayed in the same place (supplementary movie 1). Because DPP6 interacts with fibronectin in the extracellular matrix (28), DPP10 may also bind to the extracellular matrix via the extracellular domain to stay in the same place.

Kv4.2 (10 ng of cRNA) and mEGFP-DPP10-D-Extra (0.1 ng) were then co-expressed in Xenopus oocytes. Probably because DPP10-D-Extra mutants were mobile, fewer co-localized spots were found (white arrowheads in Fig. 6B) than with the combination of Kv4.2 and DPP10 (Fig. 4C). Nevertheless we were able to collect 158 countable spots. Even at this cRNA ratio (100:1), we observed a substantial number of three and four bleaching spots (Fig. 6C). The distribution of bleaching steps looks more similar to the distribution of the 1:1 cRNA ratio than that of the 100:1 ratio in Kv4.2/DPP10 (Fig. 4E). This result suggests that the dimeric
extracellular domains of DPP10 probably prevents binding of the third and fourth DPP10 subunits to Kv4.2. This is the reason why the 4:2 stoichiometry is preferred in the Kv4.2 and full-length DPP10 complex.

**DISCUSSION**

In this study, we obtained three findings as follows. First, we showed that the electrophysiological properties of Kv4.2 are affected by the expression level of DPP10 (Figs. 1, and 2). Second, approximately 70% of DPP10 on the membrane exists as dimers and the rest exists as monomers (Fig. 3). Third, the stoichiometry of the Kv4.2/DPP10 complex changes depending on the expression level of each subunit with a tendency to form a 4:2 (Kv4.2 : DPP10) channel probably due to the dimeric nature of the extracellular domain (Figs. 4, 6 and 7A). Here we discuss how the observations can be explained in accordance with the current view of the Kv4.2 multimolecular complex.

*Variable stoichiometry and function of the Kv4.2/DPP10 complex*

In electrophysiological experiments, we observed that the current amplitude of Kv4.2 increased with expression of DPP10 (2.5 ng) (Fig. 1). There are two reported mechanisms by which DPP6/10 boosts the Kv4 current. DPP6 (and probably DPP10) increases the single channel conductance of Kv4.2 (39). It has also been demonstrated that co-expression of DPP6/10 facilitates the surface expression of Kv4.2 through an effect on the ER (62). Therefore, the augmentation of Kv4.2 current amplitude observed in Fig. 1 is caused by either or both of an increase in the single channel conductance and an increase in the number of Kv4.2 channels expressed in the plasma membrane. We also observed that the current amplitude augmentation required relatively high (2.5 ng) DPP10 cRNA (Fig. 1), whereas the recovery from inactivation (Fig. 2) was affected by much lower amounts of DPP10 cRNA. These findings raise the possibility that the number of DPP10 subunits required for each type of modulation is different: i.e. a smaller number of DPP10 subunits can accelerate the recovery from inactivation while more subunits are required for the augmentation of the current amplitude.

We observed that Kv4.2 tends to form a 4:2 (Kv4.2:DPP10) complex with DPP10 but additional DPP10 subunits can bind to the 4:2 channel when the DPP10 co-expression is high (Fig. 4). According to a previous study using the tandem repeat constructs KD (Kv4.2-DPP6) and KKD (Kv4.2-Kv4.2-DPP6), the KKD (4:2) channel can be modulated further by additional DPP6 while the KD (4:4) channel cannot (49). There are a few other notable findings from the previous results: (1) the current amplitude of KD is much higher than that of KKD, suggesting high DPP expression is required to increase the
current amplitude as we showed in Fig. 1, 
(2) the conductance-voltage relationship, 
the steady state inactivation-voltage 
relationship, and time to peak are gradually 
changed from K (Kv4.2 alone), through 
KKD to KD, suggesting that the 
stoichiometry and function of Kv4.2/DPP6 
are variable as we discussed here. 
Although the conclusion of the authors of 
the previous study is that Kv4.2 requires 
four DPP6 subunits to generate I_{SA} current, 
their data also support the idea that the 
stoichiometry of Kv4.2/DPP is variable. 

A model of Kv4.2/DPP10 complex formation 

Under the TIRF microscope, we 
observed that approximately 70% of the 
DPP proteins on the membrane exist as 
dimers and the rest exist as monomers (Fig. 
3). Which form of DPP10 binds to Kv4.2 
and forms the complex? 

In the previous study using chimeras of Kv4.3/Kv1.4 and DPP10/DPP4, 
the region from the cytoplasmic N-terminus 
and the transmembrane domain of DPP10 
and the S1-S2 segments of Kv4.3 were 
shown to be sufficient for complex 
formation (47). It is also reported that the 
extracellular-domain-less DPP6 mutants 
(DPP6-D-Extra) can bind to and modulate 
Kv4.2 channels (41). On the other hand, the 
crystal structure demonstrates that the 
extracellular domain of DPP6/10 forms a 
dimeric structure (26,48). Dipeptidyl 
peptidase 4 (DPP4), which is a membrane- 
bound enzyme and does not modulate Kv4, 
shows catalytic activity when DPP4 forms a 
dimer (63). If the transmembrane domain of 
DPP binds to the S1 and S2 segments of 
Kv4, there would be four binding sites for 
DPPs in one Kv4 channel (Fig. 7A). If two 
DPP10 subunits diagonally bind to Kv4.2 
retaining a dimer structure, the dimeric 
large extracellular domain may hinder the 
additional binding of a second DPP10 dimer 
(Fig. 7Ba). Or if the DPP10 dimer can 
bind to Kv4.2 via one of the transmembrane 
domains of the dimer, up to eight DPP10 
subunits could exist in one Kv4.2/DPP10 
complex (Fig. 7Bb), although our subunit 
counting experiments demonstrated that up 
to four, not more, DPP10 subunits can bind 
to a single Kv4.2 channel (Fig. 4). Another 
possible model of binding is that a DPP10 
dimer binds to two neighboring S1-S2 
segments (Fig. 7Bc). However, this model 
does not explain why the 4:2 stoichiometry 
is favored. 

How then is the Kv4.2/DPP10 complex formed? Because DPPs can 
be exported to the plasma membrane without 
Kv4, and exist as a mixture of monomers 
and dimers (Fig. 3), one possibility is that 
monomeric DPP10 subunits bind to Kv4 
and form a dimer in the extracellular 
domain (Fig. 7C). On the other hand, the 
dimeric nature of DPP10 may prevent the 
third and fourth DPP10 subunits from 
binding to the 4:2 channels. The fact that the 
extracellular deletion mutant DPP10-D- 
Extra showed a higher number of three and
four bleaching spots supports the idea of a role of the extracellular domain in determining the stoichiometry (Fig. 6).

Is the stoichiometry of the Kv4/KChIP/DPP ternary complex variable under physiological conditions?

It is known that Kv4 forms a ternary complex with DPP and KChIP in the brain (17,35,60,64). In a previous study, we revealed that the stoichiometry of the Kv4.2/KChIP4 complex is variable with no specific preference and changes depending on the expression level of KChIP (50). We also showed that the co-expression of DPP10 does not affect the stoichiometry of the Kv4.2/KChIP4 complex. In this study, we also observed that co-expression of KChIP4 does not affect the stoichiometry of the Kv4.2/DPP10 complex. These findings suggest that KChIP and DPP bind independently of each other.

Are the expression levels and the stoichiometry of Kv4/DPP variable? In the case of KChIP2, the expression level is dynamically changed with circadian rhythm, and that could change the stoichiometry of Kv4.2/KChIP2 (65). It is unknown whether the expression level of DPP is dynamically controlled. Interestingly, the graded expression of A-type current (Kv4 current) critically controlled by DPP6 (29) suggests that the subcellular localization of DPP6 might also be graded. DPP6 is basically well co-localized with Kv4.2 in the hippocampus and the cerebellar cortex. DPP6 is also expressed in the hippocampal mossy fibers where Kv4.2 is absent (22) suggesting that the expression of Kv4.2 and DPP6 do not always overlap. DPP10 is also widely co-expressed in many brain regions with Kv4.2 and/or Kv4.3 (35). Interestingly, sublocalization of DPP10 is not perfectly overlapping with Kv4 and KChIP. DPP10 is mainly expressed in the soma while Kv4.3 and KChIP1 are expressed both in the soma and the dendrites in the hippocampus and the neocortex (35). This means that the stoichiometry of the ternary complex may be different depending on the subcellular location.

The expression pattern of DPP10 partially overlaps the distribution of DPP6 (20,60). Because the amino acid identity of the transmembrane domain between DPP6 and DPP10 is high (91%) and the transmembrane domain of DPP10 is important for the binding to Kv4.2, DPP6 may form a heteromultimeric complex with DPP10 (47,48). The combination of Kv4, KChIP and DPP6/10 in the complex would give rise to further diversity of the biophysical properties and may dynamically regulate cell excitability by a complicated stoichiometry.
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CONFLICT OF INTEREST
The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS
YK and KN designed the research. MK performed the experiments. MK and KN analyzed the data. All authors reviewed the results, wrote the manuscript and approved the final version of the manuscript.

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**FOOTNOTES**
The abbreviations used are: Kv, voltage-gated K⁺ channel; DPP, dipeptidyl aminopeptidase-like protein; KChIP, K⁺ channel interacting protein; cRNA, complementary RNA; TIRF, total internal reflection fluorescence; mEGFP, monomeric enhanced green fluorescent protein; mCherry, monomeric Cherry fluorescent protein.
FIGURE LEGENDS

FIGURE 1. The expression level of DPP10 affects the current amplitude of Kv4.2. A, Representative current traces of Kv4.2 (2.5 ng) with DPP10 (0, 0.025, 0.25 and 2.5 ng) are shown. The holding potential was -80 mV. After 500 ms hyperpolarization at -110 mV to remove inactivation, currents were elicited by depolarizing voltage steps for 1 s between -100 mV and +50 mV in 10 mV increments. Current traces of the first 150 ms are shown. B, Current amplitudes of Kv4.2 with various expression levels of DPP10 are compared (n = 10-19). *** indicates p < 0.001. C, I-V relationships of Kv4.2 without DPP10 (open circles) and with DPP10 (0.025 ng, open squares; 0.25 ng, open triangles; and 2.5 ng, open inverted triangles). Peak current amplitudes at each membrane potential taken from (A) are normalized by the peak current amplitude at +50 mV (n = 10-19).

FIGURE 2. The recovery from inactivation of Kv4.2 is gradually accelerated with increasing expression level of DPP10. A, Representative current traces showing the recovery from inactivation. Various amounts of DPP10 cRNA (0, 0.025, 0.25 and 2.5 ng) were co-injected with 2.5 ng of Kv4.2 cRNA. The currents were elicited by a two-pulse protocol (inset). The currents evoked by the prepulse was omitted and only the currents by the second test pulses are presented. B, Kinetics of the recovery from inactivation of Kv4.2 with various expression levels of DPP10 (0 ng; open circles, 0.025 ng; open squares, 0.25 ng; open triangles, 2.5 ng; open inverted triangles; n = 7-9). Peak current amplitudes during the second +40 mV step were normalized by the average of the peak current amplitude during the +40 mV prepulse. C, Time constants of the recovery from inactivation of Kv4.2 with different DPP10 expression levels. The time constants are obtained by fitting the curves taken from (B) with a single exponential function. Error bars are S.E. *** indicates p < 0.001.

FIGURE 3. Approximately 70% of DPP10 exists as dimers. A, A schematic illustration of mEGFP-DPP10. mEGFP was fused to the intracellular N-terminus of DPP10. B, A single frame from a TIRF movie from a Xenopus oocyte expressing mEGFP-DPP10. Green circles indicate the fluorescent spots of mEGFP-DPP10. Scale bar is 2 µm. C, Representative traces of fluorescence intensity taken from the fluorescent spots in (B). Green bars show the time of illumination with a 488-nm laser. Green arrows indicate the bleaching steps. D, A histogram of the observed distribution of the number of bleaching steps.

FIGURE 4. The stoichiometry of the Kv4.2/DPP10 complex has a preference of 4:2. A, Schematic illustrations of Kv4.2-mCherry and mEGFP-DPP10. mCherry is fused to the C-terminus of Kv4.2 and mEGFP is fused to the N-terminus of DPP10. B, Representative current
traces of Kv4.2 (2.5 ng) with DPP10 (2.5 ng) (top) and Kv4.2-mCherry (2.5 ng) with mEGFP-DPP10 (2.5 ng) (bottom). C, Images of fluorescent spots of Kv4.2-mCherry (left), mEGFP-DPP10 (middle) and overlaid image (right). The white arrowheads (yellow spots) indicate co-localization of Kv4.2-mCherry and mEGFP-DPP10. Scale bars are 2 µm. D, Representative fluorescence traces of one, two, three and four bleaching steps. Red bars indicate illumination with a 588-nm laser and green bars indicate illumination with a 488-nm laser. Green arrows show the bleaching steps. E, Histograms of the distributions of the number of bleaching steps. Kv4.2-mCherry and mEGFP-DPP10 are expressed with different ratios (Kv4.2-mCherry:mEGFP-DPP10 = 100:1, 10:1 and 1:1).

FIGURE 5. **Co-expression of KChIP4 does not affect the stoichiometry of the Kv4.2/DPP10 complex.** A, Schematic illustrations of Kv4.2-mCherry, mEGFP-DPP10 and KChIP4. B, Representative current traces of wild-type Kv4.2, wild-type DPP10 and wild-type KChIP4 (top), and Kv4.2-mCherry, mEGFP-DPP10 and wild-type KChIP4 (bottom). C, Kv4.2-mCherry, KChIP4 and mEGFP-DPP10 were expressed in *Xenopus* oocytes with different ratios (Kv4.2-mCherry:mEGFP-DPP10:KChIP4 = 100:1:100, 10:1:10 and 1:1:1). The histograms show the distributions of the bleaching steps from the spots of mEGFP-DPP10.

FIGURE 6. **The extracellular domain deletion DPP10 mutant shows less preference for the 4:2 stoichiometry.** A, Schematic illustrations of Kv4.2-mCherry and mEGFP-DPP10-D-Extra. B, Image of fluorescent spots of Kv4.2-mCherry (red) and mEGFP-DPP10-D-Extra (green). The white arrowheads indicate co-localization of Kv4.2-mCherry and mEGFP-DPP10-D-Extra. The scale bar is 2 µm. C, A histogram of the distributions of the number of bleaching steps from oocytes expressing Kv4.2-mCherry and mEGFP-DPP10-D-Extra with 100:1 cRNA ratio.

FIGURE 7. **Hypothetical models of Kv4.2/DPP10 complex formation.** A, The stoichiometry of the Kv4.2/DPP10 complex changes depending on the relative expression level of Kv4.2 and DPP10 subunits with a preference for 4:2. Green columns indicate Kv4.2 α subunits and pink columns indicate the transmembrane domain of DPP10. B, Possible models of the 4:2 (Kv4.2:DPP10) channel complex. a; Two DPP10 subunits bind to Kv4.2 at diagonal positions. The extracellular domains of two DPP10 subunits bind to each other and prevent an additional DPP10 subunit from binding to Kv4.2. b; A DPP10 dimer binds to a single subunit of the Kv4.2 channel. As a tetrameric Kv4.2 channel has four binding sites for DPP10, up to eight DPP10 subunits can bind to one Kv4.2 channel in this configuration, although only a single DPP10 dimer is depicted. c; Two DPP10 subunits bind to adjacent binding sites on Kv4.2. The third and fourth DPP10 subunits may be able to bind to Kv4.2 in this configuration. C, Schematic illustrations of
the DPP10 monomer-dimer transition and the Kv4.2/DPP10 complex formation in the plasma membrane. All five stoichiometries (4:0, 4:1, 4:2, 4:3 and 4:4) of the Kv4.2/DPP10 complex are depicted although the 4:2 stoichiometry is stable because of the dimeric nature of DPP10.
TABLE 1.

Calculated proportion of dimer and monomer of DPP10 on the plasma membrane

The proportions of dimer and monomer DPP10 based on the distribution in Fig. 4D. The value of fluorescence probability of mEGFP \( (p) \) was changed between 0.63 and 1.

<table>
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<tr>
<th>( p )</th>
<th>Dimer</th>
<th>Monomer</th>
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<tbody>
<tr>
<td>1</td>
<td>0.46</td>
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</tr>
<tr>
<td>0.9</td>
<td>0.54</td>
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<td>0.01</td>
</tr>
</tbody>
</table>
Figure 2
Figure 6

A

Kv4.2-mCherry  +  mEGFP-DPP10
D-Extra

B

C

100 : 1

# of spots

1  2  3  4

# of bleaching steps
Kv4.2 and Accessory Dipeptidyl Peptidase-like Protein 10 (DPP10) Subunit Preferentially Form a 4:2 (Kv4.2:DPP10) Channel Complex
Masahiro Kitazawa, Yoshihiro Kubo and Koichi Nakajo

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