Rearrangements in the Relative Orientation of Cytoplasmic Domains Induced by a Membrane-anchored Protein Mediate Modulations in Kv Channel Gating

Received for publication, June 5, 2009, and in revised form, August 3, 2009. Published, JBC Papers in Press, August 18, 2009, DOI 10.1074/jbc.M109.028761

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Interdomain interactions between intracellular N and C termini have been described for various K⁺ channels, including the voltage-gated Kv2.1, and suggested to affect channel gating. However, no channel regulatory protein directly affecting N/C interactions has been demonstrated. Most Kv2.1 channel interactions with regulatory factors occur at its C terminus. The vesicular SNARE that is also present at a high concentration in the neuronal plasma membrane, VAMP2, is the only protein documented to affect Kv2.1 gating by binding to its N terminus. As its binding target has been mapped near a site implicated in Kv2.1 N/C interactions, we hypothesized that VAMP2 binding to the N terminus requires concomitant conformational changes in the C terminus, which wraps around the N terminus from the outside, to give VAMP2 access. Here, we first determined that the Kv2.1 N terminus, although crucial, is not sufficient to convey functional interaction with VAMP2, and that, concomitant to its binding to the “docking loop” at the Kv2.1 N terminus, VAMP2 binds to the proximal part of the Kv2.1 C terminus, C1α. Next, using computational biology approaches (ab initio modeling, docking, and molecular dynamics simulations) supported by molecular biology, biochemical, electrophysiological, and fluorescence resonance energy transfer analyses, we mapped the interaction sites on both VAMP2 and Kv2.1 and found that this interaction is accompanied by rearrangements in the relative orientation of Kv2.1 cytoplasmic domains. We propose that VAMP2 modulates Kv2.1 inactivation by interfering with the interaction between the docking loop and C1α, a mechanism for gating regulation that may pertain also to other Kv channels.

Interdomain interactions between intracellularly located N and C termini have been described for various K⁺ channels, including inwardly rectifying Kir2.3 and Kir6.2 (1, 2), small conductance Ca²⁺-activated (hSK3) (3), and voltage-gated Kv2.1 (4) and Kv4.1 (5) channels. In the case of Kv2.1, two modes of interaction have been proposed: an association of the distal part of Kv2.1 C terminus (termed CTA domain; amino acids (aa) 741–853)⁴ with aa 67 and 75 of the Kv2.1 N terminal (4); or an association between the proximal part of the Kv2.1 C terminus (aa 444 – 477) and the predicted loop structure (aa 55 – 71) in the N-terminal T1 domain (6). In addition, involvement of the S4-S5 linker in this interaction has been suggested (7). Although these studies propose two different C-terminal sites, they indicate a specific loop in the N terminus of Kv2.1 (6, 8), which could be functionally related to the Shaker and Shal docking loops in the lateral part of their T1 domains (9, 10). These latter loops are responsible for the subfamily-specific association with β-subunits (Kvβ and KChIP, respectively). Further, the interaction between the N and C cytoplasmic termini (N/C interaction) of Kv2.1 has been shown to be dynamic and voltage-dependent and to involve structural rearrangements between these domains, which could affect both activation and inactivation gating of the channel (4, 6, 7). These rearrangements can be clearly detected with fluorescence resonance energy transfer (FRET) (11). A similar N/C interaction has been shown to affect gating of the closely related Kv4.1 channel (5, 12).

It is conceivable that the specific packaging of Kv2.1 cytoplasmic termini (a relatively long C terminus (>400 aa) wrapping the N terminus (<190 aa) from the outside (4)) not only supports multiple interactions between the termini but also reflects the fact that most of the interactions of the channel with intracellular and membrane-bound regulatory factors occur at the C terminus, including channel phosphorylation (13–15), clustering through a unique proximal restriction and clustering signal (16), and protein-protein interactions with both the plasma membrane SNAREs, syntaxin 1A and SNAP-25 (17–19), and the MiRP2 (KCNE3) peptide (20). For the Kv2.1 N

⁴ The abbreviations used are: aa, amino acid(s); SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptors; eCFP, enhanced cyan fluorescent protein; eYFP, enhanced yellow fluorescent protein; FRET, fluorescence resonance energy transfer; GST, glutathione S-transferase; r.m.s.d., root-mean-square deviation; MD, molecular dynamics; NTD, N-terminal domain; PDB, Protein Data Bank; WT, wild type; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; KChIP, Kv4 channel interaction protein.
terminus, on the other hand, there are only two examples of protein–protein interactions: a transient association with KChAP (21), which does not affect channel function; and an interaction with the vesicular SNARE partner VAMP2 (synaptobrevin 2), which is also present at a high concentration in the neuronal plasma membrane and enhances channel inactivation (8). Specifically, VAMP2 has been shown to associate with the extension of a docking loop in the lateral part of the T1 domain (8) near the site of interaction with the C terminus (4, 6). Thus, it is reasonable to hypothesize that interaction with VAMP2 will affect the N/C interaction, similar to proton-mediated Kir2.3 (1) and Kir1.1 (22) N/C interactions or the ATP-dependent Kir6.2 (2) N/C interaction. To date, no protein molecule that directly affects N/C interactions in a K⁺ channel has been demonstrated. Because VAMP2 was the first protein documented to affect Kv2.1 channel gating by binding to a specific N-terminal site, which is probably masked by the C terminus, we have put forward the idea that its interaction with the Kv2.1 N terminus requires conformational changes in the C terminus that will enable its access to the N terminus.

Here we endeavored to gain a mechanistic and structural understanding of the Kv2.1-VAMP2 interaction. Based on our evidence, we propose that VAMP2 modulates Kv2.1 gating by interfering with the Kv2.1 cytoplasmic N/C interaction.

**EXPERIMENTAL PROCEDURES**

**Constructs and Antibodies**—The antibodies used were: polyclonal antibodies against Kv2.1 C terminus or Kv2.1 N terminus (Alomone Laboratories, Jerusalem, Israel) and polyclonal antibody against VAMP2 (Abcam, Cambridge, UK). Kv2.1 wt type (WT) (kindly provided by Prof. R. Joho, The University of British Columbia, Vancouver, Canada) cDNAs were cloned in pBluescript. Type (WT) (kindly provided by Prof. R. Joho, The University of British Columbia, Vancouver, Canada) cDNAs were cloned in pBluescript. Kv2.1 cDNAs (amino acids 28–853 or 29–120, correspondingly) with oligonucleotide primers designed for introducing its deletion/truncation mutants; 2.5 ng/oocyte Kv2.1N/Kv1.5 and 1.5 ng/oocyte VAMP2. Two-electrode voltage clamp recordings were performed as described (25). To avoid possible errors introduced by series resistance, only current amplitudes up to 6 μA were recorded. Net current was obtained by subtracting the scaled leak current elicited by a voltage step from −80 to −90 mV. Oocytes with a leak current of more than 3 nA/1 mV were discarded. Experimental protocols and data analyses are described in the legends to Figs. 2C, 3A, and 4C and Fig. 6, D and E. In general, the voltage dependence of the channel inactivation was studied using 5- or 30-s depolarizing pre-pulses in the case of WT Kv2.1 or ΔNKv2.1 and Kv2.1ΔC1a (of which inactivation is attenuated), respectively. Previously, it had been shown that both protocols yield qualitatively similar results (19).

**Immunoprecipitation in Oocytes**—For the biochemical assays, oocytes were injected with 5–15 ng/oocyte Kv2.1 or all mutants and with 0.25–1.5 ng/oocyte VAMP2 (further detailed in the legends to Figs. 2C, Fig. 3, B and D, and Fig. 4D). Oocytes were subjected to immunoprecipitation as described (25). Briefly, immunoprecipitates from 1% CHAPS oocytes homogenates were analyzed by SDS-PAGE (12% polyacrylamide). Digitized scans were derived by PhosphorImager (GE Healthcare), and relative intensities were quantified using ImageQuant.

**In Vitro Binding of GST Fusion Proteins with His₅-tagged or [³⁵S]-Labeled VAMP2**—The fusion proteins were synthesized and reacted with VAMP2 as described (26). Briefly, purified GST fusion proteins (100–200 pmol) immobilized on glutathione-Sepharose beads were usually incubated with either 200 pmol of His₅-tagged VAMP2 or [³⁵S]-labeled VAMP2 (VAMP2 translated on the template of *in vitro* synthesized RNAs using a translation rabbit reticulocyte lysate kit (Promega) according to the manufacturer’s instruction manual) in 1 ml of phosphate-buffered saline containing a protein detergent (0.1% Triton X-100). After washing, the GST fusion proteins were eluted with 20 mM reduced glutathione in 40 μl of elution buffer (120 mM NaCl, 100 mM Tris–HCl, pH 8) and separated by 12% SDS-PAGE. The immunoblot was visualized using the ECL detection system (Pierce); proteins were stained by Ponceau.

**Protein Structure Prediction**—Two types of structure prediction methods were exploited: *homology*-based prediction was used to create a model of Kv2.1 tetramerization (T1 domains, and *ab initio* was used for modeling of various protein domains including Kv2.1 proximal N-terminal (N-tail, residues 1–30) and C1a domain (residues 411–510), and the N terminus of VAMP2 (NTD, residues 1–30) of which templates are not available.

Homology-based prediction of Kv2.1 T1 domain was performed via the SWISS-MODEL server. The first 186 amino acids of Kv2.1 were submitted to the template identification tool for best alignment template identification using the following parameters: InterPro domain scan performed against HMMPam, HMMTigr, ProfileScan, SuperFamily, BlastProDom; PSI-BLAST profile generation: iteration = 10, *E*-value = 0.0001, protein substitution matrix = BLOSUM62 11(G) 1(E), SEG filter for low complexity subsequence = activated; PSI-BLAST profile search: search data base = ExpPDB90, *E*-value = 0.00001, protein substitution matrix = BLOSUM62 11(G) 1(E), alignment = 50; HHSearch software was used to detect distantly related templates (supplemental Fig. 1A). A crystal structure-based model of the Kv3.1 T1 domain (PDB ID: 3KV, chain A) was used as a template, since: score = 96.3 bits (238),
E-value = 7e-21, identities = 46/107 (42%), positives = 65/107 (60%), gaps = 11/107 (10%). Swiss-PDB viewer was used to generate the project submitted to the SWISS-MODEL server. Energetic profile analysis of the predicted structure of Kv2.1 T1 was tested based on standard Anolea, GROMOS, and Verify3D parameters of the SWISS-MODEL outcome, which revealed significant stability with a very limited number of structures having unfavorable energy scores (supplemental Fig. 1B). The calculated energy for the derived model was \(-5982.097\) kJ/mol.

Ab initio prediction was based on the Protinfo ab initio structure prediction server. Prediction output sums up to the five most favorable structures. The obtained models were checked for Ramachandran plot. The best structures were selected and assigned with the CHARMM22 force field (Gasteiger method was used for charge assignment) with subsequent Newton-Raphson method (Truncated Newton algorithm 30,000 iterations in Vega ZZ software) (27–29) for the structure minimization. Five structures predicted by the server were aligned structurally using SWISS-PDB viewer 3.7 SP5; Magic Fit was applied to the structure backbone atoms. Significant similarities revealed during the alignment were considered as adequateness of ab initio prediction (as shown for N-tail on supplemental Fig. 1C). Ramachandran plot-based analysis was used for choosing the most favorable structure.

Docking Modeling of a Protein-Protein Interaction—Files containing atomic coordinates tetramerization domain from Kv3.1 voltage-gated potassium channel (PDB ID: 3KV7) and complexin-SNARE complex (PDB ID: 1KIL) were obtained from the Protein Data Bank server. Three-dimensional models, prepared by cutting off unnecessary information from PDB files, were used for structure alignment and protein-protein interaction analysis (docking simulation), as described below. A Windows version of Hex docking software was used for simulation of protein-protein interaction (30). The receptor-ligand choice was dependent on the simulation purpose. For Kv2.1T1-Kv2.1N–30 docking model (model set 1): receptor = T1, ligand = N1–30; for Kv2.1 N terminus-VAMP2NTD (model set 2): receptor = Kv2.1 N terminus (approved model of Kv2.1T1-Kv2.1N–30 interaction), ligand = VAMP2 NTD; for Kv2.1 N terminus-VAMP2 NTD with C1a interaction (model set 3): receptor = Kv2.1-NTD (approved model of Kv2.1 N terminus-VAMP2 NTD interaction); for Kv2.1 N terminus-C1a interaction (model set 4): receptor = Kv2.1 N terminus (approved model of Kv2.1T1-Kv2.1N–30 interaction), and ligand = C1a. Docking parameters were as follows: correlation type: shape and electrostatics; FFT mode: five-dimensional post-processing: MM minimization (Newton-like energy-based molecular mechanic refinement); grid dimension = 0.6 Å, solutions = 50 000, receptor range = 180 (for model sets 1 and 2) and 60 (model sets 3 and 4) with step size = 7.5 Å; ligand range = 180 (for model sets 1 and 2) and 60 (model sets 3 and 4) with step size = 7.5 Å; twist range = 360 with step size = 5.5 Å; distance range = 40 Å with scan step = 0.75 Å and substeps = 2; order of docking resolution, 16 for sterical scan and 25 for final search. The obtained result was clustered according to spatially similar docking orientations with clustering root-mean-square threshold = 3.0 Å and bumping threshold = 0.100. The 100 most favorable clusters were visually evaluated to make the final option.

In addition, we performed structural alignment of the obtained docking models against the tetrameric model of the Kv1.2 channel to exclude any interaction occurring at the tetramerization interfaces or sterical clashes with other neighboring subunits.

Molecular Dynamics (MD) Simulations—Multiple MD simulations were performed on the NAMD package (31) with VEGAZZ GUI and TINKER 4.2 with force field explorer GUI using the CHARMM force field. Partial charges were assigned according to the force field parameters. The structures were minimized using the first order Steepest Descent and the second order Adopted basis Newton-Raphson algorithms, 500 steps each. The protein was heated from 300 to 1000 K in 2000 steps and cooled down to 300 K in 100 steps, each of 1-fs duration with the further cooling to 300 K in 1000 1-fs time steps. Equilibration was performed for 1000 1-fs time steps. The equilibrated structure was used for the NVT thermodynamic ensemble was used for simulation. The production was performed for either 50,000 (for T1 domain) or 100,000 (for C1a) 1-fs time steps at 300 K. Every 100 steps, a snapshot was saved. We used an implicit solvent with a distance-dependent dielectric set to 80. A spherical cutoff was used to treat long range electrostatics; the nonbonded list radius was set to 14 Å, the nonbonded higher cutoff to 12 Å, and the nonbonded lower distance to 10 Å. No SHAKE constrains were applied. The obtained trajectory file was analyzed, and conformation samples were extracted. Geometric parameters such as r.m.s.d., gyration radius and molecular volume were calculated on VegaZZ software.

Protein Structure Alignment and Visualization—Molecular surfaces were computed using the DeepView/Swiss-PDB-Viewer 4.01 and Accelrys DS Viewer Pro (version 2). Protein structures were visualized using the Swiss-PDB viewer, Sirius, RasMol, PyMol, Accelrys DS viewer Pro, and Molsoft ICM browser.

FRET Analyses—Fluorescence emissions from eCFP- and eYFP-tagged proteins were collected from the animal hemisphere of the oocyte with a Zeiss inverted confocal microscope (Zeiss Axiovert LSM 510 Meta), using a ×20 0.75 N.A. objective and laser excitations of 405 and 514 nm, respectively. The level of laser intensity and the photo-multiplier tube and amplifier gain were kept the same for all experiments after initial calibration and titration of proteins. We used a spectrum-based method to remove contamination caused by donor emission and direct excitation of the acceptor (see Fig. 6, C and B). The FRET assay was performed as described previously (32). Briefly, two emission spectra were collected from each oocyte, one with 405 nm excitation and the other with 514 nm excitation. A scaled eCFP spectrum, collected from control oocytes expressing eCFP-tagged proteins only, was used to normalize the eCFP spectrum, collected from oocytes expressing both fluorophores at 405 nm excitation. This procedure allows the dissection of the eYFP emission spectrum, termed $F_{405}^{395}$, $F_{405}^{395}$, has two components, one due to direct excitation of eYFP, $F_{405}^{direct}$, and one due to FRET, $F_{405}^{FRET} \cdot F_{405}^{395}$ is normalized to the total eYFP emission with 514 nm excitation at the same oocyte, $F_{514}^{395}$. The resulting ratio, termed ratio A, can be expressed as

$$\text{Ratio A} = \frac{F_{405}^{395}}{F_{514}^{395}} = \frac{F_{405}^{direct}}{F_{514}^{395}} + \frac{F_{405}^{FRET}}{F_{514}^{395}}$$

(Eq. 1)
The direct excitation component in the calculated ratio $A$, termed ratio $A_{0}$, is determined experimentally from a large population of oocytes expressing eYFP-tagged proteins only. This allows the precise calculations of the bleed-through or the contamination of the direct excitation of eYFP by the 405 nm laser. The difference between ratio $A$ and ratio $A_{0}$ (ratio $A - ratio A_{0}$) is directly proportional to FRET efficiency.

\[
\text{Ratio } A - \text{Ratio } A_{0} = \frac{F_{\text{Å514}}}{F_{\text{Å405}}}
\]  

(Eq. 2)

**Presentation and Statistical Analysis**—The statistical significance of differences between the two groups was calculated by the use of independent sample, two-tailed $t$ test procedures; in the case of unequal variances, Mann-Whitney’s rank-sum test was elaborated. Multiple group comparisons were done using one-way analysis of variance followed by the Holm-Sidak post-hoc test for multiple comparisons. All statistical analyses were performed using SigmaStat (Systat Software). Graphical presentation of the data was made in SigmaPlot (Systat). Data are presented as means ± S.E.

**RESULTS**

**Structural Model of the N Terminus of Kv2.1**—The N terminus of Kv2.1, specifically the T1 domain, has been suggested to be the target of VAMP2 interaction (8). In the absence of any resolved structure for Kv2.1, we aimed to generate a structure of the N-terminal domain by homology modeling with SWISS-MODEL based on known crystal structures of the T1 domains of other Kv channels. As determined previously (6), the T1 domain of Kv3.1, with its high crystal structure resolution (2 Å) and its 40.541% amino acid identity to Kv2.1 T1 domain, was the best template found (supplemental Fig. 1A). Alignment of secondary and tertiary structures revealed a high similarity between the T1 domains of both channels, with an extra loop-like structure of 11 amino acids (aa 61–71), with the marginal amino acid identity to Kv2.1 T1 domain, was the best template found (supplemental Fig. 1A). This loop corresponds, but is not identical, to the docking loop identified previously by us as a site involved in VAMP2 binding and modulation of Kv2.1 inactivation gating (8).

Because the proximal part of the N termini of some Kv4.x channels has been shown to be important for interaction with their β-subunits and for determination of channel gating (12, 33, 34), we decided to include this part of Kv2.1 in our analysis of VAMP2 interaction. We used ab initio structure prediction to obtain a theoretical model of the first 30 amino acids of Kv2.1 (the “N-tail”), as homology modeling failed to predict the structure (see “Experimental Procedures”). Visual assessment revealed that with the exception of a six-amino acid sequence (aa 20–25, PMEIVR) the N-tail was unstructured and built of loops with high degrees of freedom (Fig. 1B). Next, to reconstitute the structure of the whole N terminus, we performed docking between the modeled N-tail and T1 structures, exploiting Hex software (see “Experimental Procedures”). One docking model (Fig. 1C, right panel) was chosen as the best steric (including tetrameric steric reconstitution; see “Experimental Procedures” and Fig. 1C, left panel) and energetic solution ($E_{\text{total}} = -318.9 \text{kJ/mol}$), showing a significantly higher contribution of geometric fit over electrostatic interaction (Fig. 1D and supplemental Fig. 1, D and E).

The N-terminal Domain of VAMP2 Is the Relevant Site for Interaction with Kv2.1—Interaction of VAMP2 with THE Kv2.1 N terminus could be mediated by the former’s proline-rich N-terminal domain (aa 1–18; NTD) and/or the membrane distal half (aa 60–88) of its SNARE domain, which forms the second amphipathic helix shown to interact with other proteins, especially those with a coiled-coil structure (35). However, THE interaction of the membrane distal part of the SNARE domain with the T1 domain seems sterically impossible, considering the former’s 108.07-Å-long helical structure (Fig. 2A), its close proximity to the plasma membrane, and the size and geometry of the T1 domain (36). The potential interaction of this region was further ruled out, as it is mostly unstructured in the monomeric form of VAMP2 as determined by circular dichroism (37) and solution NMR experiments (38, 39). As the structure of the VAMP2 NTD has not yet been resolved and no homology model has been successful, we created a model by ab initio prediction on the basis of the sequence of the first 29 amino acids. According to the obtained model, VAMP2 NTD is packed into a globular structure, as it is enriched with proline residues (40) (Fig. 2B).

To evaluate the role of the VAMP2 NTD in the interaction with Kv2.1 experimentally, we generated VAMP2 with a deleted NTD domain (ΔVAMP2). In PC12 cells, deletion of NTD affects neither VAMP2 expression nor its targeting to the vesicle membrane and endosomes (41), as both targeting sequences of VAMP2 (aa 31–38 and 41–50) remain intact. Here, we first showed that ΔVAMP2 is targeted to the plasma membrane of oocytes; however, 15-fold larger ΔVAMP2 mRNA concentrations were needed to obtain expression similar to that of WT VAMP2 (supplemental Fig. 2B). Next, we compared the association of ΔVAMP2 with Kv2.1 and its effect on the channel inactivation versus WT VAMP2. Co-immunoprecipitation analysis revealed that the association of ΔVAMP2 with Kv2.1 is significantly weaker than that of WT VAMP2 (Fig. 2C, right panel). Functional analysis of Kv2.1 steady-state inactivation using two-electrode voltage clamp revealed that whereas VAMP2 shifts the half-inactivation voltage ($V_{1/2}$) to hyperpolarized potentials as shown previously (8), ΔVAMP2 does not (Fig. 2C, left panel). Taken together, the biochemical and electrophysiological analyses conform to the above structural considerations that point to VAMP2 NTD as the domain interacting with Kv2.1.

**Docking Simulation of the Interaction between the N Terminus of Kv2.1 and VAMP2 NTD**—Next, we tested the proposed interaction between the Kv2.1 N terminus and VAMP2 by simulating docking of the structural model of VAMP2 NTD (Fig. 2B) onto that of the whole Kv2.1 N terminus (including both the N-tail and T1 domains; Figs. 1C and 2D). In this model, as well as in most of the obtained docking models, the docking loop of Kv2.1 T1 is the major contact surface exposed to VAMP2 NTD. Whereas electrostatic interaction was revealed to be insignificant (supplemental Fig. 3), sterically VAMP2 NTD fitted ideally into the T1 groove formed by the docking loop (aa 61–71), with the marginal amino...
FIGURE 1. Predicted structure and possible relative orientation of Kv2.1 T1 and N-tail domains, yielding the best whole N-terminal structure. A, structural alignment of Kv2.1 T1 with Kv3.1 T1 domains. Kv3.1 T1-based predicted structure was aligned in SWISS-PDB viewer 3.7 SP5. The Magic Fit algorithm was applied to the backbone structures of Kv2.1 and Kv3.1 T1 domains, and the obtained alignment was further improved by iterative Magic Fit. Backbone atom-based alignment r.m.s.d. was equal to 0.14 Å. Yellow and red structures correspond to Kv3.1 T1 and Kv2.1 T1 domains, respectively. The Kv2.1 loop (Docking loop), which deviates from the Kv3.1 structure, is depicted with amino acid side chain assignment. B, the most favorable structure of Kv2.1 N-tail (aa 1–30) of five predicted based on Ramachandran plot analysis done using Sirius software: 79.16% of non-glycine and non-proline residues (19) in most favorable region, 20.83% of non-glycine and non-proline residues (4) in additional allowed region; 4 prolines and 2 glycines. C, Kv2.1 N-tail docking onto Kv2.1 T1, obtained as described under “Experimental Procedures.” In addition, close proximity (within 5Å) of the last amino acid of the N-tail (Ser-30) and the first amino acid of T1 (Arg-31) was an important requisite condition to ensure continuity in the Kv2.1 N terminus. All approved docking versions were tested against the quaternary structure of Kv3.1 to exclude steric clashes against the other channel subunits (left panel), yielding the best monomeric docking model (right panel). Red and green structures depict the Kv2.1 T1 and N-tail domains, respectively. Highlighted amino acids and stick side chains correspond to the docking interface. D, electrostatic surface of the N terminus of Kv2.1 (constructed as in C). Red and blue colors depict negatively and positively charged surfaces, respectively. Energetic analysis revealed that the protein-protein interaction is mostly based on geometric fit (E_shape = −228.1 kJ/mol) and that the relative contribution of electrostatic forces is small (E_electrostatic = −90.8 kJ/mol), mostly due to the interface between the N-tail Lys-7 and the T1 Asp-62.
VAMP2 Modulates Kv2.1 Gating via N/C Domain Interactions

VAMP2 and presynaptic plasma membrane proteins SNAP-25 and syntaxin 1a. The distance between end amino acids of the motif is 106.07 Å. In 12 of 21 favorable docking solutions, VAMP2 NTD docked in the case of N-tail deletion, NTD tends to interact with the N-tail docking surface on Kv2.1 T1. In contrast to the model presented in Fig. 2, the docking loop (Fig. 2F) and similar to that in E, the NTD interaction with Kv2.1 T1 represents VAMP interaction with the N-tail-deleted channel. The superimposition model shows that in the absence of N-tail deletion, NTD tends to interact with the N-tail docking surface on Kv2.1 T1. Blue, green, and red depict the NTD, N-tail, and T1 chains, respectively.

acids (aa 75–85) forming a β-sheet and the C-terminal end of an α-helix (aa 118–124) facing the intracellular milieu (Fig. 2D, supplemental Table 1). Next, we checked the docking pattern of VAMP2 NTD onto T1 (in the absence of N-tail). In 12 of 21 favorable docking solutions, VAMP2 NTD filled the N-tail docking pocket on T1 which includes the docking loop (Fig. 2E), suggesting that in the absence of Kv2.1 N-tail, VAMP2 NTD may occupy the region normally filled by it (the superposition of N-tail and NTD is shown in Fig. 2F). In the other nine solutions, VAMP2 NTD docked in the Kv2.1 T1 groove, occupying the same place as in the case of the full-length N terminus (not shown). Hence, in all of the obtained docking models, the T1 docking loop was involved in the interaction with VAMP2 NTD.
Role of Kv2.1 N-tail in Kv2.1 Inactivation and Its Regulation by VAMP2—In a previous study we showed that T1 is important for the effect of VAMP2 on Kv2.1 inactivation (8). In view of the findings that in some Kv4.x channels, their N-tails participate in channel gating (12, 33, 34), and to match the above structural model of VAMP2 NTD docking onto the whole N terminus including the N-tail, we tested the involvement of N-tail in Kv2.1 inactivation and its regulation by VAMP2. Two-electrode voltage clamp analysis in oocytes showed that deletion of aa 2–28 (∆NKv2.1) causes a rightward shift of \( V_{1/2} \), suggesting that the Kv2.1 N-tail is important for Kv2.1 inactivation (Fig. 3A). Notably, upon co-expression with VAMP2, \( V_{1/2} \) shifted back to that of the WT channel, conforming to the structural prediction (see above) that in the absence of Kv2.1 N-tail, VAMP2 NTD may occupy the N-tail docking pocket on T1, thereby restoring the function of N-tail. Concomitant co-immunoprecipitation analysis in oocytes showed that binding of VAMP2 to ∆NKv2.1 is partially impaired relative to that with the WT channel (Fig. 3B). These biochemical and functional results underscore the role of the N-tail in Kv2.1 inactivation and complement the above structural analysis, implicating Kv2.1 N-tail in the interaction with VAMP2.

Kv2.1 N-Terminus Fails to Confer to Kv1.5 Ability to Interact with VAMP2—The above analyses focused on Kv2.1 N terminus. One of the key experiments indicating that the N terminus mediates the Kv2.1-VAMP2 interaction was the demonstration that upon replacement of the whole N terminus of Kv2.1 with that of the VAMP2-resistant channel, Kv1.5, the channel does not interact either physically or functionally with VAMP2 (8). Here, we sought to determine whether the Kv2.1 N terminus is an autonomous module that confers to Kv1.5 the ability to interact with VAMP2 (8). Because the Kv2.1 N terminus has been shown to confer to Kv1.5 a Kv2.1-like U-shaped inactivation (42), we expected that the chimeric channel Kv2.1N/Kv1.5, in which the N terminus of Kv1.5 is substituted with that of Kv2.1, would interact with VAMP2. Surprisingly, Kv2.1N/Kv1.5 did not interact with VAMP2 in oocytes; the N terminus of Kv2.1 failed to provide either VAMP2 modulation of inactivation (Fig. 3C) or binding (Fig. 3D) to Kv1.5, suggesting that there must be another Kv2.1 determinant that is important for the interaction with VAMP2.

VAMP2 Binds the Proximal Part of Kv2.1 C Terminus, C1a, Concurrently with Its Binding to the N Terminus—The proximal part of the C terminus of Kv2.1 (C1a; see scheme in Fig. 4A) is a site recognized for its protein-protein interactions, specifically with syntaxin 1A and the t-SNARE complex (syntaxin 1A/SNAP-25) (17, 19, 43). In our previous experiments, we noticed that although not affecting VAMP2 modulation of inactivation, deletion of almost the whole C terminus of Kv2.1 (including most of C1a) reduces VAMP2 binding to the channel (8). To substantiate the suggested notion that VAMP2 can interact physically with the Kv2.1 C terminus, a pulldown assay of recombinant His\(_{6}\)-tagged VAMP2, using an immobilized GST fusion protein corresponding to different fragments of the C terminus (see scheme in Fig. 4A), C1 (aa 415–636; GST-C1), C2 (aa 637–857; GST-C2), C1a (aa 415–527; GST-C1a), and C1b (aa 526–636; GST-C1b), was performed (Fig. 4B). The GST fusion protein corresponding to the whole N terminus of Kv2.1 (aa 1–184; GST-N) was used as a positive control. The results demonstrated direct binding of VAMP2 to the C1a domain, albeit more weakly than to the N terminus. Importantly, it was further demonstrated that GST-N pulls down the Kv2.1 C terminus (\(^{35}\)S-labeled, synthesized in reticulocyte lysate) only in the presence VAMP2 (\(^{35}\)S-labeled), suggesting that VAMP2 can link the N and C termini by binding simultaneously to both the N terminus and the C1a domain (Fig. 4E).

Role of Kv2.1 C1a in Kv2.1 Inactivation and Its Regulation by VAMP2—Next, testing a channel mutant with deleted C1a domain (aa 425–526; ∆C1a; see scheme in Fig. 4A) in oocytes revealed a rightward shift of \( V_{1/2} \), suggesting that C1a is important for Kv2.1 inactivation (Fig. 4C). Notably, upon co-expression with VAMP2, \( V_{1/2} \) shifted toward that of the WT channel; hence VAMP2 could restore the function of C1a. Furthermore, deletion of C1a partially impaired binding of VAMP2 as compared with the WT channel (Fig. 4D), suggesting that C1a stabilizes the interaction of VAMP2 with the N terminus.

Docking Simulation of the Interaction of VAMP2 with Both the N and C Termini of Kv2.1—Following the proposed simultaneous interaction of VAMP2 with both the N and C terminus, we attempted to simulate triple docking with VAMP2 NTD, the whole N terminus (comprising the N-tail and T1 domain) and the C1a domain. Because the crystal structure of the Kv2.1 C terminus has not been resolved, and we failed to find any satisfactory homologous model for C1a, we performed an \textit{ab initio} based protein prediction for C1a (aa 415–514; Fig. 5A). In otherwise unstructured protein, there were three helical structures in the modeled region: one long helix (aa 415–438), which is the extramembrane part of S6, and two small helices (aa 455–460 and 485–488; Fig. 5A). The best
chosen model of C1a was further docked onto the Kv2.1 N-terminus-VAMP2 NTD complex (shown in Figs. 1C and 5A). Interactive surface analysis revealed that C1a interfaces with T1 and VAMP2 NTD, the latter interacting with T1 with total surface of about 122 Å² (Fig. 5B). The contribution of electrostatic interaction was limited to a contact between the slightly negatively charged surface of VAMP2 NTD and the positively charged surface of C1a (data not shown). A comparison of the contact areas of VAMP NTD with C1a and with T1 showed that the latter contact is larger (168 versus 123 Å²), in accordance with the biochemical finding showing stronger interaction of VAMP2 with the N terminus than with C1a (Fig. 4B).

C1a has been shown to harbor several residues that are phosphorylated in neurons (15, 44). Interestingly, the above model predicts that their phosphorylation may be relevant to the interaction of VAMP2 with Kv2.1. First, the interaction of VAMP2 with C1a at Ser-484 (Fig. 5C) may be phosphorylation-related, as Ser-484, identified as a Kv2.1 phosphorylation site, is located close to the most negatively charged surface of VAMP2 NTD, and its phosphorylation may prevent the C1a-VAMP2 NTD interaction (data not shown). Conversely,

FIGURE 4. Role of the Kv2.1 C1a domain in the interaction of the channel with VAMP2. A, schematic representation of wild-type Kv2.1 and Kv2.1ΔC1a C-terminal deletion mutant. B, VAMP2 can bind both the N terminus and the C1a domain in vitro. Purified His₆-tagged VAMP2 was incubated with immobilized GST-fused Kv2.1 N- and C-terminal proteins. GST itself was used as a control. The glutathione-eluted proteins were separated by SDS-PAGE (visualized by Ponceau staining (lower panel)) and subjected to Western blot analysis using VAMP2 antibody (IB VAMP (upper panel)). Numbers on the right refer to the mobility of prestained molecular mass standards. C, upper panel, a representative experiment with 5 oocytes/group, demonstrating the effect of VAMP2 on the inactivation of Kv2.1ΔC1a. Currents were derived as described in the legend for Fig. 2C with 30-s depolarization prepulses. Lower panel, a bar diagram comparing mean effects of VAMP2 on V₁/₂ values of Kv2.1ΔC1a and Kv2.1, obtained from two independent experiments (10 oocytes in each group). D, digitized PhosphorImager scans of co-immunoprecipitation analysis by SDS-PAGE of [³⁵S]Met/Cys-labeled Kv2.1 and VAMP2 from homogenates of oocytes of a single frog that were co-immunoprecipitated by Kv2.1 (IP Kv2.1) antibody. Oocytes were co-injected with VAMP2 (+ VAMP2) or injected with VAMP2 only (+ VAMP2). Bars below scan depict normalized ratios (quantified by ImageQuant and corresponding to the lanes above the bars) of VAMP2 co-precipitated with the channel-to-channel. E, in the presence of in vitro synthesized ³⁵S-labeled VAMP2 (+), but not in its absence (−), GST-N, but not GST itself, can pull down the in vitro synthesized ³⁵S-labeled C terminus (C). Upper panel, digitized PhosphorImager scan. Lower panel, Coomassie Brilliant (CB) staining of the gel showing the GST-fused proteins.
VAMP2 NTD may create steric hindrance for kinase access to Ser-484 (Fig. 5C), preventing its phosphorylation. Furthermore, in the presence of VAMP2 NTD, an 8-aa motif of C1a containing threonine and multiple serines (aa 496–503; SETSSSKS, of which the serines at the extremities, 496 and 503, have been identified as Kv2.1 phosphorylation sites) faces the negatively charged surface of T1. Therefore, we can speculate that in the presence of VAMP2, the phosphorylation of this part of C1a is not favored because in this conformation access to a kinase might be impossible. In general, any phosphorylation in this region is expected to significantly impair the C1a-T1 interaction.

**FIGURE 5.** Possible scenario of interaction of C1a domain with Kv N terminus-VAMP2 NTD complex. A, C1a docking onto Kv2.1 N terminus-NTD complex. C1a docking was based on C1a *ab initio* prediction based on the Protinfo server. Sequence strip of aa 411–510 was predicted. Structural alignment of the obtained models revealed acceptable similarity, with an average r.m.s.d. = 5.31 Å; unstructured regions accounted for the most deviation. Five proposed models were tested for Ramachandran plot, and the best structure was chosen: there were 82.35% amino acids in the most favored position and 17.64% in the additional allowed region. Neither amino acid was located in the generously allowed and disallowed regions. To decrease the degrees of freedom of the interactions and exclude forbidden docking with the T1 tetramer-forming surface, we used a 60° rotation range for both C1a and the complex instead of the generally used 360° rotation range. 100 of the most favorable docking models were analyzed, applying restrictions of tetrameric channel structure and closeness of Ile-411 to the C-terminal amino acid of the S6 transmembrane helix on the aligned quaternary structure of Kv1.2 (supplemental Fig. 5); six best solutions were found, and the lowest energy model was chosen (E\text{total} = −321.7 kJ/mol, E\text{shape} = −359.4 kJ/mol, E\text{electrostatic} = 37.7 kJ/mol). Structure coloring: violet, C1a; red, T1 domain; blue, NTD; green, N-tail. B, alignment of the docking model (A) onto x-ray-based reconstituted model of the chimerical tetrameric Kv channel. C, amino acid interfaces of contact surfaces between C1a and the T1-NTD complex. In the selected model, seven amino acids of C1a (underlined in blue in Fig. 9A and marked in red in supplemental Table 2) interact with six amino acids of VAMP2 NTD (the residues contributing most to the interaction are marked by asterisks).
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VAMP2 Reduces the FRET Signal between the N and C Termi

VAMP2 Modulates the FRET Signal between the N and C Termi

FIGURE 6. Binding of VAMP2 to eCFP-Kv2.1-eYFP (C-Kv-Y) channel reduces FRET between the N and C Termini. A, schematic description of C-Kv-Y. B, a and b, confocal images of oocytes expressing C-Kv-Y taken with 514 nm (a) and 405 nm (b) laser excitation. Note the lighter blue shade in comparison with image c, c, confocal image of an oocyte expressing eCFP alone, excited with 405 nm. d and e, confocal images of an oocyte expressing eYFP alone, excited with 514 nm (d) and 405 nm (e). Note the very weak emission of eYFP caused by 405 nm excitation. C, normalized spectra of C-Kv-Y excited with 405 and 514 nm. Briefly, the standard eCFP emission spectrum (green line) was subtracted from the emission spectrum of a FRET oocyte expressing C-Kv-Y taken with 405 nm excitation laser (red line). The difference in the intensities in the eYFP region (after dichroic mirror) was then normalized to the total eYFP emission spectrum (taken with 514 nm excitation laser (yellow line)) and is denoted as ratio A. The ratio A component caused by the direct excitation of eYFP (denoted as ratio A_0) was measured directly from control oocytes expressing eYFP only (see image b, e). The difference between ratio A and ratio A_0 (ratio A_0 - ratio A) directly proportional to FRET efficiency, was determined as described (51). D and E, VAMP2 reduces ratio A concomitantly, causing a hyperpolarizing shift in the inactivation curve. In one representative experiment of seven, oocytes injected with C-Kv-Y mRNA alone or together with 1.5 ng/oocyte VAMP2 mRNA (17 oocytes/group) were tested (with 5-s prepulses) for the effect of VAMP2 on the voltage dependence of C-Kv-Y inactivation (D) prior to analysis of their FRET signals (E). VAMP2 reduced the ratio A of C-Kv-Y from 0.34 ± 0.02 to 0.28 ± 0.01 (E). F, summary of normalized values of [ratio A - ratio A_0] from seven experiments (left and middle bars; 115 and 150 oocytes, respectively). In three of seven experiments oocytes were also co-injected with 20 ng/oocyte ΔNVAMP2 as a negative control (right bar; 33 oocytes). *p < 0.05. a.u., arbitrary units.

Previously that Kv2.1 exhibits stronger intramolecular FRET than FRET between adjacent subunits (11); therefore we envisaged that this labeling method would yield greater changes in FRET. To this end, we expressed the channel in oocytes and measured the FRET signal in the presence or absence of co-expressed VAMP2. ΔNVAMP2, which does not interact with the channel, was used as a negative control. We monitored changes in FRET as reporters of rearrangements in the relative orientation/distance between the N and C termini within the same subunit.

The spectral properties of eCFP and eYFP are well suited for measuring molecular rearrangements by this technique (45, 46). Because of the overlap in eCFP and eYFP spectra, the measured eYFP emission caused by FRET is always contaminated by both direct excitation of eYFP and by eCFP emission in the eYFP range (32, 47). To overcome this problem, a sensitive spectral method for FRET efficiency quantification was used, as described in the legend for Fig. 6, B and C (see “Experimental Procedures”). Prior to the FRET analysis, oocytes were subjected to electrophysiological analysis, where C-Kv-Y currents were recorded and the inactivation effect of VAMP2 was assessed (a representative experiment is shown in Fig. 6, D and E). In all experiments, the FRET signal was confirmed to be specific and independent of eCFP or eYFP fluorescence intensity (48); see supplemental Fig. 4A. Moreover, the co-expression of VAMP2 had no effect on channel expression level, and thus the change in FRET between groups was found to be specific and independent of channel plasma membrane expression level (supplemental Fig. 4B).

When C-Kv-Y was expressed in oocytes, we found a strong FRET signal, reflecting the very close proximity between the termini (see Fig. 6E for a representative experiment; the FRET efficiencies, expressed as the mean value of [ratio A - ratio A_0], ranged between 0.18 and 0.27 in seven experiments). When C-Kv-Y was co-expressed with VAMP2, we observed a significant decrease in the FRET signal (~10%; Fig. 6F), reflecting the distancing or rearrangement of the relative orientation of the termini. In contrast, the presence of ΔNVAMP2 (injected at a 15-fold greater mRNA concentration than the WT VAMP2, as discussed above) neither interacted with Kv2.1 (Fig. 3A and B).
nor caused any significant change in the FRET signal (three independent experiments) (Fig. 6F).

Molecular Dynamics and Docking Simulations: Unfolded C1a Domain Tends to Interact with the Docking Loop of T1 Domain and with the Kv2.1 N-tail—The N and C termini of Kv2.1 have been shown to interact with each other (4). Specifically, deletion of a particular segment within either C1a (aa 448–481) or the N terminus (aa 59–75) has been shown to disrupt the N/C interaction (6), so the C1a domain was proposed to be able to interact with the T1 domain’s docking loop (shown schematically in Fig. 7A). However, because of space restrictions (C1a is attached directly to the S6 transmembrane helix at the top of T1), this interaction is probably only possible upon unfolding of the C1a domain in the absence of VAMP2 or due to specific phosphorylation of C1a. To model the interaction of the unfolded C1a domain with the docking loop of T1 and the N-tail, we performed MD simulation on the predicted C1a domain in order to generate unfolded structures, which we could then attempt to dock to the T1 domain. We performed four medium resolution unfolding MD simulations (See “Experimental Procedures” for parameters). During the simulation, we observed a steep increase in the gyration radius of the protein and in the r.m.s.d. from the native structure of C1a over simulation time (Fig. 7B), indicating rapid protein unfolding. As the simulation progressed, the unfolded domain showed a steep increase in its polar surface area, with significant exposure of positively charged surfaces (supplemental Fig. 5). Considering the concentration of negative partial charges on the surface of T1 exposed to the intracellular milieu, especially in the docking loop, interaction between the unfolded C1a and the Kv2.1 N terminus appears to be highly energetically favorable. This potential interaction was tested by attempting to dock the unfolded C1a domain to the T1-N-tail structure (Fig. 7D).

As we did not have information about the precise conformation of natively unfolded C1a, we used, as a proof of concept, a partially unfolded structure of C1a produced after 25 ps of unfolding MD simulation that showed a slightly greater than 2-fold increase in gyration radius compared with the folded structure. The chosen docking demonstrated interaction of the C1a unfolded structure with T1, especially around the docking loop, and with the N-tail (Fig. 7, A and C).

Finally, to elucidate the potential mobility of the T1 structure and the possible effects of protein-protein interactions on its conformation, we performed five MD simulations of the T1 domain. A T1 domain derived from the x-ray structure was subjected to 50 ps of MD simulation (see “Experimental Procedures”). Analysis of the trajectory file revealed significant over-
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FIGURE 8. Molecular dynamics simulation of conformational changes within the structure of T1 domain. A, representative conformational changes at the indicated time steps of MD simulation. A gray circle is drawn around the docking loop, which is enlarged on the left panel. Each color corresponds to a time point color-coded above the figure. B and C, changes in r.m.s.d. (B) and gyration radius (C) during the MD simulation production stage are demonstrated for five individual initial velocities (independent simulation runs).

all stability of the T1 structure (Fig. 8A, right panel), with minor oscillations in the gyration radius over the course of the simulation (Fig. 8, B and C). However, we did observe some r.m.s.d. from the native structure (Fig. 8B), which could indicate significant atom rearrangements within a limited space. A closer look at the structure using multiple structural alignments revealed slightly increased mobility in the regions of aa 31–54 and 130–141 (the latter is the helical region bridging T1 with T1-S1 linker), whereas the docking loop exhibited significant mobility and could be considered the major contributor to the r.m.s.d. observed during the simulation (Fig. 8A, left panel).

DISCUSSION

Kv2.1 N Terminus Plays a Critical but Not Solo Role in the Interaction of Kv2.1 with VAMP2—In our previous work, we demonstrated that VAMP2 can enhance inactivation of the Kv2.1 channel via direct interaction with its N-terminal T1 domain (8). One of the key experiments in that work demonstrated that upon replacement of the whole N terminus of Kv2.1 with that of Kv1.5 (the latter channel does not interact either physically or functionally with VAMP2), Kv2.1 loses its physical and functional interaction with VAMP2 (8). Following the establishment of this crucial role for the Kv2.1 N terminus, here we found that the N terminus of Kv2.1 by itself is unable to confer to Kv1.5 the ability to interact functionally or physically with VAMP2 (Fig. 3, C and D). This led us to seek another part of the channel that plays a role in the VAMP2 interaction. This part was identified as the proximal quarter of the C terminus, the C1a domain. Using a computational biology approach dealing with protein structure and protein-protein interaction modeling of the VAMP2- and Kv2.1-interacting domains, combined with electrophysiological and biochemical studies of VAMP2 and Kv2.1 deletion mutants, we suggested that although the N terminus of Kv2.1 is the main target of the VAMP2 interaction, it has to cooperate with the C terminus, most probably with C1a, to realize this interaction.

Involvement of Kv2.1 N/C Interaction in the Effect of VAMP2 on Channel Gating—There is a body of evidence for N/C interactions as a mechanism for the regulation of Kv channel gating (see the Introduction). Regarding Kv2.1, it has been shown that FRET between its N- and C-terminal tags decreases upon channel activation, suggesting a voltage-dependent relative rearrangement between the termini, which mediates channel gating (11). The Kv2.1 C terminus is relatively long, and in the tetrameric channel the C termini wrap the “hanging gondola” of the T1 domains from the outside. Thus, the Kv2.1 C terminus may contain more than one interaction site with the N terminus. Though the precise location of this site(s) has not been identified conclusively, it was proposed that one site could reside at the very distal part of the C terminus. This site was suggested to interact with the lateral part of the T1 domain, at the stretch which overlaps the docking loop, and to be involved in determining Kv2.1 activation gating (4). Recently, however, it has been suggested that a Kv2.1 N/C interaction, governed by a 34-aa motif in the C1a domain and a 17-aa motif that overlaps the T1 docking loop, regulates trafficking, gating, and phosphorylation-dependent modulation of the channel (6).

Our results substantiate a Kv2.1 N/C interaction, governed by C1a and the T1 docking loop (Fig. 7C), which plays a role in the channel gating (discussed below). Furthermore, the binding of VAMP2 to both the T1 docking loop and C1a domain, which can occur simultaneously (Figs. 4E and 5), suggests changes in this N/C interaction in the presence of VAMP2. Taken together with the decreased FRET between Kv2.1 N- and C-terminal tags, which occurs in the presence of VAMP2 (Fig. 6F) in correlation with the VAMP2 effects on the inactivation of the channel (Fig. 6D), we have proposed a mechanism of regulation of Kv2.1 gating by VAMP2, and probably other channel-interacting proteins, which involves changes in the C1a/T1 docking loop interaction (discussed below).

Mapping of Interaction Sites on Both VAMP2 and Kv2.1 Molecules Provides Important Insight into Understanding the Mechanism Underlying Channel Gating and Its Modulation by
VAMP2 Interaction—Understanding the mechanism underlying the effect of VAMP2 on Kv2.1 channel inactivation required precise mapping of the interaction sites of both molecules. First, we showed that the VAMP2 interaction site with the channel is its NTD, as deletion of VAMP2 NTD abolished the effect of VAMP2 on Kv2.1 channel inactivation and impaired the physical association of the two proteins (Fig. 2C). Interaction of the VAMP2 distal SNARE motif with the channel was proven to be sterically impossible by theoretical estimation of their dimensions and geometry.

Second, according to our results, the Kv2.1 N-terminal interaction site is the groove formed by the T1 docking loop (aa 61–71; Fig. 2D). In a previous study, involvement of this loop in the VAMP2 interaction was suggested by showing that a deletion next to it (aa 74–77) attenuates both the VAMP2 association with and effect on inactivation (8). In the present work, we showed that the N-tail (aa 1–30) is also involved in the interaction. Whereas deletion of the N-tail caused a rightward shift of $V_{1/2}$ (Fig. 3A), co-expression of VAMP2 inverted it completely. Moreover, the physical association of VAMP2 with the channel was attenuated by this deletion (Fig. 3B). These data fit the modeling suggesting that upon deletion of Kv2.1 N-tail, VAMP2 NTD can interact with the site on T1 that is normally occupied by the N-tail (Fig. 2, D versus E; summarized in Fig. 2F). Taken together, we believe that the N-tail is important for establishing the correct conformation of the N-terminal binding site for VAMP2. In addition, we also suggest a role for the N-tail in Kv2.1 inactivation gating (see further on).

Third, we found that the Kv2.1 C1a site can also participate in the interaction with VAMP2. C1a bound VAMP2 in vitro (Fig. 4B), and its deletion attenuated the association of VAMP2 with Kv2.1 (Fig. 4D). Furthermore, the rightward shift of $V_{1/2}$ caused by the deletion was shifted back toward the $V_{1/2}$ of the WT channel upon co-expression with VAMP2, similar to the deletion of the N-tail (compare Fig. 3A with Fig. 4C). Finally, the docking of C1a onto the Kv2.1 N terminus in the absence of VAMP2 NTD also indicates an interaction of C1a with the VAMP2 binding site on T1 (Fig. 7A).

In summary, the docking simulations showed that not only VAMP2 NTD, but also both the N-tail and C1a domain, can...

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**FIGURE 9.** Proposed mechanism of interaction between VAMP2 and Kv2.1 channel includes the interaction with both of the cytoplasmic termini of the channel. A, the amino acid sequence of C1a domain used for modeling and subsequent docking experiments is shown. Two predicted $\alpha$-helices are indicated as blue boxes. The residues predicted to be important for the interaction with T1 and VAMP2 NTD are underlined. B, a schematic illustration of the mechanism of interaction between VAMP2 and the N and C termini of Kv2.1. The C terminus wraps T1, occluding the access to the VAMP2-interacting site (the groove at the outer lower portion of the T1 domain) on the N terminus (left side). Upon interaction with VAMP2, the C terminus moves away and unveils the VAMP2-interacting site, allowing the N terminus of VAMP2 to bind T1 (right side). Only one VAMP2 molecule per two Kv2.1 subunits is shown, though the exact stoichiometry of the interaction was not determined. VAMP2 NTD and Kv2.1 T1 are shown as electrostatic meshes (negatively charged surfaces are presented as a variation of red and positively charged surfaces as a variation of blue). The remaining domains of both molecules are depicted schematically.
interact with the T1 docking loop; all of these interactions had similar effects on channel inactivation. Taken together, the notion emerges that the interaction with VAMP2 presumably restricts the movement of the docking loop, stabilizing the T1 domain structure and thereby affecting channel gating. Several lines of evidence support this notion. First, it was shown previously by T1-T1 cross-linking experiments that T1-T1 interface immobilization prevents the channel from being completely open upon membrane depolarization and consequently inhibits Kv2.1 channel activation (49). Later, a similar T1-T1 interface “clamping” mechanism was employed to explain the ability of KChIP to slow inactivation and speed up recovery from inactivation of the Kv4.x channels (34). Our findings from MD simulation of the T1 domain also revealed that significant mobility around the T1 domain; the C1a domain interacts with T1, including the docking loop, blocking access to the VAMP2-NTD interface, as proposed previously (8). Although VAMP2 may also remain bound to the C terminus, this is not essential for its effect, as has been shown by C-terminal truncations (8) and here by C1a deletion (Fig. 4C). Of significance is the drawing apart of the Kv2.1 intracellular termini by VAMP2, which almost certainly underlies the unique mechanism of Kv2.1 channel inactivation. Taking into account that the N-tail or C1a affect inactivation similarly to VAMP2 (in the absence of the N-tail or C1a V_{1/2} shifts rightward, opposite to the leftward shift by VAMP2; Figs. 3A and 4C), we can put forward a general hypothesis that the inactivation of Kv2.1 is dependent on both C1a and N-tail interactions with the T1 docking loop, conferring T1-T1 interface stabilization by restricting movement of the T1 domain and affecting gating.

Our FRET experiments were performed under resting conditions, suggesting that depolarization was not needed to unveil the N-terminal site. However, this does not exclude the possibility that depolarization, which may trigger the outward movement of the Kv2.1 C terminus in order to produce relative rearrangements between the N and C termini (as proposed by Kobrinsky et al. (11)), can make the N terminus even more accessible to the interaction with VAMP2, so that VAMP2 can easily “catch” the channel when it begins to open and clamp it in its partially activated closed state, which is the favorable condition for unique Kv2.1 U-type inactivation (50). Future in vivo dynamic FRET experiments may help assess this possibility.

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**VAMP2 Modulates Kv2.1 Gating via N/C Domain Interactions**

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