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Phosphorylation Induces Conformational Rigidity at the C-terminal Domain of AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) Receptors

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Abstract

The intracellular C-terminal domain (CTD) of AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptor undergoes phosphorylation at specific locations during long-term potentiation (LTP). This modification enhances conductance through the AMPA receptor ion channel and thus potentially plays a crucial role in modulating receptor trafficking and signaling. However, because the CTD structure is largely unresolved, it is difficult to establish if phosphorylation induces conformational changes that might play a role in enhancing channel conductance. Herein, we utilize single molecule Förster Resonance Energy Transfer (smFRET) spectroscopy to probe the conformational changes of a section of the AMPA receptor CTD, under the conditions of point-mutated phosphomimicry. Multiple analysis algorithms fail to identify stable conformational states within the smFRET distributions, consistent with a lack of well-defined secondary structure. Instead, our results show that phosphomimicry induces conformational rigidity to the CTD and such rigidity is electrostatically tunable.

TOC Graphic:

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Supporting Information

Predicted 3D structures of the peptides, CV analysis on smFRET results of the PM peptide under varying salt concentrations, reversibility of charge-shielding effects on the PM peptide, schematic explaining possible mechanism of ionic strength affecting conformational flexibility of the PM peptide, representative smFRET trajectories and statistics of smFRET measurements.

Introduction

Ionotropic glutamate receptor proteins form ligand-gated ion channels across neuronal membranes in the mammalian central nervous system, thereby modulating fundamental neuronal functioning as well as cognitive development by mediating excitatory neurotransmission.¹⁻⁶ Despite sharing a common tetrameric structure,⁷⁻⁹ the ionotropic glutamate receptor family of proteins exhibit diverse functional properties.^{1-2, 4} AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors are known to undergo a number of chemical modifications at the intracellular CTD that regulate receptor trafficking and gating, thereby affecting activation of the ion-channel.¹⁰⁻¹⁴ Hence, unraveling the dynamic structure-function interplay during modifications at the CTD of AMPA receptors is pivotal to the field of targeted neuro-therapy, especially for treating several neurological and psychiatric disorders.^{4-5, 15-25}

Crystallography provides structural information about the extracellular domains of AMPA receptors,⁸ but the structure of the intracellular CTD still remains unresolved, perhaps due to its flexible nature.^{13, 26} Electrophysiological measurements provide information on functional responses of the receptor channels in the activated state by measuring the ion channel conductance.^{13, 27-29} Still, detailed structural information about the CTD relating to the activated-deactivated states of AMPA receptors could be crucial, given the possibility of conformational heterogeneity and dynamics of proteins under physiological conditions.³⁰⁻³³ It is likely that ensemble measurements are not ideal to unravel inherent heterogeneity of CTD conformations given they yield statistically and temporally averaged information.

Functional studies show that Ca^{2+} mediated phosphorylation of the intracellular CTD (at Ser818, Ser831, and Thr840) within the AMPA GluA1 subunit plays an important role during long-term potentiation (LTP) of the synapses^{11, 16-18, 34-35} and enhances channel conductance.^{10, 27-29, 36-37} Phosphorylation at Ser818 and Thr840 has been shown to influence synaptic plasticity.^{5, 10, 27-29, 38-39} Interestingly, the synaptic activity of AMPA receptors are also known to be influenced by phosphorylation at transmembrane AMPA receptor regulatory proteins (TARPs), followed by their interaction with the lipid bilayer. Thus intracellular phosphorylation plays a significant role in controlling AMPA receptor activity in multiple possible ways.⁴⁰⁻⁴¹ However, studying effects of phosphorylation *in-situ* poses challenges in terms of controlling multiple-site phosphorylation and kinase interference.²⁸ Point mutation by glutamic acid eliminates these challenges and has been shown to mimic phosphorylation without altering the functional responses.^{28, 42}

Here, utilizing smFRET spectroscopy, we studied the conformational behavior of a 34 amino acid long section of the membrane proximal CTD, containing three known phosphorylation sites, under the conditions of phosphomimicry with glutamic acid. smFRET is a well-established technique for exploring conformational dynamics of biomolecules.⁴³⁻⁴⁸ smFRET is finding increased applicability in elucidating structural dynamics of biomolecules by incorporating multiple spectroscopic parameters in the measurement,⁴⁹⁻⁵⁰ by combining smFRET with simultaneous electrophysiological or force-spectroscopy based measurements,⁵¹⁻⁵² or by applying state determination algorithms to resolve complex mechanisms.^{33, 53-54} In this work, smFRET spectroscopy was used to probe conformational changes in point-mutated phosphomimetic CTD compared to its native form, where both the native and phosphomimetic (PM) peptides were labeled with a pair of FRET dyes. We also investigated the effect of ionic strength on the PM peptide to determine the role of electrostatics in the structural changes to the peptide. Common state analysis algorithms, which identify as many as seven well-defined conformations in other glutamate receptor domains and even the full receptors,^{44-45, 55} fail to converge on reproducible conformational state assignments for the AMPA receptor CTD. Instead, the smFRET results, coupled with circular dichroism spectroscopy, suggest that introduction of phosphomimicry causes the native C-terminal peptide to become rigid and that the conformational rigidity is electrostatically tunable.

Methods

Labeling and purification

Both peptides were custom synthesized by Peptide 2.0 Inc. and ~95% purity was ensured with HPLC purification. The sequence for the native unphosphorylated peptide is EFCYKSRSESKRMKGFSLIPQQSINEAIRTSTLC. The underlined residue indicates Cys17Ser point mutation from the native sequence, which is a common mutation strategy^{33, 47} to block unwanted Cys labeling by fluorophores. One Cys residue (Cys34) was also introduced at the end of the sequence to bookend another label at the C-terminal end of the sequence, thereby ensuring specific labeling at both ends of the peptide construct. Similar mutation was introduced in the PM peptide construct, along with the mutations at Ser10Glu, Ser23Glu, and Thr32Glu for phosphomimicry, making the final sequence as EFCYKSRSEEKRMKGFSLIPQQEINEAIRTSELC. Both peptides also had a biotin tag at the N-terminus to ensure immobilization through biotin-streptavidin interaction, for single molecule acquisition. The peptides, received as a lyophilized powder, were dissolved in standard 1X phosphate buffered saline (Santacruz Biotechnologies; 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 1.8 mM KH₂PO₄). Stock concentrations of 150 μ M were prepared and stored as aliquots at -20 °C. On the day of experiments, Alexa 555 maleimide ester (Life technologies) and Alexa 647 maleimide ester (Life technologies) were added to a 2–3 μ M solution of the peptide at a 1:1:4 concentration ratio of peptide: Alexa 555(donor): Alexa 647(acceptor). Cys3 and Cys34 were the only Cys residues in both peptides. Thus, site-specific labeling with the donor-acceptor fluorophores was achieved, through the cysteine-maleimide interaction. The labeled sample was left at 4–6 °C for ~1 hr. The sample was then diluted to 2–3 nM with respect to the peptide concentration and loaded into a microfluidic chamber for single molecule acquisition. The biotin tag ensured selective

binding of the peptides and the unbound fluorophores were washed out of the chamber by a copious buffer rinse.

Sample chamber preparation

Glass coverslips (no.1, 22 × 22 mm, Fischer Scientific) were sonicated sequentially for 10 minutes each in acetone, soapy water, and Millipore water and then cleaned for 90 seconds in a bath of TL1 solution composed of 4% (v/v) H₂O₂ (Fisher Scientific, Radnor, PA) and 13% (v/v) NH₄OH at 75 °C. The slides were then washed with Millipore water and dried with Nitrogen. The dried slides were cleaned for 2 minutes under O₂ plasma (PDC-32G; Harrick Plasma; medium power). Afterward, plasma cleaned slides were submerged in a Vectabond-acetone solution (2% vol/vol, Vector Laboratories, Burlingame, CA) for 5 minutes for aminosilanization of the coverslips. Subsequently, they were dipped into Millipore water to quench the aminosilanization reaction. A PEG-BiotinPEG solution containing 5 kDa mPEG succinimidyl carbonate (25% w/w, Laysan Bio, in molecular biology grade (MB) water, GE Lifesciences), 5 kDa biotin terminated PEG (2.5% w/w in MB water, NOF corp.), and sodium bicarbonate (Sigma-Aldrich) was prepared. A custom-sized silicon template (43018M, Grace BioLabs) was placed on the coverslip, and 35 – 40 μL of the PEG-BioPEG solution was added inside the well of the templates to ensure PEGylation of a desired area on the coverslip. These slides were allowed to incubate in a dark, humid environment. Afterward, excess PEG-BioPEG solution was rinsed off with Molecular Biology Grade water and dried with Nitrogen gas. HybriWell custom chambers (43018C, Grace BioLabs) were placed on top of the PEGylated side of the slides and two custom silicone ports (460003, Grace BioLabs) were pressed to the surface of the chamber to allow for the flow of solution through the chamber.

Protein Immobilization

A 0.2 mg/mL solution of streptavidin in 1X PBS buffer was pipetted in two 18 μL aliquots, into the sample chamber through the inlet port and allowed to equilibrate for 10 minutes. About 180 μL of previously prepared and labeled peptide solution (2–3 nM), was pipetted into the sample chamber and then allowed to equilibrate for 20 minutes, prior to flushing the system with an excess buffer solution, to wash off unbound peptides and free dyes. For higher salt concentration experiments, a final flow of corresponding buffer solutions was carried out and the sample chamber was left to equilibrate for 10–15 minutes prior to acquisitions.

smFRET Data Acquisition

All smFRET trajectories were acquired using a home-built confocal microscope described in previous work.^{44, 46} In this system, the excitation light is focused onto the sample via an oil immersion objective (100 X 1.3 NA, Carl Zeiss) with a power density of ~50 μW/cm² and the emitted light is collected with the same objective and split by a 640 nm high-pass dichroic mirror, (640 DCXR, Chroma Technology) in order to collect the donor and acceptor signals with two respective avalanche photodiodes (SPCM-AQR-15, Perkin Elmer). Band-pass filters (NHPF-532.0, Kaiser Optical Systems) were placed in front of the respective photodiodes in order to exclude the excitation light, tuning the light to 570 and 670 nm respectively. Using a scanning x-y-z piezo stage (P-517.3CL, Physik Instrumente), a 10 μm

area was scanned with both 532 nm CW laser (Compass 315M-100SL, Coherent) and 637 nm laser (OBIS-FP 637 LX, Coherent) to determine the locations of proteins exhibiting FRET. Individual proteins were then selected and excited with 532 nm CW laser and photon counts were collected by the respective photodiodes. All experiments were performed under an oxygen scavenging and photo-stabilizing buffer (ROXS) composed of 1 mM methyl viologen, 1 mM ascorbic acid, 1% w/w glucose oxidase, 0.1% v/v catalase and 33% w/w D-(+)-glucose (all from Sigma-Aldrich) in 1X PBS buffer. For higher salt concentration experiments, ROXS was prepared in PBS buffer containing corresponding salt concentrations.

Data analysis

All data processing and analysis were performed in MATLAB (R2017a, MathWorks). Donor and acceptor signals for each protein were smoothed with the wavelet denoising technique⁵⁶⁻⁵⁷ and then the FRET efficiencies (E_A) were calculated from the denoised donor and acceptor fluorescence intensities (F_A and F_D respectively) with the equation (Equation 1) given below. Each trajectory was post-processed to remove trajectories exhibiting multistep photobleaching or abnormally low signal to noise, which was based on a normal distribution. Trajectories not exhibiting anti-correlation between the donor and acceptor intensities were also discarded. The donor-acceptor distances (r) were also calculated from the FRET efficiencies using Eq.2, where, R_0 ($= 51 \text{ \AA}$)^{33, 44} is the Förster radius for the donor-acceptor fluorophores.

$$E_A = \frac{F_A}{F_A + F_D} \quad (1)$$

$$E_A = \frac{R_0^6}{R_0^6 + r^6} \quad (2)$$

State identification using STaSI and vbFRET

Step Transition and State Identification (STaSI)⁵³ and Variational Bayesian FRET (vbFRET)⁵⁸ analyses were applied to the denoised smFRET trajectories for both the peptides to identify states. STaSI analyzes piecewise signals based on a mathematical function termed as minimum description length (MDL), which reaches a minimum as the error in state fitting is minimized and simultaneously, the sparsest approximation of the fitting model is attained. vbFRET is a popular state identification analysis, specifically designed for temporal data sets such as smFRET trajectories. It utilizes an approximation technique known as “variational Bayes” to fit the smFRET data to a likely model and finds the best fit by optimizing two parameters, namely, “maximum likelihood” and “maximum evidence”.

Circular Dichroism (CD) spectroscopy

CD spectra for both the peptides were acquired using a Jasco J-810 spectropolarimeter. Native and PM peptide solutions (75 μ M) were prepared in 1X PBS buffer as previously mentioned. Additionally, two separate samples of the PM peptide were prepared with higher salt concentrations (2X: [NaCl] = 274 mM, [KCl] = 5.4 mM; 5X: [NaCl] = 685 mM, [KCl] = 13.5 mM). After preparation, all the peptide solutions were allowed to equilibrate for 15 minutes at room temperature prior to data collection. Measurements were conducted at room temperature, from wavelengths of 180 to 250 nm, with a scan speed of 20 nm/min, in a 0.01 cm quartz cuvette, using 20 μ L of each solution. Data were obtained in millidegrees and was averaged over 10 accumulations with a data pitch of 0.1 nm. Millidegrees was converted to molar residue ellipticity ($[\theta]$) using the equation $[\theta] = \frac{\theta}{l \times N \times c \times 10}$ where θ is ellipticity in degrees reported by the instrument, l is the pathlength of the cuvette in cm, N is the number of residues in the protein, and c is the concentration of the protein in g/cm³. Data was graphed from 195 nm to 250 nm due to the presence of salt causing noise below 195 nm.

Results and Discussion

Point mutation with glutamic acid was utilized to mimic phosphorylation

In order to study the effects of phosphorylation, a small section was selected from the membrane proximal CTD of the AMPA receptor (Figure 1A).^{27-28, 35, 59} The selected 34-amino acid native peptide sequence was point-mutated (Ser818Glu, Ser831Glu, and Thr840Glu) to mimic phosphorylation. A pictorial depiction of the 34-amino acid native and PM peptide sequences, along with the biotin tags and labeling sites, is presented in Figure 1B. Both the native and the PM peptide constructs were labeled at Cys3 and Cys34 with a pair of FRET-compatible fluorophores in order to probe their conformational changes utilizing smFRET spectroscopy. Cys3 and Cys34 are the only Cys residues in the peptides. Thus the possibility of non-specific labeling was eliminated. It is important to note that the labeling procedure generates a mixture of donor-only labeled and acceptor-only labeled peptides, along with the desired donor-acceptor labeled peptides. Improperly labeled peptides are excluded during data acquisition and analysis, by applying filters that ensure anti-correlation between donor and acceptor fluorescence intensity, and single step photobleaching of the fluorophores.^{46, 56-57}

smFRET data reveals conformational rigidity introduced by phosphomimicry

FRET efficiency was calculated (see Methods) from single donor and acceptor fluorescence intensity trajectories (Figure 2). After denoising using wavelet decomposition,⁵⁶⁻⁵⁷ the smFRET trajectories were combined to generate histograms that depict the distribution of smFRET efficiencies. smFRET efficiency histograms for the native peptide and the PM peptide are shown in Figure 2A. It is evident that both the peptides explore a significant population around 0.80 – 1.00 FRET efficiency, indicated by the peaks at 0.96 (native; Figure 2A, top) and at 0.98 (PM; Figure 2A, bottom); and around 0.60 FRET efficiency region indicated by the peaks at 0.56 (native) and 0.58 (PM). However, the native peptide explores a large number of conformations with intermediate FRET efficiency in contrast to the PM peptide.

De novo structure prediction algorithms, PEP-FOLD⁶⁰ and Bhageerath⁶¹ were used to predict the 3D structure of both the peptides. All predicted structures, along with the estimated donor-acceptor distances for each structure, are presented in the Supporting Information. The prediction algorithms did not yield a single conformational solution, but instead multiple conformers for both peptides. However, some of the possible conformations associated with the native peptide showed less α -helical content and more irregular structure in contrast to the PM peptide, supporting the smFRET conclusions that the CTD is flexible, enabling it to explore multiple conformations. Two conformers, which correspond to higher (>0.80) and lower (<0.60) FRET efficiencies, are shown in the insets of Figure 2A (see Figure S1 in the Supporting Information for all the predicted structures).

Common state identification algorithms fail to converge on conformational assignments for both the native and PM peptides (Figure 2B). Assignment of states and quantification of transitions between the states are the most common ways to analyze single molecule data through complementary theoretical simulations⁶²⁻⁶⁴ or rigorous mathematical fitting algorithm.^{53-54, 58, 65-66} STaSI⁵³ and vbFRET⁵⁸ were applied to the smFRET trajectories for the native and the PM peptide. However, for both the peptides, STaSI-generated MDLs do not yield distinct minima for an optimum state assignment (Figure 2B) and vbFRET suggests the best fits at 23 states for the native and 12 states for the PM peptide (Figure 2B, inset). Thus, neither of the methods provided a consistent and reasonable solution for state assignment.

To quantify the variation in smFRET efficiency and thereby conformational fluctuations of the peptides, the coefficient of variation (CV) for each single molecule trajectory is calculated and their cumulative probability is plotted in Figure 2C. The CV measures the variability of a data set with respect to its mean. A steeper rise in cumulative probability at lower CV indicates lower variability, as depicted by the PM peptide. In contrast, single molecule trajectories corresponding to the native peptide exhibit a more gradual rise in cumulative probability, indicating the presence of conformational dynamics. Thus, both the smFRET distributions in Figure 2A and the variability data in Figure 2C support the conclusion that phosphomimicry induces rigidity to the flexible native peptide that likely does not have a well-defined secondary structure (Figures 2B).

Variation of salt concentration

Without quantitative structural information, it is difficult to directly measure phosphorylation induced structural changes, but it is possible to test the hypothesis that electrostatics contribute to conformational rigidity (Figure 3). As discussed earlier, Ca^{2+} dependent protein kinase phosphorylates the CTD of the AMPA receptor at three specific sites thereby enhancing channel conductance.²⁸ It has been speculated that the introduction of a phosphate group in the C- terminus alters the shape of this region, affecting the ion channel gating. Electrostatic interactions in proteins play an important role in the dynamic interplay of structure and function.⁶⁷⁻⁶⁸ Phosphorylation at Ser818, Ser831 and Thr840 of the CTD introduces negative charges to the native peptide structure, whereas here, phosphomimicry through glutamic acid point mutation introduces negatively charged residues in the native sequence. Environmental parameters such as pH and ionic strength

also affect the structural properties of proteins by the dielectric screening of the backbone charges, thereby tuning long-range interactions, which are crucial for the stability of the secondary structure. Thus, the increased negative charge on the peptide surface due to phosphorylation (and phosphomimicry) can be responsible for its increased conformational rigidity.

To understand the role of electrostatics in the conformational rigidity introduced by phosphomimicry, we measured smFRET responses from the PM peptide under varying salt concentrations. Figure 3A presents the smFRET histograms (normalized with respect to maximum occurrence) corresponding to the PM peptide at three different salt concentrations compared with that of the native peptide. Here 1X salt concentration corresponds to 137 mM NaCl and 2.7 mM KCl solution in standard PBS buffer (pH = 7.4). It is evident from Figure 3A that with increasing salt concentration, the PM peptide explores multiple intermediate smFRET efficiencies, similar to that of the native peptide. At higher salt concentrations, the charges on the peptide backbone are more effectively screened, which disrupts the long-range electrostatic interactions responsible for the stability of secondary structural motifs.

To compare the conformational flexibility gained by the PM peptide at increased ionic strength, the standard deviation (SD) of each smFRET efficiency trajectory was calculated. Figure 3B shows the distributions of SD of smFRET efficiency for the PM peptide under each of the 1X, 2X and 5X conditions and the native peptide under 1X condition. Under 2X condition, the PM peptide does not exhibit a significant difference in smFRET efficiency variation as compared to that of the PM peptide under 1X condition. However, at 5X, the smFRET distribution for the PM peptide gets broader and becomes qualitatively similar to the native peptide distribution. The increased conformational flexibility of the PM peptide at higher salt concentrations is also evident from the cumulative probability distributions of CV (Figure S2). Variability in the smFRET efficiency of the PM peptide starts increasing with increasing salt concentrations, and at 5X salt condition, it almost identical to that of the native peptide.

The smFRET efficiency distributions were divided into three sub-regions (region I: $E_A > 0.80$, region II: $0.60 < E_A < 0.80$ and region III: $E_A < 0.6$) and the respective populations explored by both the peptides were compared (See Table S1 in the Supporting Information). The PM peptide (1X) is rigid with most of the population distributed over region I and region III, which correspond to donor-acceptor distances of 20–40 Å and 48–55 Å, respectively. This is in contrast to the native peptide that is more flexible and explores intermediate conformations in region II, corresponding to donor-acceptor distances of 41–47 Å. Furthermore, the PM peptide, under 2X and 5X salt conditions, explores a significant percentage of region II smFRET efficiencies by gaining conformational flexibility, similar to the native peptide.

Thus, it is evident that the PM peptide loses its structural rigidity due to the electrostatic screening of charges. Such electrostatic screening of the peptide backbone charges, due to increased ionic strength of the medium, can significantly affect the protein structure. In order to test if the high ionic strength of the medium causes permanent conformational changes to the PM peptide, the salt concentration was periodically altered on the same sample. The

corresponding smFRET efficiency histograms are presented in Figure S3 in the Supporting Information. The increased conformational flexibility of the PM peptide, induced by higher ionic strength and manifested in a higher population of intermediate smFRET efficiencies, is a reversible effect as evident from Figure S3.

Ensemble CD spectra show significant changes in secondary structural elements due to phosphomimicry that are not modulated by increasing salt concentrations

Ensemble Circular Dichroism (CD) spectroscopy was utilized to compare secondary structural elements associated with both the peptides. Figure 3C shows CD spectra for the native peptide under 1X and the PM peptide under 1X, 2X, and 5X salt conditions. The CD spectrum for the native peptide is weak, with random-coil shape and little α -helix or β -sheet character, consistent with the notion that the CTD of the native AMPA receptor has a disordered and flexible structure.⁸ Small peptides exhibiting weak CD signals are not uncommon.⁶⁹⁻⁷⁰ The PM peptide shows stronger, yet still random-coil type CD signal in contrast to the native peptide, supporting the conclusion that phosphomimicry induces rigidity. Interestingly, increasing salt concentration show no return of the CD spectra to the native signal. The CD result is in seeming contradiction to the smFRET data shown in Figure 3A because it indicates that the phosphomimicry induced secondary structural changes are retained even at higher ionic strength.

The smFRET and CD results can be explained by a mechanism that involves an alteration in the secondary structure by phosphomimicry, followed by stabilization of the peptide structure through electrostatic interactions. Thus, the negative charges introduced through Glu mutations (or, phosphate groups) render rigidity to the PM peptide. Proteins undergoing phosphorylation and other conformational modifications have been reported to show significant changes in their secondary structures, accompanied by formation of salt-bridges between the phosphate group (or, other negatively charged groups) and nearby positively charged amino acid residues (e.g. arginine, lysine) that stabilizes their structures.⁷¹⁻⁷⁴ For the PM peptide, all the three Glu mutations have Arg residues nearby. Specifically, Glu23, positioned at the hinge random coil joining the two helices, can control the relative motion of the two helical arms by interacting with the nearby Arg12 and Arg29, thereby modulating conformational rigidity. Increased ionic strength shields these attractive electrostatic interactions, thereby making the PM peptide flexible like the native peptide. This trend is reflected in our smFRET data as previously discussed (Figure 3A and 3B). Additionally, it has also been shown in some studies⁷⁵⁻⁷⁸ that even though high ionic strength can shield the charges on the peptide backbone (introduced by the functional groups on amino acid side chains) and minimize their contribution towards the protein conformation, the effect on secondary structure (and thereby CD signal) is largely insignificant. Hence, even if the conformational flexibility is regained through electrostatic changes, the secondary structure of the PM peptide does not resemble that of the native peptide at high salt concentrations. This conclusion is also evident from the fact that the variability of smFRET efficiency can be reversibly controlled by altering salt concentration (Figure S3). These results not only show that phosphomimicry causes conformational rigidity in the native structure, but also corroborates that such rigidity gained by phosphomimicry at the native C-terminal peptide is electrostatically tunable, without significant alterations to the secondary structure. Figure S4

in the Supporting Information represents a schematic to explain the above-mentioned mechanism.

Conclusion

Our study provides the first mechanistic insight on the phosphorylation induced structural changes at the membrane proximal section of the CTD of AMPA receptors. Utilizing point mutated phosphomimicry, we have demonstrated that phosphorylation introduces rigidity to the native peptide structure, and that this conformational change is electrostatically tunable. We speculate that such rigidity can cause the ion channel to stay open longer, enhancing ion flow, which has been demonstrated in previous channel conductance studies.²⁷ Furthermore, enhanced ion flow then could lead to charge buildup in the intracellular domain, which could in turn cause the CTD to regain conformational flexibility. However, a detailed mechanistic study of phosphorylation of the full-length CTD under the membrane proximal condition is needed to provide further insight into the role of phosphorylation in controlling the activity of AMPA receptors. Additionally, our results confirm that the biological function of the CTD and other disordered peptides and proteins can be driven by a change in conformational rigidity rather than through distinct structural states. Furthermore, our single molecule method can also be implemented to study other modes of phosphorylation that affect AMPA receptor activity, whether directly or through TARP phosphorylation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Phosphorylation sites at the CTD of AMPA receptors were mutated with glutamic acid to mimic phosphorylation.

A) Structure and position of the C-terminal peptide under study with respect to the full tetrameric AMPA receptor protein are shown. The section of the protein being studied is zoomed in on. **B)** Sequences of the peptide sections under study showing labeling sites and biotin tag. [Structure reference: Homology model of GluA1 by Jenkins et. al.²⁸ based on crystal structure reported with PDB ID: 3KG2⁸]

Figure 2. Insertion of phosphorylation-mimicking glutamic acid residues reduces the flexibility of the native unphosphorylated C-terminal peptide.

A) Top: smFRET histogram (blue) of the native peptide (84 molecules) and representative predicted 3D structures corresponding to high and low FRET efficiency conformations. Bottom: smFRET histogram (orange) of the PM peptide (49 molecules) and representative predicted 3D structures corresponding to high and low FRET efficiency conformations. Insets: Representative smFRET trajectories (green: raw trajectories, black: denoised trajectories) [3D structures generated using de novo structure prediction algorithms PEP-

FOLD⁶⁰ and Bhageerath.⁶¹]. **B)** MDL plots generated from STaSI⁵³ analysis of smFRET trajectories for the native (blue) and the PM (orange) show that the MDL values do not reach distinct minima. Inset: state assignments by vbFRET⁵⁸ also show unresolvable states for both native (blue) and PM (orange). **C)** Coefficient of variation (CV) of individual smFRET trajectories are calculated and their cumulative probabilities are plotted for the native peptide (blue) and the PM peptide (orange), under 1X salt conditions. Inset: magnified section of the plot to show the difference in variability of the native and the PM peptide even at low CV.

Figure 3: Rigidity gained by PM peptide is electrostatically tunable.

A) smFRET histograms for the native and PM peptide under various salt concentrations (1X (orange) (49 molecules), 2X (green) (56 molecules) and 5X (purple) (41 molecules)) are shown in comparison to that of the native peptide (blue) (84 molecules). [NaCl] and [KCl] represents molar concentrations of sodium chloride and potassium chloride respectively. μE_A and $E_{A,max}$ denote apparent average smFRET efficiency and most probable smFRET efficiency respectively. **B)** Standard deviations of smFRET efficiency combined as a histogram; for the native peptide at 1X (blue) and PM peptide under 1X (orange), 2X

(green), and 5X (purple) salt conditions. C) CD spectra of the native peptide (blue) at standard buffer condition (1X) and the PM peptide at 1X (orange), 2X (green) and 5X (purple) salt concentrations. No significant change in the secondary structure of PM is observed under increasing ionic strength, despite the obvious increase in flexibility as shown by smFRET analysis. despite the obvious increase in flexibility as shown by smFRET analysis.