PKC SUMOylation inhibits the binding of 14–3–3\(\tau\) to GluK2

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ABSTRACT
Phosphorylation and SUMOylation of the kainate receptor (KAR) subunit GluK2 have been shown to regulate KAR surface expression, trafficking and synaptic plasticity. In addition, our previous study has shown that a phosphorylation-dependent interaction of 14–3–3\(\tau\) and GluK2a-containing receptors contributes to the slow decay kinetics of native KAR-EPSCs. However, it is unknown whether SUMOylation participates in the regulation of the interaction between 14–3–3\(\tau\) and GluK2a-containing receptors. Here we report that SUMOylation of PKC, but not GluK2, represses the binding of 14–3–3\(\tau\) to GluK2a via decreasing the phosphorylation level of GluK2a. These results suggest that PKC SUMOylation is an important regulator of the 14–3–3 and GluK2a protein complex and may contribute to regulate the decay kinetics of KAR-EPSCs.

KEYWORDS
14–3–3\(\tau\); GluK2; phosphorylation; PKC SUMOylation

Introduction
Kainate receptors (KARs) are ionotropic glutamate receptors consisting of tetrameric arrangements of 5 subunits (GluK1–5).\(^1,2\) At the presynaptic-terminal KARs can modulate neurotransmitter release, and at the postsynaptic membrane they contribute to fast excitatory synaptic transmission.\(^3\)–\(^6\) Slow decay kinetics are one of the important gating features of KARs.\(^7\)–\(^9\) Our previous study showed that protein kinase C (PKC) regulates the properties of GluK2a receptors by controlling phosphorylation-dependent binding of 14–3–3 to GluK2a, and such modulation may contribute to the slow decay kinetics of KAR-excitatory postsynaptic currents (EPSCs).\(^10\) Additionally, phosphorylation of KARs by PKC can regulate KAR-mediated synaptic transmission and long-term depression.\(^11\)–\(^13\)

SUMOylation is a form of posttranslational modification (PTM) in which the small ubiquitin-related modifier (SUMO) can be covalently attached to lysine residues on target proteins, and regulates target proteins function. Previous studies show that GluK2 can be modified by SUMOylation at lysine residue (K886).\(^14\)–\(^16\) In neurons, SUMOylation participates in regulating ion channel function, membrane protein endocytosis, neuronal differentiation, synaptic transmission and cell survival.\(^14\)–\(^20\) In particular, dynamic control of synaptic SUMOylation regulates KAR synaptic transmission and plasticity.\(^14\)–\(^16\) Previously, both phosphorylation and SUMOylation of KARs have been shown to individually regulate KAR surface expression,\(^13\),\(^14\) but it is unclear whether the binding of 14–3–3 to GluK2a is modulated by SUMOylation and consequently contributes to the slow kinetics of KAR-EPSCs.

Here, we show that SUMOylation inhibits the binding of 14–3–3 to GluK2a but does not change the level of mRNA and protein expression of GluK2a and 14–3–3\(\tau\). We also report that SUMOylation of PKC, but not GluK2, suppresses 14–3–3\(\tau\) binding to GluK2a. In addition, PKC SUMOylation reduces GluK2 phosphorylation. In view of our previous data showing that PKC can be SUMOylated and its SUMOylation inhibits its activity,\(^20\) these results suggest that PKC SUMOylation represses the binding of 14–3–3 to GluK2a by reducing the phosphorylation level of GluK2a.

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Results

SUMOylation inhibits the binding of 14–3–3ζ to homomeric GluK2a receptors

Our previous study revealed that decay kinetics of GluK2a-containing receptors are modulated by closely associated 14–3–3ζ proteins in a phosphorylation-dependent manner.15 To assess whether SUMOylation modification affects the binding of 14–3–3ζ to GluK2a, we expressed Flag-tagged 14–3–3ζ and GluK2a, either alone or together with GFP-SUMO1 in HEK293T cells. As assessed by coimmunoprecipitation and Western blot analysis, the interaction between 14–3–3ζ and GluK2a was significantly attenuated in SUMO1-cotransfected cells compared with a control group that was not cotransfected with SUMO1 (Fig. 1A and B), indicating that SUMOylation inhibited the binding of 14–3–3ζ to GluK2a. To provide further support for the inhibitory effect of SUMOylation, we examined the effect of overexpressed wild-type sentrin/SUMO-specific protease 1 (SENP1) and a catalytically inactive mutant SENP1 (SENP1m) in HEK293T cells. As shown in Figs. 1C and D, the binding of 14–3–3ζ to GluK2a was enhanced by overexpression SENP1, but not SENP1m (Fig. 1C and D). To exclude the possibility of a transcriptional mechanism involved in the inhibitory effect of SUMOylation, we examined the mRNA and protein expression of GluK2a and 14–3–3ζ in HEK293T transfected with plasmids as indicated. Western blot analysis and quantitative RT-PCR showed that overexpression of either SUMO1 or SENP1 did not change the mRNA (Fig. 2B and D) or protein levels of 14–3–3ζ and GluK2a (Fig. 2A and C), suggesting that transcriptional mechanism did not involve in regulating the interaction between 14–3–3ζ and GluK2a.

GluK2a SUMOylation does not alter the binding of GluK2a to 14–3–3ζ

Previous studies have shown that SUMOylation of GluK2 at lysine 886 regulates endocytosis of KAR and modifies synaptic transmission.11-13 To explore the potential underlying mechanisms for SUMOylation mediated suppression of the binding of GluK2a to 14–3–3ζ, we transfected either wild type or the SUMOylation-deficient mutant GluK2a (GluK2a K886R) with Flag-tagged 14–3–3ζ and GFP-SUMO1 in HEK293T cells. This revealed that both GluK2a and GluK2a K886R were present with no significant difference in 14–3–3ζ immunoprecipitates from the 2 groups (Fig. 3A and B), demonstrating that SUMOylation of GluK2a had no major effect on modulating the interaction of 14–3–3ζ and GluK2a.

PKC SUMOylation inhibits the binding of 14–3–3ζ to GluK2a by decreasing GluK2a phosphorylation

We previously showed that Ser-846 and Ser-868 residues were major 14–3–3ζ-binding sites in the GluK2a subunit, and 14–3–3ζ proteins modulated the kinetics of GluK2a containing kainate receptors in a PKC phosphorylation-dependent manner.10 Moreover, PKC SUMOylation suppressed its enzymatic activity both in vivo and in vitro.20 Based upon our previous study and current findings that SUMOylation inhibits the binding of GluK2 to 14–3–3ζ, we therefore hypothesized that SUMOylation of PKC may repress the binding of GluK2 to 14–3–3ζ by altering the status of GluK2 phosphorylation. To test this hypothesis, we transiently transfected HEK293T cells with GluK2a, Flag-tagged 14–3–3ζ, HA-tagged PKCα or HA-tagged PKCα K465R (the SUMOylation-deficient K465R PKCα) with or without GFP-SUMO1. Cell lysates were immunoprecipitated using an anti-Flag antibody, followed by immunoblotting using an anti-GluK2 antibody. As expected, PKCα K465R promoted the binding of 14–3–3ζ to GluK2a either with or without coexpression of SUMO1 (Fig. 4A and B). Similar results were also obtained using a reverse coimmunoprecipitation strategy (Fig. 4C and D). Together, these data indicate that SUMOylation of PKC represses the binding of GluK2 to 14–3–3ζ.

Finally, we compared the phosphorylation status of GluK2a in cotransfected HEK293T cells. As shown in Figs. 5A and B, the level of GluK2a phosphorylation was significantly reduced by expression of GFP-SUMO1 in the group of cells cotransfected with wild type PKC (Fig. 5A and B). However, GFP-SUMO1 did not alter GluK2a phosphorylation in the groups of cells cotransfected with SUMOylation-deficient K465R PKC (Fig. 5A and B). Taken together, these results suggest that PKC SUMOylation suppresses the binding of 14–3–3ζ to GluK2a via decreasing GluK2a phosphorylation.
Here, we show that inhibition of the binding between GluK2a and 14–3–3τ by SUMOylation is not due to the changes of mRNA or protein expression of GluK2a and 14–3–3τ. Additionally, the binding of GluK2 to 14–3–3τ is modulated by SUMOylation of PKC but not GluK2. Taken together, our results suggest that PKC SUMOylation inhibits the binding of 14–3–3τ to GluK2a by reducing the phosphorylation level of GluK2a.

Our previous study has shown that Ser-846 and Ser-868 residues, the major phosphorylation sites of PKC within the GluK2a C-terminal tail, are the binding
Figure 2. SUMO/deSUMOylation do not alter the level of mRNA and protein expression of GluK2α and 14–3–3ε. (A, C) Western blot analyses of cell lysates from HEK293T cells transfected with GluK2α, Flag-tagged 14–3–3ε and other proteins as indicated. The blot is representative of 3 independent experiments. (B, D) The relative 14–3–3ε and GluK2α mRNA levels in HEK239T cells which were transfected with GluK2α, Flag-tagged 14–3–3ε and other proteins as indicated by quantitative real-time PCR. Data are shown as means ± SEM from 3 independent experiments.

Figure 3. GluK2α SUMOylation does not change the binding of GluK2α to 14–3–3ε. (A) Western blot analyses of immunoprecipitates and cell lysates from HEK293T cells cotransfected with either GluK2 or SUMOylation mutant site of GluK2 (GluK2 k886R) with Flag-tagged 14–3–3ε. Cell lysates were prepared 24 h post-transfection and immunoprecipitated with anti-Flag antibody followed by Western blot with an anti-GluK2 or anti-Flag antibodies. (B) Quantification of Western blots in (A). The blot is representative of 3 independent experiments. Data are means ± SEM.
sites of 14–3–3τ on GluK2. More importantly, PKC modulates desensitization kinetics of GluK2a receptors by regulating phosphorylation-dependent binding of 14–3–3τ to GluK2a. In addition, SUMOylation of GluK2 regulates endocytosis of KARs and modifies synaptic transmission. However, our study found that PKC SUMOylation repressed the binding of 14–3–3τ to GluK2, but GluK2 SUMOylation did not alter the binding of GluK2a to 14–3–3τ (Fig. 3 and Fig. 4).

Based on our previous reports that SUMOylation of PKC inhibits its activity, those results suggest that the level of GluK2 phosphorylation is decreased via PKC SUMOylation and consequently results in decreased the binding of GluK2 to 14–3–3τ.

In summary, we report that SUMOylation of PKC, but not GluK2, represses the binding of 14–3–3τ to GluK2.
14–3–3τ to GluK2a via reducing the phosphorylation of GluK2a. Because a protein can be modified by more than one type of PTM, recent studies have provided evidence for functional cross-talk and complex interplay among SUMOylation, phosphorylation, and ubiquitination for several proteins.22-27 Phosphorylation of GluK2 by PKC, PKA, and CaM-KII regulates KAR expression, trafficking, and excitatory synaptic transmission.21,28,29 GluK2 can also be modified by Ubiquitin and SUMO, and consequently regulates its degradation and membrane expression, trafficking.14-16,30 Our results show that SUMOylation of PKC, but not GluK2, inhibits the binding of 14–3–3τ to GluK2a through reducing the phosphorylation of GluK2a (Fig. 4 and 5). Previous studies have shown that 14–3–3 proteins are an important regulator of GluK2a-containing KARs and contribute to the slow decay kinetics of native KAR-EPSCs.10 Taken together, these results suggest that PKC SUMOylation is an important regulator of 14–3–3 and GluK2a protein complex and may contribute to regulate the decay kinetics of KAR-EPSCs. Therefore, it is important for future studies to further determine the function of PKC SUMOylation on decay kinetics of KAR-EPSCs.

Materials and methods

Plasmids and antibodies

pcDNA3.1-GluK2a, Flag-14–3–3τ, GFP-SUMO1, RGS-SENP1 and RGS-SENP1m were described previously.10,19,27 GluK2a mutation (GluK2a K886R) was generated using the QuikChange site-directed mutagenesis kits and confirmed by DNA sequencing. Antibodies used were as follows: rabbit anti-GluK2/3 polyclonal antibody (53518) was from AnaSpec; rabbit anti-GluK5 polyclonal antibody (06-315) was from Millipore; mouse anti-Flag (F1804) was from Sigma-Aldrich; rabbit anti-SUMO1 (4940) was from Cell Signaling Technology.
Quantitative real-time PCR

Total RNA was extracted from HEK293T cells using TRIzol reagents and reverse transcribed to obtain single-strand cDNA using a Reverse Transcription System as described previously. Reactions were performed in a 20 µl final volume with Power SYBR Green PCR Master Mix and 0.2–0.5 mM primers using the Applied Biosystems 7500 fast Real-Time PCR System according to the manufacturer’s instructions. Results are the average of at least 3 independent biologic replicates. Primers used are: GluK2a: Forward: 5’-AGCGTGGGCTCAAA-CATAAG-3’ and Reverse: 5’-TTTCTTTACCTGG-CAACCTTCT-3’; 14–3–3ζ: Forward: 5’-GGACTATC GGAGAAAGTGG-3’ and Reverse: 5’-TCCTGCAC TGTCTGATGTCC-3’; GAPDH: Forward: 5’-CATG GCCCTCCTGTTCC-3’ and Reverse: 5’-GCCTGCTT CACCACCTTCTT-3’.

Cell culture, transfection, and protein preparation

HEK293T cells were cultured and maintained at 37°C in a 5% CO2 humidified incubator supplemented with Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (Fisher Scientific). Cells were grown to 70–90% confluency and transfected using Lipofectamine 2000 in accordance with manufacturer’s instructions.

Immunoprecipitation and western blot

Immunoblotting was performed as described previously with modifications. After 24 h of transfection, HEK293T cells were lysed in lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 20 mM NEM (Sigma-Aldrich) and protease inhibitor cocktail (Sigma-Aldrich), and phosphatase inhibitor cocktail (Roche) were added to cell lysates for the detection of phosphorylated GluK2a. The lysates were immunoprecipitation was performed using standard approaches. Briefly, lysates containing 1 mg of proteins were incubated with 1 mg of antibodies overnight at 4 °C, followed by the incubation with 15 µl of protein A/G-agarose at 4 °C for 3 h. The beads were subsequently washed 3 times with lysis buffer and then boiled for 10 min in sample buffer and further analyzed by immunoblotting. Protein densities on Western blots were analyzed by ImageJ software. Relative band densities were determined by normalizing the immunoprecipitated band density to that of the lysate band. All experiments were repeated at least 3 times.

Statistical analysis

Data are expressed as mean ± SEM with statistical significance assessed by Student’s t test for 2 group comparisons or one-way analysis of variance tests for multiple comparisons. The value of *P* < 0.05 was considered statistically significant difference.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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