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SHORT COMMUNICATION



Multiple basic amino acid residues contribute to phosphatidic acid-mediated inhibition of rice potassium channel OsAKT2

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ABSTRACT

Anionic phospholipid phosphatidic acid (PA) behaves as an important second messenger involved in many cellular processes, such as development, cytoskeletal dynamics, vesicle trafficking, and stress response. Recently, it was reported that PA can directly bind with the rice Shaker K⁺ channel OsAKT2 to inhibit its channel activity. Two adjacent arginine residues (R644 and R645) in ANK domain were identified as a PA-binding site essential to the PA-mediated inhibition of OsAKT2. However, there may be still other PA-binding sites unidentified in OsAKT2. Here, using a PA biosensor (PAleon), we found that the exogenous PA treatment significantly increased the PA level at the plasma membrane of *Xenopus* oocytes which were used to express OsAKT2 for electrophysiological assays. As reported previously, exogenous PA markedly inhibited OsAKT2 K⁺ currents. Replacement of two adjacent basic residues (R190 and K191) in the S4 voltage sensor by glycine completely abolished the time-dependent K⁺ currents of OsAKT2, but this variant was insensitive to PA treatment. In addition, we also identified other two adjacent arginines (R755 and R756) located in the cytosolic domain as a PA-binding site, which were also essential to the PA-mediated inhibition of OsAKT2. These results provide a more comprehensive understanding of the PA-K⁺ channel interaction mechanism. Combining the findings here with the previous study, we propose that multiple basic residues (R190/K191, R644/R645, and R755/R756) in different domains of OsAKT2 contribute to PA-mediated regulation of OsAKT2.

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Phospholipids not only play essential roles in membrane composition but also function as important secondary messengers in organismal growth, development, and response to the environment.^{1,2} Several kinds of anionic phospholipids have been shown to be involved in modulating the gating of ion channels in animal cells, especially in neurons and cardiac myocytes.^{3,4} Phospholipids regulate ion channels in two well-documented ways: (1) some phospholipids can bind to specific sites that control channel gating; (2) the composition of phospholipids can influence the physical membrane environment to modulate ion channels nonspecifically.⁴ Because phospholipids are the main components of cell membrane, they are in close proximity to membrane ion channels or channels' modulators located in membrane micro-domains.

Phosphatidylinositol biphosphate (PIP₂) is the first known and remains the most prominent signaling lipid to affect ion channels. In animal cells, it can bind to and regulate numerous ion channels including inwardly rectifying potassium (Kir) channels, KCNQ, voltage-gated Ca²⁺ channels, and voltage-gated potassium (K_v) channels.² The PIP₂-mediated modulation of plant ion channels was also identified. PI(4,5)P₂ applied to the cytosolic side of excised membrane patches of *Xenopus* oocytes can inhibit the rundown of the *Arabidopsis* outward rectifying K⁺ channel SKOR and two inward rectifying K⁺ channels (KAT1 from *Arabidopsis* and LKT1 from tomato),⁵ whereas PI(4,5)P₂ inhibits K⁺-efflux channel activity in NT1 tobacco cultured cells.⁶ Light stimuli can induce the

accumulation of PI(4,5)P₂ on the plasma membrane, and PI(4,5)P₂ can inhibit the anion channel activity in guard cells to promote stomatal opening.⁷ In addition, enhancing PIP₂ level increases the abundance of active aquaporins at the plasma membrane of tobacco protoplast.⁸

Besides PIP₂, phosphatidic acid (PA) is also a versatile lipid second messenger in cell signal transduction and plays crucial roles in many physiological processes, such as development, lipid metabolism, and stress response.⁹ PA is generated by two major pathways: hydrolysis of the structural lipid phosphatidylcholine by phospholipase D (PLD) enzymes, or phosphorylation of the diacylglycerol (DAG) by DAG kinase.^{10,11} The physical properties of PA, such as its dual deprotonation and capacity to form hydrogen bonds, promote its direct binding with some basic amino acid residues in target proteins.¹² Many kinds of functional protein have been identified as PA targets, including metabolic enzymes, kinases, phosphatases, and transcription factors, etc.¹³ PA binding can alter the catalytic activity or membrane association of the target proteins. PA is also proposed to be involved in K⁺ channel modulation. In *Streptomyces lividans*, PA functions in stabilizing and folding of the tetrameric potassium channel KcsA.¹⁴ Hite et al. revealed that PA modulated rat voltage-gated potassium (K_v) channel in both specific and nonspecific ways.¹⁵ PA can also bind to and activate TREK-1 K⁺ channel and consequently to control the excitability of nerves.¹⁶ In some instances, PA also competes with PIP₂ binding with ion channels and reverse PIP₂

-mediated effects.³ In plants, PA treatment significantly inhibits the inward K^+ current of guard cells,¹⁷ and PLD α 1 and PLD δ are essential to ABA-induced inhibition of guard cell K^+ channel.¹⁸ McLoughlin et al. found that PA could bind with the K^+ channel β -subunit KAB1 under saline condition.¹⁹ Despite these observations, the underlying mechanism by which PA modulates plant K^+ channels remains unclear. Recently, PA was reported to modulate a rice Shaker K^+ channel OsAKT2 which is primarily expressed in shoot phloem tissue.²⁰ OsAKT2 operated as a weakly rectifying K^+ channel which can prevent the H^+ /sucrose-symport-induced membrane depolarization. This feature may stabilize membrane potentials to promote sucrose phloem loading and translocation. Disruption of OsAKT2 results in delayed growth of rice seedlings under short-day conditions. PA directly binds with two adjacent arginine residues in the ANK domain of OsAKT2, which is essential to PA-mediated inhibition of OsAKT2.²⁰ This study reveals a direct link between phospholipid signaling and plant K^+ channel modulation. The PA-mediated inhibition of AKT2 may play an important role in both growth regulation and stress response. Firstly, PA is a key intermediate in glycerolipid metabolism, so its accumulation may inhibit AKT2 thus decreasing phloem sugar loading and consequently restricting lipid biosynthesis which depends on the carbohydrate supply. Secondly, as a second messenger, the stress-induced PA may target AKT2 subunit thus inhibiting guard cell K^+ uptake and stomatal opening, or consequently suppressing sucrose phloem loading and plant growth. The reduction of both water loss via stomata and plant growth can improve plant tolerance to stress.

It has been shown that when expressed in *Xenopus* oocytes, OsAKT2 is significantly inhibited by the addition of PA in bath solution.²⁰ However, it remains unclear whether the exogenous PA treatment can induce PA accumulation at plasma membrane. Thus, we expressed a PA biosensor (named PAleon²¹) in *Xenopus* oocytes and employed FRET to monitor the PA level. As shown in Figure 1, there were no FRET/CFP ratio signals at the plasma membrane of the water-injected oocytes,

irrespective of the presence or absence of exogenous PA. However, application of 100 μ M 16:0–18:2 PA to the PAleon-expressing oocytes resulted in a dramatic increase of FRET/CFP ratio. This result indicated the exogenous PA treatment was effective in enhancing the PA level of plasma membrane.

Some PA-targeted proteins, such as MAP65-1²² and SnRK2.4,²³ contain multiple basic amino acid residues located in different regions to bind with PA. In addition, many basic residues have been proposed to be important for the PIP₂-dependent activation of KCNQ K^+ channel.²⁴ Thus, there might be other unidentified PA-binding sites in OsAKT2 besides the R644/R645 residues identified in the previous study.²⁰ Considering that PA was proposed to specifically interact with some arginine residues in the S4 transmembrane domain (voltage sensor) of the KvAP K^+ channel to keep the channel pore closed,¹⁵ we investigated whether some basic amino acid residues in S4 voltage sensor are involved in PA-mediated inhibition of OsAKT2 (Figure 2a). Using site-direct mutagenesis, we generated two OsAKT2 variants (R190G/K191G and K193S) and determined the effect of PA on their activity. The R190G/K191G mutations completely abolished the time-dependent K^+ currents and convert OsAKT2 to be an “open-leak” channel. PA treatment did not affect the activity of R190G/K191G variant, whereas the wild-type OsAKT2 was significantly inhibited by the same PA treatment (Figure 2b,c). However, the K193S variant without instantaneous-activated currents was still sensitive to PA (Figure 2d). These results suggest that the R190/K191 residues may also play an essential role in the PA-mediated inhibition of OsAKT2. A plausible interpretation is that the R190G/K191G mutation in S4 makes the voltage sensor constitutively activated and eliminates the PA-mediated inhibition of voltage sensor activation.

In previous study, although replacement of the R644/R645 residues by glycine significantly inhibited the interaction between PA and the C-terminal domain of OsAKT2 (OsAKT2_{533–855}), the R644G/R645G variant still showed some affinity to PA in lipid dot-blot assay.²⁰ Thus, besides R644 and R645 residues, there might be still other binding sites in this

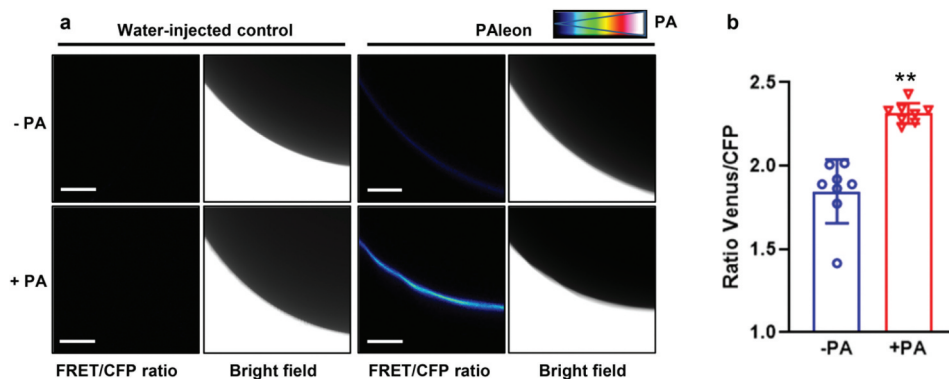


Figure 1. PA treatment significantly induces the PA accumulation at plasma membrane of *Xenopus* oocytes. (a) Representative FRET ratio images of *Xenopus* oocytes expressing PAleon (a PA biosensor²¹) under exogenous PA treatment. The N-terminal domain (1–250 Aa) of RbohD specifically binding with PA was inserted between a cyan fluorescent protein (CFP) and a VENUS yellow fluorescent protein, generating the PA biosensor named PAleon.²¹ The water-injected oocytes were used as background control. Photographs were taken at 0 h (-PA) or 1.5 h (+PA) after the addition of 100 μ M PA (16:0–18:2) to the culture medium. The FRET/CFP ratio images were calculated pixel by pixel as the ratio of FRET image divided by the CFP image, and were pseudocolor-coded according to the scale at upper right. Bars represent 100 μ m. CFP, cyan fluorescent protein; FRET, fluorescence resonance energy transfer. (b) The ratio of FRET (Venus) to CFP fluorescence derived from the plasma membrane of PAleon-expressing oocytes like those in (a). The data bars were presented as means $\pm$ SD ($n = 8$). Asterisks indicate significant difference from -PA control at $P < 0.01$ by Student's t test.

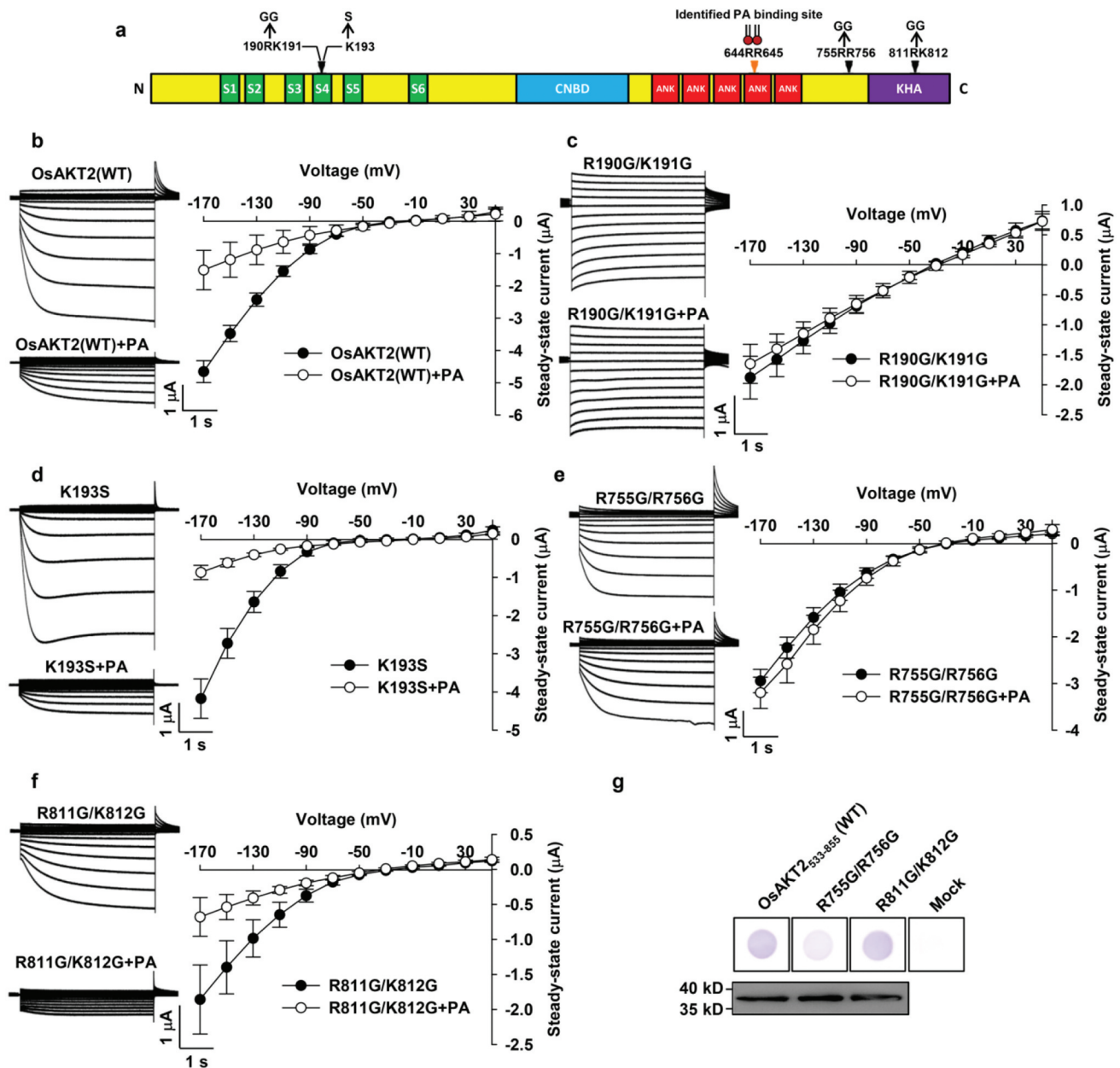


Figure 2. The effect of PA on the activities of OsAKT2 variants. (a) Schematic diagram shows the site-directed mutagenesis of putative PA-binding sites in OsAKT2. The colorful boxes represent various functional domains in OsAKT2. S1 to S6, six transmembrane domain; CNBD, cyclic nucleotide-binding domain; ANK, ankyrin repeat domains. The orange triangle indicates the R644/R645 residues which had been identified as the PA-binding site in previous study,²⁰ while the black triangles indicate the location of other possible PA-binding sites. Arrows indicate site-directed amino acid mutations. (b) to (f), the effects of PA treatment on the activities of various variants, including WT ($n = 6$), R190G/K191G ($n = 4$), K193S ($n = 6$), R755G/R756G ($n = 8$) and R811G/K812G ($n = 5$). Two electrode voltage clamp experiments were performed on the *Xenopus* oocytes expressing OsAKT2 variants before and after incubation in bath solution containing 100 μM PA (16:0–18:2). The bath solution contains 30 mM KCl, 2 mM CaCl_2 , 1 mM MgCl_2 , and 10 mM HEPES (pH 7.2 with Tris), and the osmolality of the solution was adjusted to 240–260 mOsm kg^{-1} with mannitol. The holding potential was -30 mV and test potentials ranged from -170 to $+50$ mV (20 mV increments). The data points for current-voltage curves are presented as means $\pm$ SD. (g) The lipid dot-blot assays show the PA binding of the OsAKT2 variants, including OsAKT2₅₃₃₋₈₅₅ (WT), R755G/R756G and R811G/K812G. For Mock condition, no protein was added into the incubation buffer. Equal amounts of proteins were used and checked by immunoblot assay. The protein bound to PA was visualized by immunoblotting using alkaline phosphatase-conjugated secondary antibodies.

region. Two sites containing adjacent basic amino acid residues were selected as the candidates. One consists of the R755/R756 residues located in the junction between ANK domain and KHA domain, while the other points to the R811/K812 residues in the KHA domain. We generated two OsAKT2 variants (R755G/R756G and R811G/K812G) and determined their sensitivity to PA. As shown in Figure 2e,f, R755G/R756G variant

exhibited no sensitivity to PA treatment, whereas R811G/K812G mutation did not affect the PA-mediated inhibition of OsAKT2. Lipid dot-blot assay was performed to determine the effect of the site-directed mutations on the PA-OsAKT2 binding. The R755G/R756G mutations resulted in a significant decrease of PA-binding ability of OsAKT2₅₃₃₋₈₅₅, whereas the R811G/K812G mutation did not affect the interaction between

PA and OsAKT2₅₃₃₋₈₅₅ (Figure 2g). These results indicate that the R755/R756 residues are also a PA-binding site essential to PA-mediated inhibition of OsAKT2.

Combining the findings here with the previous study, we proposed that multiple basic amino acid residues binding to PA contributed to the PA-mediated inhibition of OsAKT2. These findings further confirmed that OsAKT2 K⁺ channel is a target of PA signaling, and presented a more comprehensive understanding of the PA-K⁺ channel interaction mechanism.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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