Coupling between the voltage-sensing and phosphatase domains of Ci-VSP

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The *Ciona intestinalis* voltage sensor–containing phosphatase (Ci-VSP) shares high homology with the phosphatidylinositol phosphatase enzyme known as PTEN (phosphatase and tensin homologue deleted on chromosome 10). We have taken advantage of the similarity between these proteins to inquire about the coupling between the voltage sensing and the phosphatase domains in Ci-VSP. Recently, it was shown that four basic residues (R11, K13, R14, and R15) in PTEN are critical for its binding onto the membrane, required for its catalytic activity. Ci-VSP has three of the basic residues of PTEN. Here, we show that when R253 and R254 (which are the homologues of R14 and R15 in PTEN) are mutated to alanines in Ci-VSP, phosphatase activity is disrupted, as revealed by a lack of effect on the ionic currents of KCNQ2/3, where current decrease is a measure of phosphatase activity. The enzymatic activity was not rescued by the introduction of lysines, indicating that the binding is an arginine-specific interaction between the phosphatase binding domain and the membrane, presumably through the phosphate groups of the phospholipids. We also found that the kinetics and steady-state voltage dependence of the S4 segment movement are affected when the arginines are not present, indicating that the interaction of R253 and R254 with the membrane, required for the catalytic action of the phosphatase, restricts the movement of the voltage sensor.

INTRODUCTION

The *Ciona intestinalis* voltage sensor–containing phosphatase (Ci-VSP) is a voltage-dependent phosphatidylinositol phosphatase (Murata et al., 2005; Murata and Okamura, 2007; Halaszovich et al., 2009), which bears homology to two different types of proteins. The first 239 amino acids resemble the voltage-sensing domain (VSD) of potassium-selective voltage-gated channels. The second half of the protein is the phosphatase domain (PD), which shares high homology with the tumor suppressor enzyme known as PTEN (phosphatase and tensin homologue deleted on chromosome 10) (Li et al., 1997; Steck et al., 1997).

When expressed in *Xenopus* oocytes, Ci-VSP produces nonlinear capacitive currents similar to those observed upon expression of voltage-gated channels after ionic current blockade. It is accepted that these currents, called sensing currents, are mainly produced by the movement of "sensing" charges located in the putative fourth transmembrane segment (S4) of the predicted secondary structure of this protein (Murata et al., 2005; Murata and Okamura, 2007). The conformational change induced by the charge movement

produces the activation of the phosphatase activity, presumably by moving the PD close to the membrane (Murata et al., 2005; Murata and Okamura, 2007; Halaszovich et al., 2009).

The PD of Ci-VSP and PTEN exhibits high homology. In particular, these proteins have almost identical sequences in their catalytic region (Worby and Dixon, 2005; Murata and Okamura, 2007; Iwasaki et al., 2008). The presence of a glycine in position 365, instead of an alanine as in PTEN, allows Ci-VSP to dephosphorylate $PI(4,5)P_2$ (Iwasaki et al., 2008). This reaction leads to the production of $PI(4)P$ (Halaszovich et al., 2009). Sequence alignment of the two proteins shows another similarity in a 16–amino acid region located between the VSD and the PD (positions 240–255) of Ci-VSP. This region displays 50% identity with the N-terminal 16 amino acids of PTEN (Fig. 1) and is known to be essential for phosphatase activity. The simultaneous substitution of arginines 11, 14, and 15 and lysine 13 to alanine abolishes PTEN activity by impairing the binding of the phosphatase, decreasing 76-fold its affinity for the membrane (Campbell et al., 2003; Das et al., 2003). In addition, mutation of R15 to alanine occurs in human cancers and is reported to reduce PTEN activity (Steck

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et al., 1997; Furnari et al., 1998). Individual mutations of the positively charged amino acids 13, 14, and 15 have been found to decrease the activity of PTEN in vitro assays (Furnari et al., 1998; Campbell et al., 2003).

As for Ci-VSP, the deletion of eight amino acids between positions 240 and 247 (segment ΔN-L) produced the loss of the phosphatase activity of this enzyme when expressed in *Xenopus* oocytes (Murata et al., 2005). On the other hand, the deletion of the amino acids between 248 and 255 (segment ΔC-L) did not affect the phosphatase activity (Murata et al., 2005). These observations seem to indicate that the segment ΔN-L is required for function. However, the expression of a glutathione *S*-transferase–tagged mutant of Ci-VSP, in which the VSD and the segment ΔN-L were deleted, displayed phosphatase activity. To reconcile this contradiction, a plausible explanation is that the amino acids from position 240 to position 255 are spatially arranged, forming a structured, geometrically constrained binding site for phospholipids, rather than constituting a motif mediating an unspecific electrostatic interaction with the plasma membrane. In the case of PTEN, the N terminus of the protein forms a binding site specific for $PI(4,5)P_2$. Based on both the homology with PTEN and the effect of the described deletion on the activity of the phosphatase, we propose the existence of a phospholipid binding motif (PBM) in Ci-VSP between positions 240 and 255. From this viewpoint, we hypothesize that the mechanism of coupling between the VSD and the PD occurs when the movement of VSD brings the PBM close to the lipid bilayer, promoting the binding of the motif to the phospholipids. The consequence of this binding is that the PD is brought in proximity to its substrate, enabling the phosphatase activity. In this study, we disrupt the coupling between the VSD and the PD by replacing arginines with alanines in the PBM, resulting in an impaired phosphatase activity of Ci-VSP.

MATERIALS AND METHODS

Mutagenesis and expression of Ci-VSP

Different mutants of Ci-VSP were generated by site-directed mutagenesis. The mutations were introduced by PCR amplification of the plasmid with primer containing the desired mutation. Mutations were verified by sequencing. Two groups of mutants were created to study either phosphatase activity (monitored by recording KCNQ2/3 ionic currents) or sensing current of Ci-VSP. For phosphatase activity, the arginines 253 and 254 were mutated to alanines simultaneously, generating 2-alanines mutant. Also, the pair located in positions 245 and 246 were simultaneously mutated by site-directed mutagenesis. For sensing current recording, the same mutations were produced by using the mutant C363S of Ci-VSP, which bears no enzymatic activity (Murata et al., 2005). In addition, the mutant R253A-R254A-C363S was further mutated to introduce a cysteine replacing the glycine in position 214, allowing the labeling of Ci-VSP with a fluorescent probe (see below). The DNA was linearized with XbaI (New England Biolabs, Inc.). The linearized DNA was transcribed using SP6 RNA polymerase (Applied Biosystems). 50 nl of 0.5–1 µg/µl RNA was injected per oocyte. The DNA of KCNQ2 and KCNQ3 (provided by T. Jentsch, Leibniz-Institut für Molekulaire Pharmakologie, Berlin-Buch, Germany) was linearized with MluI and HpaI, respectively (New England Biolabs, Inc.). The linearized DNA was transcribed using SP6 RNA polymerase (Applied Biosystems). The oocytes were incubated at 18°C in a solution containing 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 5 mM HEPES, pH 7.5.
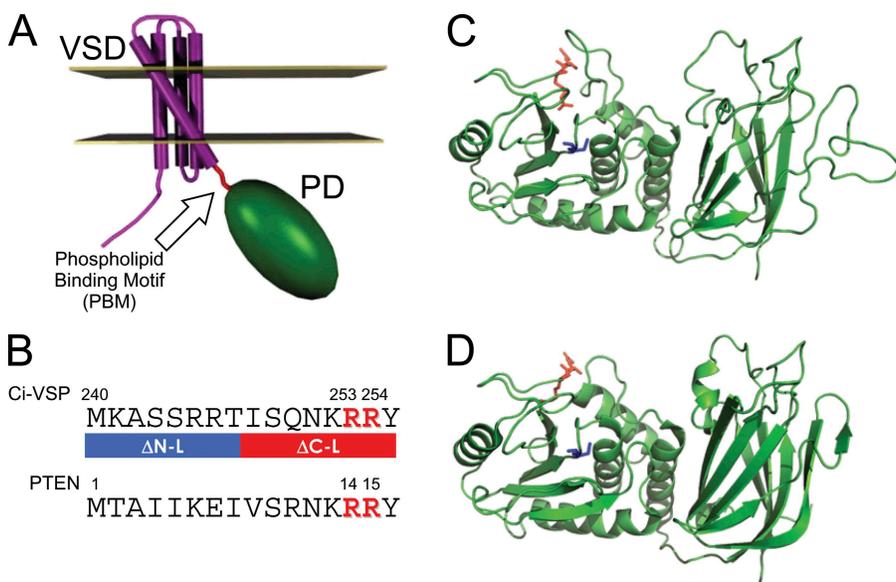
Figure 1. (A) Cartoon representation of the structure of Ci-VSP. The N terminus of Ci-VSP spans four transmembrane segments forming the VSD, and the fourth segment (S4 segment) of the VSD is connected to the PD by the PBM. (B) The proposed PBM of Ci-VSP shares 50% identity with the first 16 amino acids of the PTEN. (C) Homology model of the PD of Ci-VSP. This homology model was created by the online package 3D-jigsaw using residues 240–576 of Ci-VSP (http:// bmm.cancerresearchuk.org/~3djigsaw/). The obtained model comprises residues 253–574. (D) Crystal structure of PTEN. The crystal structure of PTEN is constituted by residues 14–351 (Lee et al., 1999). Arginines 253 and 254 of Ci-VSP (C) and 14 and 15 of PTEN (D) are displayed in red. These residues are implicated in the formation of the putative PBM in these proteins. Cysteine 363 of Ci-VSP and 124 in PTEN are displayed in blue. These residues are essential for the activity of the catalytic site.
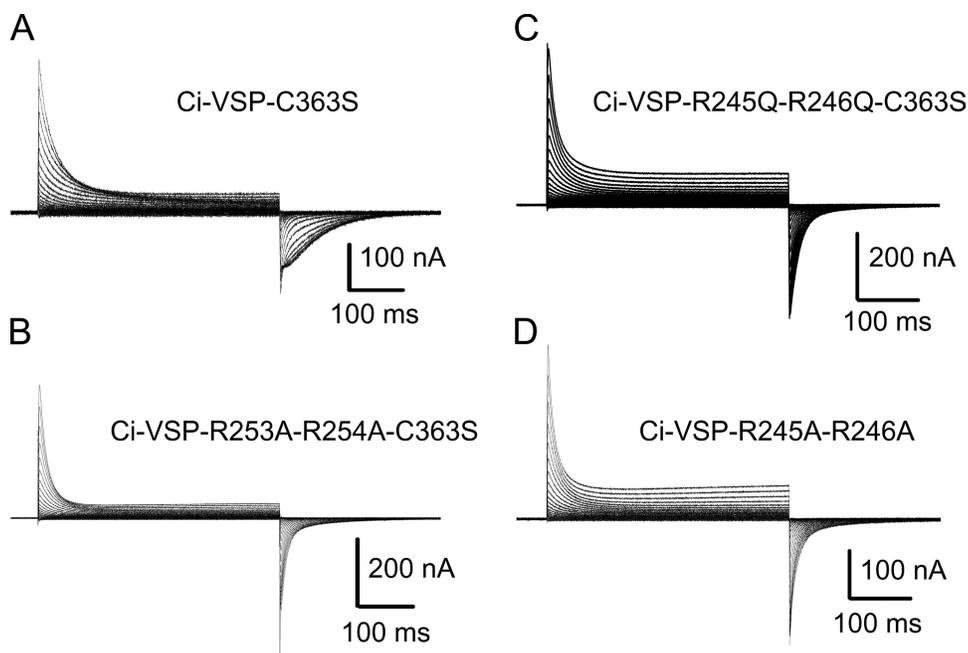
Figure 2. Sensing currents from oocytes expressing different mutants of Ci-VSP. Sensing currents were recorded by applying voltage pulses of 400 ms, ranging from -100 to 140 mV. The HP was -60 mV. (A) Sensing currents for the phosphatase-inactivated mutant Ci-VSP C363S. When pulsing to positive potentials from the HP, all the mutants, R253A-R254A-C363S (B), R245Q-R246Q-C363S (C), and R245A-R246A (D), displayed sensing currents similar to those observed with the mutant C363S. In contrast, during the repolarization to the HP, all the PBM mutants displayed faster OFF sensing currents.

Electrophysiology and fluorescence recordings

Sensing and ionic currents were measured 2–4 d after injection. Currents were recorded at room temperature (or at the indicated temperatures) with the cut-open oocyte voltage clamp technique (Stefani and Bezanilla, 1998). For sensing currents, the external recording solutions for experiments contained 120 mM NMG-Mes (methylsufonate), 10 mM HEPES, and 2 mM $CaCl_2$, pH 7.4, whereas internal solutions contained 120 mM NMG-MES, 10 mM HEPES, and 2 mM EGTA, pH 7.4. For ionic currents from oocytes injected with KCNQ2/KCNQ3, the external recording solutions contained 103 mM NMG-MES, 12 mM K^+-Mes, 20 mM HEPES, and 2 mM $CaCl_2$, pH 7.4. The internal solutions contained 120 mM K^+-Mes, 20 mM HEPES, and 2 mM EGTA, pH 7.4. The blockage of the ionic current was done by replacing both the external and the internal solution with TEA-containing ones. The external blocking solution contained 115 mM TEA-MES,

20 mM HEPES, and 2 mM $CaCl_2$, pH 7.4. The internal blocking solution contained 115 mM TEA-Mes, 20 mM HEPES, and 2 mM EGTA, pH 7.4. There was no subtraction of leak components during the acquisition. When needed, the subtraction was performed offline. Electrophysiological and fluorescence data were filtered at 1–5 kHz and sampled at 2–20 kHz. The currents were measured from a holding potential (HP) of -60 and $+80$ mV in response to various test potentials (unless otherwise stated). Test pulses were at least 800-ms long and were applied with a 10-s interval. Data acquisition and analysis were performed with in-house programs.

The experimental setup was as described previously (Cha and Bezanilla, 1998), with some modifications. In brief, the setup consisted of a microscope (BX51WI; Olympus) with optical filters suitable for tetramethyrhodamine-5-maleimide (TMRM; Invitrogen) excitation and emission acquisition. A 150-W tungsten halogen
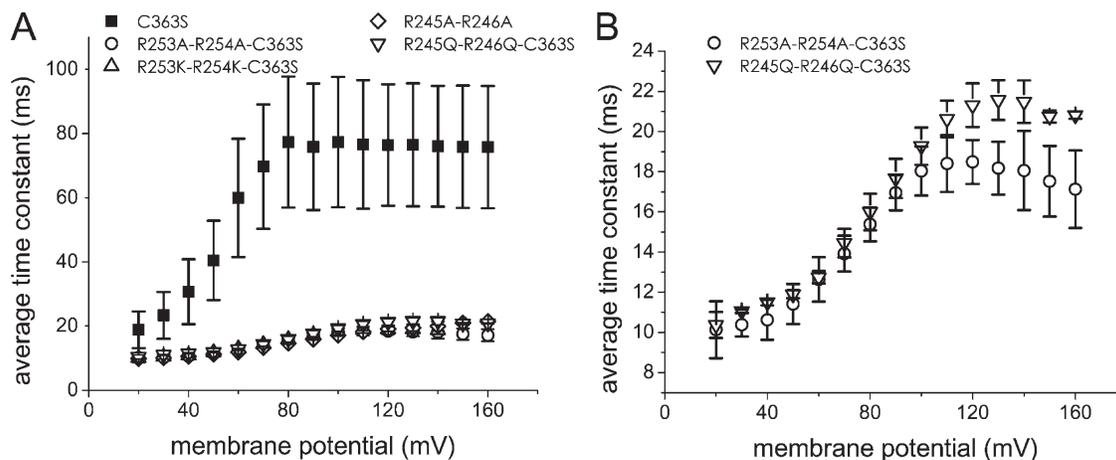
Figure 3. Average time constant of the OFF sensing current of different mutants of Ci-VSP. (A) The OFF sensing current recorded from the PBM mutants (open symbols) R253A-R254A-C363S ($n = 5$), R253K-R254K-C363S ($n = 4$), R245Q-R246Q-C363S ($n = 4$), and R245A-R246A ($n = 4$) were significantly faster than those recorded from the C363S mutant (filled squares; $n = 5$) for all potentials tested above $+20$ mV (HP = -60 mV). (B) The mutant R245Q-R246Q C363S displayed slower OFF sensing current than those from the mutant R253A-R254A-C363S for pulses above $+100$ mV.

lamp, powered by a regulated power supply (Condor F15-15-A+), was used as an excitation source. The excitation and light collection was performed with a LUMPlanFl 40× water-immersion objective with an NA of 0.80. Light measurements were performed by using a PhotoMax-201-PIN photodiode (Dagan) controlled by a PhotoMax 200 amplifier (Dagan). A Dagan CA-1B was used for voltage clamping in the cut-open oocyte voltage clamp configuration.

RESULTS

Mutant of the PBM shows faster OFF sensing currents

Sensing currents were recorded from oocytes expressing the C363S Ci-VSP mutant containing two additional mutations: R253A and R254A (Fig. 2 B). When pulsing to positive voltages with respect to the HP (HP = −60 mV), the ON sensing currents were similar to those observed from oocytes expressing the phosphatase-inactivated mutant C363S (Murata et al., 2005), which has an intact PBM. On the other hand, the OFF sensing current observed for the R253A-R254A-C363S mutant during repolarization to the HP clearly displayed faster kinetics (Fig. 2 B) compared with those recorded from oocytes expressing Ci-VSP C363S (Fig. 2 A). Analysis of the time constants of the OFF sensing currents revealed that those

from the R253A-R254A-C363S mutant were at least two-fold faster for depolarizing pulses above +30 mV, and fourfold faster for potentials above +60 mV (Fig. 3). These results indicate that during repolarization, the movement of the S4 segment is somehow hindered when arginines 253 and 254 are present. Similar observations were made when recording sensing currents from the mutants R245Q-R246Q-C363S (Figs. 2 C and 3 A) and R245A-R246A (Figs. 2 D and 3 A). Detailed analysis of the time constant of the OFF sensing current revealed those from the mutant R245Q-R246Q-C363S to be slightly slower than those recorded from the R253A-R254A-C363S mutant (Fig. 3 B).

The coupling between voltage and phosphatase activity is impaired by the double mutation R253A-R254A

The phosphatase activity of Ci-VSP was estimated by measuring the maximum activation and kinetics of the potassium-selective channels KCNQ2/3, whose activities are highly dependent on PIP$_2$ (Zhang et al., 2003). Okamura and colleagues (Murata et al., 2005; Murata and Okamura, 2007) have reported that the currents of KCNQ2/3 (Fig. 4 A) are strongly depressed when coexpressed with Ci-VSP wild type (WT) (Fig. 4 B). When we coexpressed
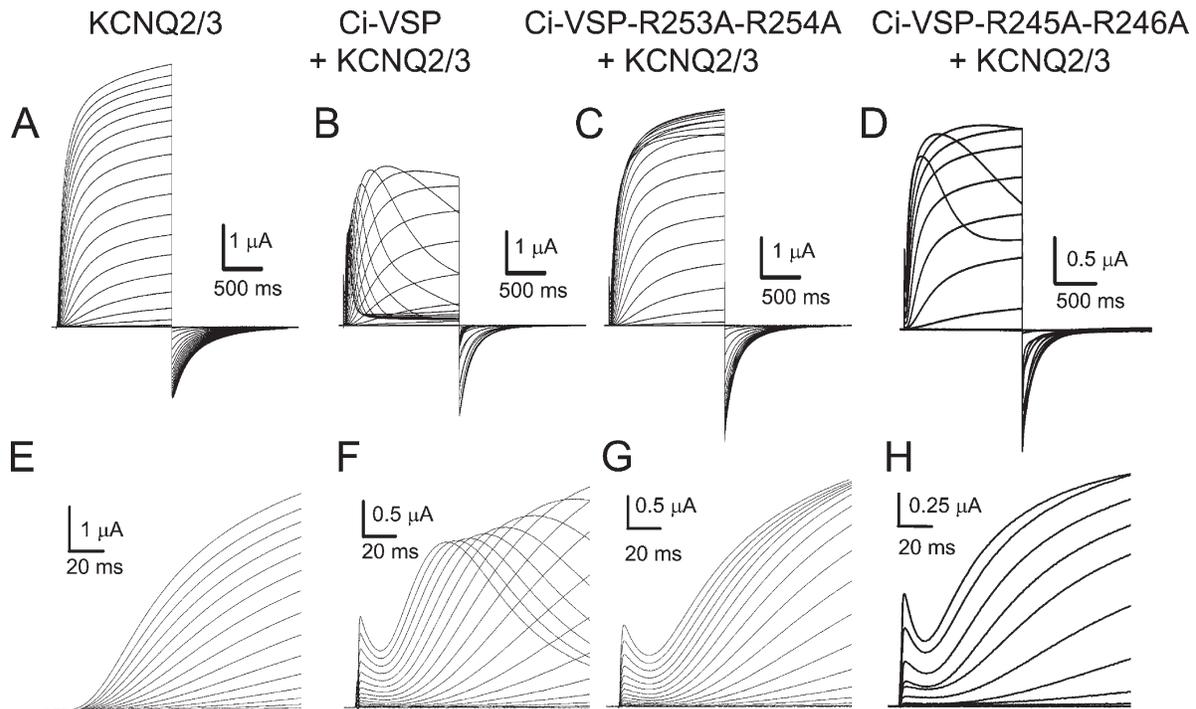
Figure 4. Phosphatase activity of Ci-VSP monitored by the activity of the potassium-selective, voltage-, and PI(4,5)P$_2$-dependent channels KCNQ2 and KCNQ3. (A) Currents of KCNQ2/3 expressed without Ci-VSP. (B) When KCNQ2/3 were expressed with the WT form of Ci-VSP, a strong decrease in the activity was observed at potentials above +20 mV. (C) Coexpression with the mutant R253A-R254A caused a slight decrease in the current of KCNQ2/3, only observable at very positive potentials. (D) Likewise, coexpression with the mutant R245A-R246A caused an initial decrease in the current of the channels at potentials above +80 mV. However, the effect on the ionic current was less profound than that observed with the mutant R253A-R254A. (E) Transient initial currents were not observed when mRNA coding for Ci-VSP was not injected in the oocytes along with the mRNA for KCNQ2/3. (F–H) In contrast, transient initial currents were observed in the presence of the phosphatase. These currents are the sensing currents of Ci-VSP. Pulses range from −100 to +100 mV. HP = −90 mV. For A–C and E–G, the interval between pulses was 10 mV. For D and H, it was 20 mV.

KCNQ2/3 with the mutant Ci-VSP R253A-R254A, the ionic currents showed minimal depression during the depolarizing pulse. This observation indicated that the mutation R253A-R254A did impair the voltage dependence of the phosphatase activity of Ci-VSP (Fig. 4 C). We were concerned that the severely diminished phosphatase activity could be a consequence of a difference in expression level of Ci-VSP. To address this issue, we verified that the Ci-VSP was present, taking advantage of the observation that when expressed, the sensing currents are seen as a clear initial transient-outward current (Fig. 4, F–H). The peak amplitude of the sensing current from Ci-VSP shows no difference between WT, the R253A-R254A mutant, and any other catalytically active mutant tested during this study (unpublished data). Therefore, the lack of phosphatase activity is not related to the difference in the expression level of the PBM mutants with respect to the WT Ci-VSP. Sensing currents were not observed in oocytes expressing only KCNQ2/3 (Fig. 4 E). Further confirmation was obtained by replacing K^+ by TEA^+ in the recording solutions. The blockage of the ionic current in oocytes expressing KCNQ2/3 alone revealed no transient current upon depolarization (Fig. 5, A and C). In contrast, when both KCNQ2/3 and Ci-VSP R253A-R254A were coexpressed (Fig. 5 B), the replacement of K^+ by TEA^+ blocked the ionic current, but the initial transient current remained (Fig. 5 D). This observation confirmed that the initial transient current corresponded to the sensing current of the R253A-R254A

mutant Ci-VSP, confirming that it was indeed expressed, although it had an impaired voltage-dependent phosphatase activity.

The phosphatase activity depends on an arginine-specific interaction

Next, we attempted to rescue the coupling between the VSD and the PD of Ci-VSP by replacing the basic arginine residues in 253 and 254 by lysine in an attempt at maintaining the charges. Instead, we found that the Ci-VSP mutant R253K-R254K showed a drastic impairment in the voltage dependence of the phosphatase activity (Fig. 6 A). As before, we confirmed that Ci-VSP was expressed along with KCNQ2/3 (Fig. 6 B). The ON sensing currents from the mutant Ci-VSP R253K-R254K-C363S did not show major differences from those of either the C363S or the R253A-R254A-C363S mutants (Fig. 6 C). However, the OFF sensing currents exhibited faster kinetics (Fig. 6 A) when compared with the mutant C363S (Fig. 2 C). It has been shown, theoretically (Green, 2005) and experimentally (Tang et al., 2007, 2008, 2009), that the guanidinium group of arginines strongly interacts with phosphate groups in membranes. Furthermore, recently published articles have shown that the N terminus of PTEN specifically binds $PI(4,5)P_2$ (Campbell et al., 2003; Walker et al., 2004; Redfern et al., 2008), indicating that the PBM of PTEN forms a binding site for this phosphoinositide phospholipid. In the case of Ci-VSP, our result indicates that lysine cannot replace

KCNQ2/3

A

1 μA
20 ms

C

1 μA
20 ms

+TEA⁺

Ci-VSP-R253A-R254A + KCNQ2/3

B

0.2 μA
20 ms

D

0.1 μA
20 ms

+TEA⁺
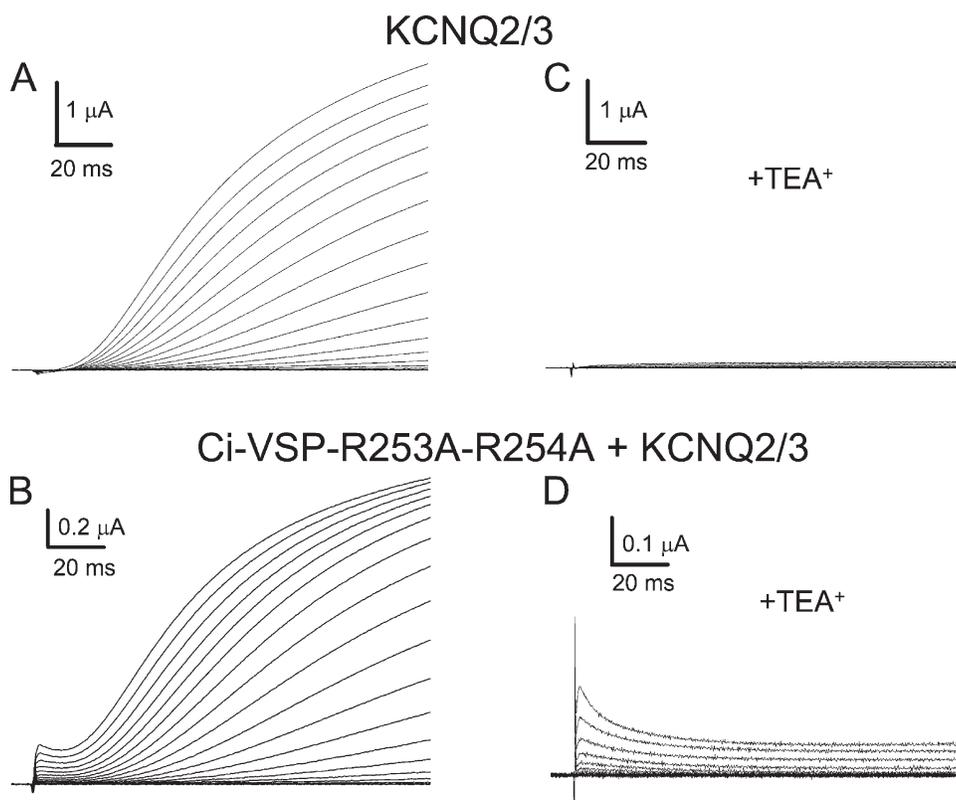
Figure 5. Block of ionic current from oocytes expressing KCNQ2/KCNQ3 alone (A and C) and coexpressed with Ci-VSP R253A-R254A (B and D). (A) The K^+ currents of KCNQ2/3 when depolarizing the oocytes. (B) Coexpression of the mutant R253A-R254A and KCNQ2/3 yielded K^+ currents and, in addition, sensing currents from Ci-VSP. (C) Oocyte expressing KCQ2/3 alone shows no currents after replacing K^+ for TEA^+ in the internal and external solutions during cut-open recording. (D) In contrast, when KCNQ2/3 was coexpressed with the R253A-R254A mutant of Ci-VSP, TEA treatment showed the transient current characteristic of the sensing currents of Ci-VSP. Pulses range from -100 to $+100$ mV. HP = -90 mV.
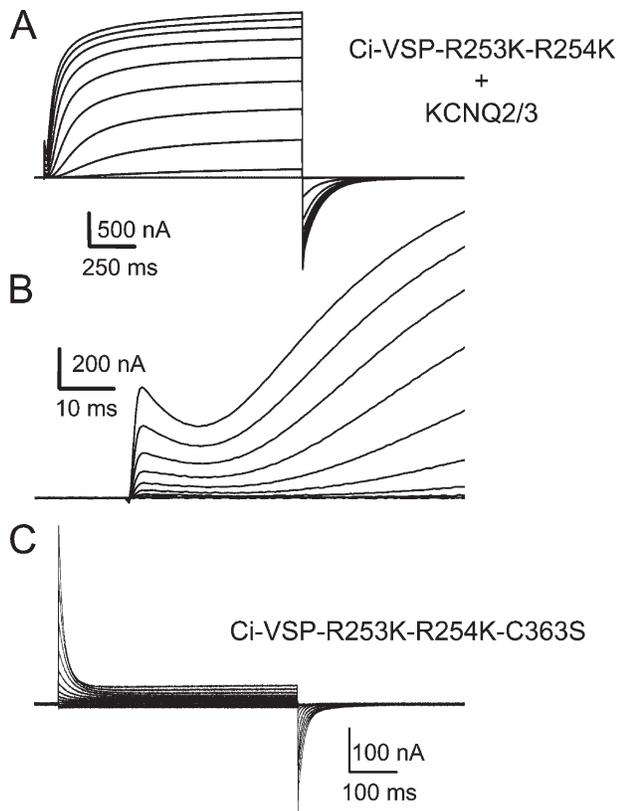
Figure 6. (A) The ionic current through KCNQ2/3 was not decreased after depolarization when coexpressed with the mutant R253K-R254K. (B) Ci-VSP is shown to be expressed because its sensing currents can be observed immediately after depolarization. (C) The phosphatase-inactivated mutant R253K-R254K-C363S was expressed alone to study sensing currents. The R253K-R254K-C363S mutant exhibits ON sensing currents that look very similar to those observed from the C363S mutant, although the OFF currents did not display the typical change in mode, observed with the C363S mutant (see Fig. 3). Pulses range from -100 to $+100$ mV. HP = -90 mV for ionic currents and -60 mV for sensing currents.

arginines in evoking voltage-dependent phosphatase activity. These combined observations strongly suggest that the interaction of the PBM of Ci-VSP with the membrane is mediated by an arginine-specific interaction rather than a purely electrostatic one. Consistently, this leads us to propose that the PBM is likely to form a structured binding site for phosphatidylinositol phospholipids. However, as we have not really measured binding, we cannot exclude the possibility that the replacement of arginines disrupts coupling by a structural change in the PD.

The arginines at positions 245 and 246 are important for coupling voltage to the PD activity

The pair of arginines in positions 253 and 254 is homologous to arginines 14 and 15 in PTEN (Fig. 1). However, an important difference between Ci-VSP and PTEN is the presence of another pair of arginines (R245 and R246) in the PBM. In contrast, the homologous positions
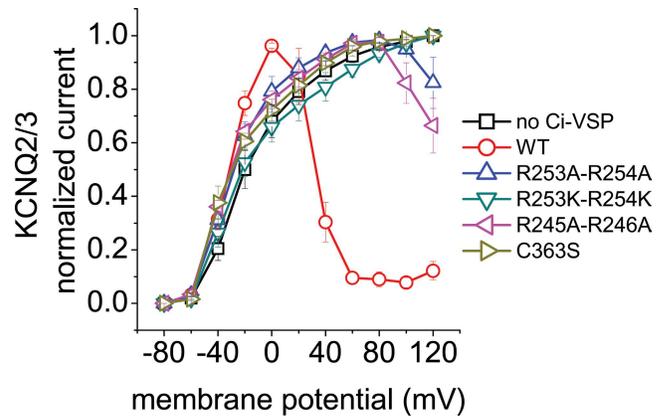
Figure 7. The activity of the Ci-VSP expressed as the average of the amplitude of the KCNQ2/3 current after 1.5 s of depolarization normalized to the maximum current. When compared with the C363S mutant, the only values significantly different are those from the R253A-R254A-C363S mutant and the R245A-R246A mutant for potentials above $+100$ and $+80$ mV, respectively.

in PTEN are occupied by a lysine and a glutamate. Replacing arginines 245 and 246 with either alanines or glutamines produces OFF sensing currents, which are clearly faster than those from the mutant C363S, but slower than those of the R253A-R254A-C363S mutant at potentials above 100 mV (Figs. 2 D and 3). This indicates that arginines 245–246 may also be involved in the interaction between the PBM and the membrane. Consistent with this idea, we found that the mutation of these residues to alanines also impairs the voltage-dependent phosphatase activity of Ci-VSP, as reported by the potassium current of KCNQ2/3 (Figs. 4 D and 7). The effect of the mutation R245A-R246A on the voltage-dependent phosphatase activity was qualitatively similar to the one caused by the mutation R253A-R254A. However, the decrease in phosphatase activity of the R245A-R246A mutant was less dramatic than the decrease produced by the R253A-R254A mutant, suggesting that the arginines 245–246 may not be sufficient to efficiently couple the sensor with the phosphatase.

The interaction of the PBM restricts the movement of the VSD

Recently, we have shown that prolonged polarization of the membrane at positive potentials leads the VSD of Ci-VSP into a relaxed state (Villalba-Galea et al., 2008). Also, we have shown the relaxation of the VSD to be a conformational change that is transmitted along the whole S4 segment of Ci-VSP (Villalba-Galea et al., 2009). In light of these findings, we hypothesize that any interaction that influences the movement of the sensing charges may have a direct impact on the relaxation of the VSD. By replacing the glycine at position 214 by a cysteine, it is possible to attach a fluorescent probe in that site (TMRM) and thus monitor the movement of the S4 segment from the resting to the active states (Kohout et al., 2008; Villalba-Galea
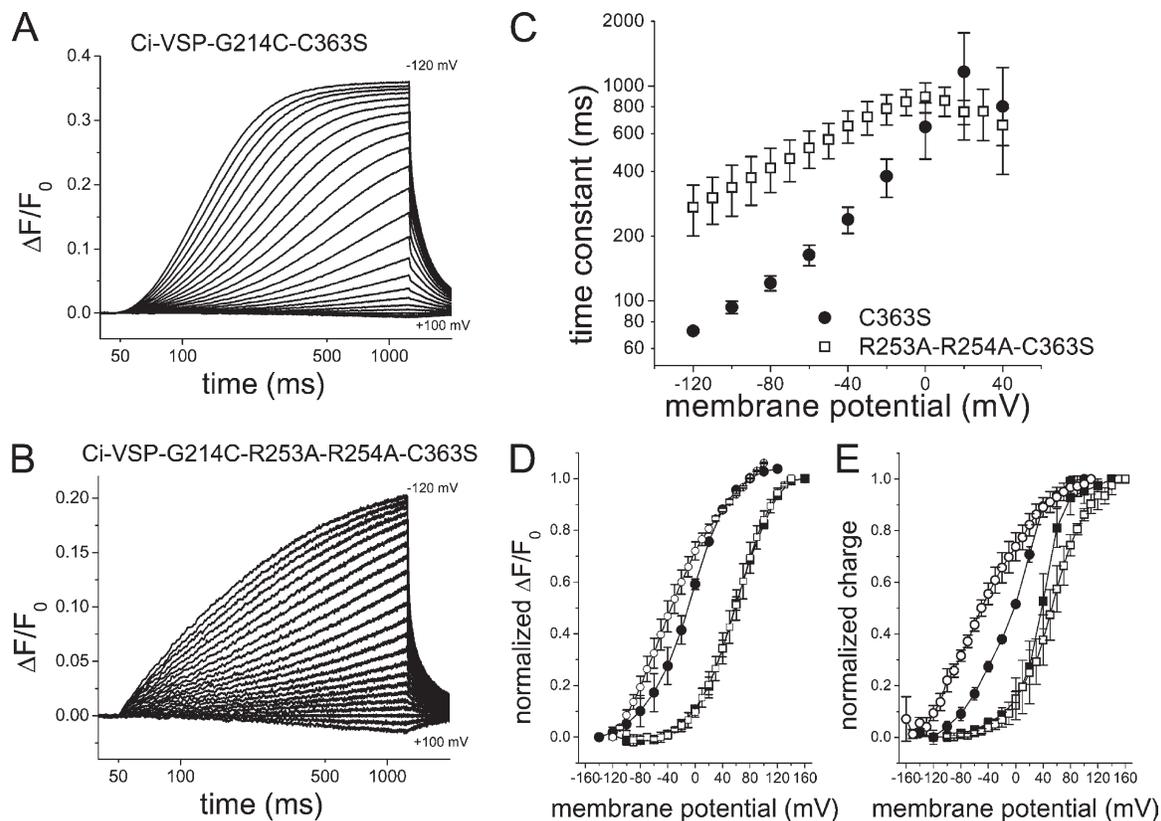
Figure 8. The mutants G214C-C363S and G214C-R253A-R254A-C363S were expressed in oocytes and labeled with TMRM. Changes in the fluorescence emission of TMRM observed from the mutant G214C-R253A-R254A-C363S (B) were slower than those observed from the mutant G214C-C363S (A) when a conditioning pulse to +80 mV was applied for 5 s to produce the relaxation of the VSD. The differences in the time constant of the fluorescence were significant for all the potentials tested below −10 mV (C), reaching up to fourfold below −100 mV. The voltage dependence of the normalized amplitude of the fluorescence change showed no difference between G214C-C363S (D; filled squares) and G214C-R253A-R254A-C363S (D; open squares) when the HP = −60 mV. However, the amplitude of the fluorescence change from the G214C-R253A-R254A-C363S mutant showed a clear shift toward negative potentials (D; open circles) with respect to the G214C-C363S mutant (D; filled circles) when the membrane was conditioned to +80 mV for 5 s. Likewise, the voltage dependence of the charge movement displays a small shift toward positive potentials for the mutant G214C-R253A-R254A-C363S (E; open squares) with respect to the mutant G214C-C363S (E; filled squares). Prepulsing the membrane to +80 mV for 5 s produces a deeper relaxation of the VSD of the mutant G214C-R253A-R254A-C363S, as reflected by a larger shift toward negative potentials (E; open circles) with respect to the shift observed in the mutant G214C-C363S (E; filled circles).

et al., 2008), and from the resting to the active and relaxed states by the quenching changes of the probe (Villalba-Galea et al., 2008). Recordings of fluorescence from the mutant G214C-R253A-R254A-C363S displayed no difference when the HP was −60 mV and positive depolarizing pulses were applied (unpublished data). However, we found that the recovery from relaxation of the VSD is significantly slower in the G214C-R253A-R254A-C363S mutant (Fig. 8 B) compared with the G214C-C363S (Fig. 8 A). When a conditioning prepulse to +80 mV was applied for 5 s to populate the relaxed state, during the subsequent repolarization, the fluorescence traces of the mutant G214C-R253A-R254A-C363S displayed average time constants up to fivefold slower than those observed in the mutant G214C-C363S for all the negative potentials tested (Fig. 8 C). As the kinetics of the fluorescence signal reflects the recovery from the relaxed state (Villalba-Galea et al., 2008), these observations are consistent with the

idea that the PBM interacts with the membrane and restricts the movement of the S4 segment. At the same time, the VSD of the G214C-R253A-R254A-C363S mutant undergoes a shift into a deeper relaxation state during prolonged depolarization, as measured with the fluorescence changes and the net charge movement. This is shown as a larger shift toward negative potentials of the voltage dependence of the fluorescence and the charge movement in the mutant G214C-R253A-R254A-C363S (Fig. 8, D and E, respectively) compared with the mutant G214C-C363S.

DISCUSSION

The striking sequence similarity of the PD of Ci-VSP with PTEN has prompted detailed research on Ci-VSP. For instance, Iwasaki et al. (2008) showed that essential residues are conserved in the active sites of both phosphatases. In the present work, we attempt to understand

how the coupling of the VSD and the PD occurs, taking advantage of the similarities between the N terminus of PTEN and the linker between the VSD and the PD of Ci-VSP. The binding of PTEN onto membranes is a key step in the mechanism of its activity. It is known that the N terminus of PTEN contains four positively charged residues that are essential for its binding to the lipid bilayer (Das et al., 2003). This charge-containing motif is referred to as PBM. In recent years, several studies have shown that the PBM specifically binds phosphoinositol phospholipids, in particular $PI(4,5)P_2$ (Campbell et al., 2003; Iijima et al., 2004; Walker et al., 2004; Redfern et al., 2008). Three of these residues are identically conserved in the linker between the VSD and the PD of Ci-VSP. This linker shares 50% identity with the N terminus of PTEN and overall homology showing above 30% identity. Although the importance of the PBM in the binding of PTEN to the membrane has been accepted, the role of the N terminus of PTEN on the catalytic activity of this phosphatase has been controversial. Recently, it has been proposed by Iijima et al. (2004) that the first 16 amino acids of PTEN occlude the active site of PTEN. Then, upon binding to $PI(4,5)P_2$, the active site is released, enabling catalysis (Iijima et al., 2004). On the other hand, previous work has shown that the binding to phosphoinositides enhances the activity of PTEN and even produces changes in the selectivity for its target (Campbell et al., 2003). These changes have been proposed to be linked to binding-induced conformational changes in the phosphatase (Campbell et al., 2003; Redfern et al., 2008). However, the catalytic capability of PTEN does not depend on conformational changes induced by the binding itself because the deletion of the residues 1–16 in PTEN can dephosphorylate soluble substrates (Iijima et al., 2004). In any of these cases, the key process in the activity of PTEN is the binding of the PBM on to the membrane. For these reasons, and others to follow, we propose that the amino acids located between positions 240 and 255 form, as in PTEN, a PBM.

In Ci-VSP, the activation of the phosphatase activity takes place after depolarization (Murata et al., 2005; Murata and Okamura, 2007; Kohout et al., 2008). The hypothesis is that the movement of the S4 segment brings the PD close to the membrane, triggering the activity of the PD (Murata and Okamura, 2007). Our findings agree with this idea; however, they provide insight on the coupling mechanism. Because the PD is constitutively active (Murata et al., 2005), the mechanism of activation of the phosphatase relies on the position of the PD with respect to the substrate. Based on our observations, we hypothesize that the displacement of the S4 during depolarization moves the PBM, allowing it to bind to the membrane locating the PD in optimal orientation and distance from its target for catalysis.

As we show in this work, the OFF sensing currents of the C363S display a strong dependence on the potential at which the membrane is depolarized (Fig. 3). For pulses below +60 mV, the OFF currents display monotonic kinetics, while pulsing above +60 mV the OFF currents have a more complex kinetics displaying a plateau before the final decay of the current. This change in mode was not observed in the R253A-R254A-C363S mutant. Instead, the rate of the OFF currents observed for the R253A-R254A-C363S mutant shows less than twofold change with respect to the membrane potential reached during the preceding depolarization pulse. In contrast, for the C363S mutant, a clear increase (over fourfold) in the time constant of the decay of the sensing current during repolarization is observed depending on the potential during the depolarizing pulse. When depolarizing above +60 mV, the rate of the OFF current did not change. This observation suggests that the PBM locks the PD in an active conformation, presumably mechanically isolating it from the VSD. It is important to point out that this group of observations reflects a different situation to what has been reported with the VSP from *Danio rerio*. The inactivation of the phosphatase activity by mutating the cysteine 302 to a serine (C302S) in the VSP from *Danio rerio* (which is equivalent to mutation C363S in Ci-VSP) speeds up the ON and OFF sensing currents when pulsing to positive potentials (Hossain et al., 2008). It should be pointed out that our comparison of the kinetic of the sensing currents is made on mutations using a common background, the inactive mutant C363S.

The results obtained when replacing the first pair (245, 246) of arginines in the PBM were not anticipated because the first pair of arginines is not present in PTEN. In 2005, Okamura and collaborators showed that the deletion of amino acids 240–247 (segment ΔN-L) disrupts the voltage-activated phosphatase activity in Ci-VSP, but not when deleting the segment 248–255, as reported by the activity of either GIRK2 or KCNQ2/3 (Murata et al., 2005). Essentially, both segments display the same number of charged residues, one lysine and two arginines. In contrast, when these arginines are replaced by alanines, a lower level of voltage-dependent phosphatase activity is observed when compared with the WT Ci-VSP. An explanation to reconcile these observations would be that the PBM forms a structured motif that is essential for the activity of Ci-VSP. In this case, deletions in the motif could produce structural distortions that would decrease or abolish the activity of the phosphatase. Furthermore, the replacement of the second pair of arginines for lysines yields a much lower level of activity than observed with the mutant of the first pair. These observations are consistent with the existence of a binding site for phospholipids formed by the PBM of Ci-VSP. Recently, it has been shown by the group of A. Gericke that the iso-coulombic mutations in the motif NKR in the PBM of PTEN (residues 12–14) have a strong detrimental impact in the ability of this

phosphatase to bind to $Pi(4,5)P_2$-containing membranes (Redfern, R.E., A.D. Hill, A. Ross, and A. Gericke. 2009. The lysine at position 13 of pten's N-terminus is necessary for its preferred interaction with $Pi(4,5) P_2$. *Biophys J.* Abstr. 547a). Furthermore, the "scrambling" of the amino acids in the sequence NKR impairs the binding of PTEN to the membrane (Redfern, R.E., A.D. Hill, A. Ross, and A. Gericke. 2009. The lysine at position 13 of pten's N-terminus is necessary for its preferred interaction with $Pi(4,5) P_2$. *Biophys J.* Abstr. 547a). These observations strongly suggest that any modification in the order or identity of the amino acid forming the binding site will produce a distortion of the conformation of this binding site impairing the coupling between the VSD and the PD. In addition, the existence of specific interactions between the phosphate groups of phospholipids and arginines, forming stable complexes that are stronger than those predicted from electrostatics interactions (Green, 2005), has been proposed. Along with these ideas, polyarginine peptides are known to be able to cross lipid bilayers very efficiently (Mitchell et al., 2000; Tang et al., 2007, 2008, 2009; Yi et al., 2007). These considerations lead us to propose that the PBM forms a binding site, presumably specific for $PI(4,5)P_2$. This putative binding site would be made up of at least four key residues: arginines 245, 246, 253, and 254. The data indicate that arginines 253 and 254 are essential, and we speculate that they interact with the phospholipids, whereas arginines 245 and 246 are necessary, but we have no equivalent data to support or disprove that they also might make direct contact with the phospholipids. We are aware that before a direct interaction of any of the four arginines and the phospholipids is proved with binding experiments, our proposals remain speculative.

The changes in kinetics of the gating current tails are consistent with the idea that the four arginines of the PBM, and in particular those in positions 253 and 254, are essential for the immobilization of the PBM. On the other hand, the ionic current mediated by KCNQ2/3 showed an initial decrease in their amplitude when depolarized above +80 mV in the presence of the mutant R245A-R246A. After this phase, the current seemed to reach a steady value. We find this observation to be consistent with the idea that the putative binding site of the PBM may require the presence of arginines 245 and 246 for optimal performance of the phosphatase. If one considers again the idea that the PBM binds to $PI(4,5)P_2$, it is plausible that the dephosphorylation of $PI(4,5)P_2$ will eventually destabilize the interaction between the PBM and the membrane. In fact, the mutant R245A-R246A, which has an active PD, displayed faster OFF sensing currents than those observed with the mutant R245Q-R246Q-C363S, which has an inactive PD, at a potential above +80 mV. Furthermore, we have previously reported that the VSD of Ci-VSP undergoes relaxation at positive membrane potentials (Villalba-Galea et al.,

2008). The effect of the relaxation can be observed at both ends of the S4 segment (Villalba-Galea et al., 2009). Therefore, it is likely that after the PBM is bound to the membrane and the PD is placed in position for catalysis, the conformational change of the S4 segment during relaxation may affect, by mechanical stress, the interaction between the PBM and the membrane. We speculate that arginines 245 and 246 have an accessory function in the putative binding site, increasing the stability of the bound conformation, therefore locking or securing the binding of the PBM, mechanically isolating them and the PD from the VSD.

The binding of the PBM also has important consequences in the behavior of the VSD. Because the relaxation of the VSD is transmitted along the whole S4 segment, the binding of the PBM could affect the relaxation of the VSD. In the current work, we showed that the fluorescence emission from TMRM, covalently bound to the S4 in position 214 of the mutant G214C-R253A-R254A-C363S, unquenched significantly slower during the recovery from relaxation (Fig. 8 B) than the fluorescence observed from the mutant G214C C363S (Fig. 8 A). A simple explanation for this observation is that during positive pulses, the electric field moves the S4, leading to the binding of the PBM. Then, upon binding, the S4 movement is restricted, affecting the relaxation as well. The alternative scenario, when there is no binding by the PBM, as seen with Ci-VSP bearing the R253A-R254A mutation, the unbound PBM will increase the freedom of the S4 to move. In this latter situation, upon a prolonged polarization at positive potentials, the outcome will be a deeper relaxation, seen as a larger shift in the voltage dependence of Q-V and an increase in the time required to recover to the resting state. Both conditions are observed in the G214C-R253A-R254A-C363S mutant. These observations strongly suggest that the binding of the PBM constrains the movement of the S4 segment during depolarization of the plasma membrane.

In conclusion, the movement of the S4 segment activates the phosphatase in Ci-VSP by coupling this movement to the PD via the PBM that requires at least arginines 254 and 255. We propose that these arginines interact with the phospholipids, placing the PD in optimal orientation for catalysis. As a consequence, the binding of the PBM also restricts the movement of the VSD, preventing it from undergoing a deeper relaxation.

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