Ci-VSP Is a Depolarization-activated Phosphatidylinositol-4,5-bisphosphate and Phosphatidylinositol-3,4,5-trisphosphate 5′-Phosphatase*5

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Christian R. Halaszovich, Daniela N. Schreiber, and Dominik Oliver1

From the Department of Neurophysiology, Institute of Physiology and Pathophysiology, Philipps-Universität Marburg, 35037 Marburg, Germany

Phosphoinositides are membrane-delimited regulators of protein function and control many different cellular targets. The differentially phosphorylated isoforms have distinct concentrations in various subcellular membranes, which can change dynamically in response to cellular signaling events. Maintenance and dynamics of phosphoinositide levels involve a complex set of enzymes, among them phospholipases and lipid kinases and phosphatases. Recently, a novel type of phosphoinositide-converting protein (termed Ci-VSP) that contains a voltage sensor domain was isolated. It was already shown that Ci-VSP can alter phosphoinositide levels in a voltage-dependent manner. However, the exact enzymatic reaction catalyzed by Ci-VSP is not known. We used fluorescent phosphoinositide-binding probes and total internal reflection microscopy together with patch-clamp measurements from living cells to delineate substrates and products of Ci-VSP. Upon activation of Ci-VSP by membrane depolarization, membrane association of phosphoinositide-converting enzymes to the plasma membrane (6, 7). Although irreversible manipulation of [PI] is particularly important because effects of long-term alteration of PI levels by overexpression or knockdown of PI-converting enzymes (e.g. Refs. 4 and 5) may be difficult to interpret. Thus, effects may be ambiguous because of different PI-dependent processes occurring on multiple time scales. Furthermore, homeostatic mechanisms and toxicity of long-term PI changes may limit the manipulations that can be utilized experimentally. So far, manipulation of PI levels remained difficult.

Recently, an elegant approach for rapid disturbance of [PI] was developed based on chemically induced recruitment of PI-converting enzymes to the plasma membrane (6, 7). Although being extremely powerful for the identification of PI-dependent processes, a disadvantage of this method may be the inherently irreversible change in [PI].

Via dynamic concentration changes that result from breakdown or interconversion of PI isoforms, PIs act as bona fide second messengers (reviewed in Ref. 1). PI dynamics can occur in response to a huge variety of cellular events, including the activation of plasma membrane receptors.

Knowledge of cellular PI signaling has dramatically increased over the last years. Much of this progress derives from the characterization of a complex set of enzymes that generate, interconvert, and degrade the various PI isoforms (2) and from the introduction of genetically encoded lipid probes that allow the tracking of the dynamics of specific PI isoforms by live cell fluorescence microscopy (3).

Further research into two aspects of PI signaling appears particularly promising. First, PI homeostasis and dynamic modulation require the tight spatiotemporal regulation of lipid kinases and phosphatases, which is largely unexplored. The second issue concerns analysis of downstream targets of PI signaling. Because cellular events that change [PI] usually involve other signaling pathways, defining the role of PIs is often not straightforward.

Both aspects of PI-related cell biology should benefit from methods for isoform-specific alteration of [PI]. Rapid and reversible manipulation of [PI] is particularly important because effects of long-term alteration of PI levels by overexpression or knockdown of PI-converting enzymes (e.g. Refs. 4 and 5) may be difficult to interpret. Thus, effects may be ambiguous because of different PI-dependent processes occurring on multiple time scales. Furthermore, homeostatic mechanisms and toxicity of long-term PI changes may limit the manipulations that can be utilized experimentally. So far, manipulation of PI levels remained difficult.

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1 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–4 and Table I.

1 To whom correspondence should be addressed: Inst. of Physiology and Pathophysiology, Deutschhaussstr. 2, 35037 Marburg, Germany. Tel.: 49-6421-286-6444; Fax: 49-6421-286-2306; E-mail: oliverd@staff.uni-marburg.de.

2 The abbreviations used are: PIs, phosphoinositides; PI(3,4,5)P3, phosphatidylinositol-3,4,5-trisphosphate; VSD, voltage sensor domain; TIRF, total internal reflection; CHO, Chinese hamster ovary; mRFP, monomeric red fluorescent protein; GFP, green fluorescent protein; YFP, yellow fluorescent protein; AMP-PCP, adenosine 5′-(β,γ-methylene)triphosphate; PLCγ3, phospholipase Cγ3, pleckstrin homology domain; OSBP, oxysterol-binding protein; PIP5K, phosphatidylinositol-4-phosphate 5-kinase; PAO, phenylarsine oxide; PI3K, phosphatidylinositol 3-kinase.

*49-6421-286-6444; Fax: 49-6421-286-2306; E-mail: oliverd@staff.uni-marburg.de.

Ci-VSP upon depolarization has been suggested (9). For use as an analytical tool, it is essential to understand the exact enzymatic activity because any PI isoform affected by Ci-VSP may have its own cellular signaling role. So far, this issue has not been addressed, and stereospecificity is unknown.

We monitored the concentrations of possible substrates and products of Ci-VSP with fluorescently labeled PI probes by imaging changes in their membrane association with total internal reflection (TIRF) microscopy. Our data indicate that in vivo, Ci-VSP is a depolarization-activated PI 5′-phosphatase that converts PI(4,5)P₂ to PI(4)P and PI(3,4,5)P₃ to PI(3,4)P₂.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection**—Chinese hamster ovary (CHO) cells were plated onto glass bottom dishes (WillCo Wells B. V., Amsterdam, The Netherlands) and transfected with jetPEI transfection reagent (Polyplus Transfection, Illkirch, France). Detailed information on all vector constructs used is given in supplemental Table I. All experiments involving Ci-VSP reported here were done on cells selected for membrane localization of mRFP-Ci-VSP and corresponding gating currents.

**Fluorescence Microscopy**—TIRF imaging was done with a BX51WI upright microscope (Olympus, Hamburg, Germany) equipped with a TIRF condenser (numerical aperture of 1.45; BX51WI microscope through fiber optics). GFP and RFP fluorescence illumination was achieved with a monochromator (TILL Photonics GmbH, Gräfelfing, Germany). Wide-field fluorescence illumination was achieved with a monochromator (Polychrome IV, TILL Photonics GmbH) coupled to the BX51WI microscope through fiber optics. GFP and RFP fluorescence was excited at 488 and 569 nm, respectively, and YFP at 510 nm. The laser shutter for TIRF illumination, the monochromatic light source, and image acquisition were controlled by TILLvisION software (TILL Photonics GmbH). The sample interval for image acquisition was 3 s. Confocal visualization of the membrane localization of mRFP-Ci-VSP was done on a Leica TCS SP2 confocal microscope.

**Electrophysiology**—Cells were whole cell voltage-clamped with an EPC-10 amplifier controlled by PatchMaster software (HEKA, Lambrecht, Germany). For measuring gating currents, linear leak and capacitance were subtracted using a P-/H11002 protocol (leak holding potential of −60 mV). To synchronize electrophysiological and optical measurements, the EPC-10 amplifier was triggered from the TILL imaging system. Patch pipettes were pulled from quartz glass to an open pipette resistance of 1.5–4.0 megohms when filled with intracellular solution containing 135 mM KCl, 2.5 mM MgCl₂, 2.41 mM CaCl₂, 5 mM EGTA, 5 mM HEPES, and 3 mM Na₂ATP, pH 7.3 (with KOH). In some experiments, ATP was replaced with AMP-PCP, or ATP was omitted. In the latter case, the MgCl₂ concentration was adapted to keep the free Mg²⁺ concentration constant. The extracellular solution contained 144 mM NaCl, 5.8 mM KCl, 0.7 mM NaH₂PO₄, 5.6 mM glucose, 1.3 mM CaCl₂, 0.9 mM MgCl₂, and 10 mM HEPES, pH 7.4 (with NaOH). Experiments were performed at room temperature (≈24°C).

**RESULTS**

**Degradation of PI(4,5)P₂ by Ci-VSP in Living Mammalian Cells**—To analyze the enzymatic activity of Ci-VSP in vivo, we transfected mRFP-tagged Ci-VSP into CHO cells. Localization of RFP indicated effective targeting of Ci-VSP to the plasma membrane (Fig. 1A). In voltage-clamped RFP-positive CHO cells, voltage steps induced gating currents (Fig. 1B) similar to the currents generated by the VSD of Ci-VSP in Xenopus oocytes (8, 9). The gating currents thus indicated a functional VSD in our expression system.

The effect of Ci-VSP on membrane [PI] was assessed by measuring membrane association of genetically encoded GFP-tagged PI-binding domains (3). Briefly, the fraction of membrane-bound probe depends on the particular [PI] such that an increase in [PI] leads to translocation from the cytosol to the cell membrane, whereas a decrease induces dissociation from the membrane. We monitored changes in membrane association of PI probes using TIRF microscopy. In this technique, an evanescent field originating at the interface between glass and aqueous solution decays exponentially into the cytoplasm (length constant of <100 nm) and selectively excites fluorophores at the cell membrane attached to the glass substrate. Thus, fluorescence emission reports the amount of membrane-bound fluorophores with only a minor contribution from cytosolic fluorophores, and changes in fluorescence intensity directly reflect dynamics of [PI]. Cells under observation were patch-clamped, enabling activation of Ci-VSP with voltage steps.

We first examined the impact of Ci-VSP on the concentration of its proposed substrate, PI(4,5)P₂, using the PI(4,5)P₂-specific probe PLCδ₁-PH-GFP (10, 11). Upon depolarization, membrane fluorescence declined (Fig. 1, C and D), indicating dissociation of the probe from the membrane due to depletion of PI(4,5)P₂. Upon repolarization, fluorescence recovered, indicating that [PI(4,5)P₂] returned to the initial concentration. In contrast, depolarization failed to affect membrane fluorescence when PLCδ₁-PH-GFP was monitored in the absence of Ci-VSP or when Ci-VSP was coexpressed with constitutively membrane-anchored Lyn-GFP (Fig. 1D) (12). These control experiments confirmed that TIRF signals reported Ci-VSP-depend-
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ent changes in plasma membrane \([\text{PI}(4,5)\text{P}_2]\). These changes resulted from enzymatic activity intrinsic to the molecule’s cytoplasmic phosphatase domain because mutation of a cysteine (C363S) in the catalytic CX$_2$R motif common to all homologous lipid phosphatases (8, 13) completely abolished voltage-dependent changes in membrane fluorescence (Fig. 1E).

As an additional test for the reliability of our approach, we measured signals from PLC$_{\delta_1}$-PH-GFP in response to activation of the G$_q$-coupled muscarinic M1 acetylcholine receptor. Activation of the M1 acetylcholine receptor displaces PLC$_{\delta_1}$-PH-GFP from the membrane due to depletion of PI(4,5)P$_2$ and generation of inositol 1,4,5-trisphosphate (14, 15). Indeed, application of a muscarinic agonist induced a robust decrease in TIRF, validating our experimental approach (supplemental Fig. 1).

PI-binding domains may often not be perfectly PI isoform-specific (16). To scrutinize the conclusion that Ci-VSP-induced changes in membrane association of PLC$_{\delta_1}$-PH reported actual changes in \([\text{PI}(4,5)\text{P}_2]\), we used the C terminus of the tubby protein, a structurally unrelated PI(4,5)P$_2$-binding domain (17). As shown in Fig. 1D, depolarization of cells coexpressing tubby-GFP and Ci-VSP induced strong and reversible reduction of membrane fluorescence, confirming the consumption of PI(4,5)P$_2$ by Ci-VSP.

To characterize the voltage dependence of Ci-VSP enzymatic activity, PLC$_{\delta_1}$-PH TIRF signals were recorded at different potentials (Fig. 2). The reduction of membrane fluorescence and thus the degree of PI(4,5)P$_2$ breakdown increased with more depolarized potentials. Fitting the fluorescence-voltage relation with a Boltzmann function yielded half-maximal fluorescence loss at \(+40.2 \pm 3.5\) mV and steepness (s) of \(17.9 \pm 1.7\) mV, similar to the voltage dependence of the VSD derived from gating currents (8). Thus, these results are consistent with a previously proposed model of Ci-VSP, where depolarization acts on the membrane-spanning VSD and thereby activates the PI(4,5)P$_2$-degrading enzymatic activity of the cytoplasmic domain (9).

Interestingly, voltage-dependent dissociation of the PI(4,5)P$_2$ probe tubby-Cterm occurred at less depolarized potentials (\(V_{1/2} = -4.6 \pm 2.9\) mV, \(s = 12.1 \pm 0.9\) mV) (Fig. 2B). This indicated that unbinding of tubby-Cterm occurred at less complete depletion of PI(4,5)P$_2$ and suggested a lower binding affinity compared with PLC$_{\delta_1}$-PH.

Voltage Activation of Ci-VSP Generates Excess PI(4)P—It was suggested that degradation of PI(4,5)P$_2$ results from phosphatase activity of Ci-VSP (9). However, dephosphorylation has not been shown directly, and it is unknown whether the 4’- or 5’-phosphate is cleaved.

To identify the exact enzymatic reaction, we sought to measure concentration changes in the putative product of PI(4,5)P$_2$ dephosphorylation using PI(4)P-specific OSBP-PH (18, 19). In cells that coexpressed Ci-VSP and OSBP-PH-GFP, membrane fluorescence increased when the cells were depolarized, indicating generation of PI(4)P upon activation of Ci-VSP. Upon repolarization to \(-60\) mV, membrane fluorescence and thus \([\text{PI}(4)\text{P}]\) returned to basal levels (Fig. 3A). When OSBP-PH was

![FIGURE 1. Depletion of PI(4,5)P$_2$ by activation of Ci-VSP. A, confocal images of a CHO cell coexpressing mRFP-Ci-VSP (red, upper panel) and constitutively plasma membrane-localized Lyn-GFP (green, lower panel). Colocalization of Ci-VSP with Lyn-GFP indicated effective targeting of Ci-VSP to the plasma membrane. B, whole cell gating current measured from a cell transfected with mRFP-Ci-VSP and PLC$_{\delta_1}$-PH-GFP (upper trace). Note the absence of gating currents measured from another cell without Ci-VSP expression (lower trace). Scale bar = 100 ms and 100 pA. C, representative images of voltage-dependent membrane association of PLC$_{\delta_1}$-PH-GFP obtained with TIRF microscopy versus epi-fluorescence from a cell coexpressed with mRFP-Ci-VSP. D, TIRF microscopy fluorescence signals measured in response to step depolarization in cells expressing mRFP-Ci-VSP plus the PI(4,5)P$_2$ probes PLC$_{\delta_1}$-PH-GFP, or tubby-Cterm-GFP, or constitutively membrane-localized Lyn-GFP. No voltage-dependent changes in membrane fluorescence were observed in the absence of Ci-VSP (lower traces). E, mutation of a cysteine in the catalytic CX$_2$R motif of CI-VSP abolishes any voltage-dependent changes in membrane association of PLC$_{\delta_1}$-PH-GFP. Mean responses to depolarization to \(+80\) mV are shown from cells coexpressing PLC$_{\delta_1}$-PH-GFP with either wild-type (wt) Ci-VSP (n = 6) or Ci-VSP(C363S) (n = 7). Correct processing of CI-VSP(C363S) and function of the VSD were verified for all cells by measuring gating currents (inset; scale bar = 100 ms and 100 pA).]
monitored in the absence of Ci-VSP, voltage had no effect on the TIRF signal (data not shown).

Similar to PI(4,5)P₂ degradation, elevation of [PI(4)P] as deduced from changes in OSBP-PH-GFP fluorescence was more pronounced at stronger depolarization (Fig. 3, A and B). The fluorescence-voltage relation was characterized by a $V_{1/2}$ of 11.0 ± 1.1 mV and a steepness (s) of 20.7 ± 2.3 mV, similar to the voltage dependence of PI(4,5)P₂ degradation by Ci-VSP. The moderate difference in the voltage dependence of PLC₅-PH most likely reflects distinct affinities of both PI probes for their respective lipid ligands (9). Thus, the findings are consistent with synchronous and quantitatively reciprocal changes in both PI isoforms, suggesting that Ci-VSP acts as a PI(4,5)P₂ phosphatase at the 5'-position and thereby generates PI(4)P.

Recovery of PI(4,5)P₂ and PI(4)P Levels Depends on PI Kinase Activity—Given that Ci-VSP dephosphorylates PI(4,5)P₂ to PI(4)P, the observed recovery of [PI(4,5)P₂] upon repolarization may result from rephosphorylation of PI(4)P. Recovery would then report the activity of endogenous PI(4)P 5-kinase (PIPK) converting PI(4)P to PI(4,5)P₂. In contrast, if PI(4,5)P₂ depletion by Ci-VSP resulted from a different enzymatic activity (i.e. Ci-VSP acting as a kinase or lipase), replenishment of PI(4,5)P₂ would depend on phosphatase activity or require resynthesis of PI(4,5)P₂ from phosphatidylinositol, respectively. The latter involves sequential activity of PI 4-kinases and PIP5Ks, as examined in detail for refilling of PI(4,5)P₂ pools after depletion by PLC (20, 21).

To further probe for the involvement of PIP5Ks, we measured PI(4,5)P₂ and PI(4)P dynamics after removal of intracellular ATP and in the presence of a competitive kinase inhibitor. Thus, ATP was omitted from the pipette solution or replaced with its non-hydrolyzable analog AMP-PCP. Experiments were done 5 min after patch rupture, sufficient for equilibration of a readily diffusible solute like ATP between the cytosol and the pipette (24). When the patch pipette contained no ATP, recovery of [PI(4,5)P₂] as monitored by PLC₅-PH-GFP was incomplete and slowed in comparison with control experiments with
Pi(3,4,5)P3 Phosphatase Activity of Ci-VSP—Because the isolated phosphatase (PTEN) homology domain of Ci-VSP shows PI(3,4,5)P3 phosphatase activity in vitro (8), we next investigated whether Ci-VSP is also capable to hydrolyze PI(3,4,5)P3 in the living cell. [PI(3,4,5)P3] was monitored as membrane association of the GFP-tagged PI(3,4,5)P3-binding probes ARNO-PH-GFP (25, 26) and Btk-PH-GFP (27).

Under resting conditions, the PI(3,4,5)P3 level of the plasma membrane is usually too low to detect membrane association of the PI(3,4,5)P3 probes (data not shown; see Ref. 28). Therefore, we coexpressed a constitutively active mutant of the phosphatidylinositol 3-kinase 110-kDa subunit (PI3K(K227E)) (29), which induced robust membrane association of all PI(3,4,5)P3 probes, indicating increased basal [PI(3,4,5)P3]. For TIRF experiments, cells were selected that coexpressed mRFP-Ci-VSP, PI3K(K227E), and one of the GFP-labeled PI(3,4,5)P3 probes and that displayed clear membrane localization of GFP fluorescence. As a control, addition of the PI3K inhibitor wortmannin (0.2 μM) resulted in the release of Btk-PH-GFP from the membrane (supplemental Fig. 3), indicating that TIRF is a useful indicator of [PI(3,4,5)P3].

Upon depolarization, ARNO-PH-GFP and Btk-PH-GFP consistently dissociated from the membrane (Fig. 5A). This finding indicated degradation of PI(3,4,5)P3 by Ci-VSP, without revealing, however, the stereospecificity of dephosphorylation. Accordingly, mutation of the conserved cysteine in the CXXR motif of the cytoplasmic phosphatase domain (C363S) completely abolished dissociation of the PI(3,4,5)-binding domain Btk-PH-GFP (Fig. 5B).

Surprisingly, when using a third PI(3,4,5)P3-binding probe, Akt1-PH-GFP (30, 31), a different result was obtained: average membrane association increased upon activation of Ci-VSP (Fig. 5C). However, the observed changes were variable, as either increase or decrease was measured from different cells. In contrast to ARNO-PH and Btk-PH (26, 32), Akt1-PH also binds to PI(3,4,5)P3 with high affinity (16, 33). Therefore, increased membrane association may result from the conversion of PI(3,4,5)P3 to PI(3,4)P2. Some variability between cells is not inconsistent with this interpretation, as the behavior of the probe must depend on the initial relation between [PI(3,4,5)P3] and [PI(3,4)P2], which may vary, e.g. because of variable expression of PI3K. We therefore used another PI-binding probe, TAPP1-PH, which binds to PI(3,4)P2 with high specificity both in vitro and in the living cell (34–36). Upon coexpression with mRFP-Ci-VSP and PI3K(K227E), depolarization invariably induced a strong increase in membrane association of TAPP1-PH-YFP, indicative of robust generation of PI(3,4)P2 by Ci-VSP.

To examine whether Ci-VSP can dephosphorylate PI(3,4,5)P3 to PI(3,4)P2 under physiological conditions, we repeated these experiments without exogenous expression of PI3K. The plasma membrane concentration of PI(3,4,5)P3 was acutely elevated by application of recombinant human insulin-like growth factor I (purchased from Sigma), which activates cellular PI3K via endogenous insulin-like growth factor receptors (28). When Ci-VSP was coexpressed with Btk-PH-GFP, depolarization...
and PI(3,4)P$_2$ respectively, as determined by TIRF measurements (supplemental Fig. 4B). This indicates that Ci-VSP dephosphorylates endogenously produced PI(3,4,5)P$_3$ at the 5’-position, Ci-VSP generates two downstream PIs, PI(4)P and PI(3,4)P$_2$, respectively. Previous reports suggested that Ci-VSP may deplete both PI(4,5)P$_2$ (9) and PI(3,4,5)P$_3$ (8). Recently, Iwasaki _et al_. (37) used a biochemical approach to determine the catalytic activity of Ci-VSP. Consistent with the results presented here, the recombinant cytoplasmic domain of Ci-VSP was shown to dephosphorylate PI(4,5)P$_2$ at the 5’-position _in vitro_. However, although PI(3,4,5)P$_3$ cleavage was confirmed (37), the exact reaction has not been determined previously. Because the Ci-VSP catalytic domain has considerable homology to the PI(3,4,5)P$_3$ 3’-phosphatase PTEN (8), conversion of PI(3,4,5)P$_3$ to PI(4,5)P$_2$ was suggested (37). In contrast, we have found that PI(4,5)P$_2$ is depleted by Ci-VSP even at increased PI(3,4,5)P$_3$ availability (data not shown). Instead, PI(3,4)P$_2$ is produced, indicating cleavage at the 5’-phosphate group. This difference in the behavior of PTEN is not entirely surprising, given that another PTEN homolog, PLIP, has 5’-phosphatase activity for its preferred substrate, PI(5)P, despite high sequence conservation of the catalytic site (38). Also, the related SAC1-like polyspecific PI phosphatase domains that share the CX$_3$R motif have 5’-phosphatase activity (39).

In summary, PI dynamics induced by Ci-VSP may be quite complex, involving detectable concentration changes in four PI isoforms, yet Ci-VSP can be usefully employed to study PI(4,5)P$_2$ dependence of effector proteins, as already demonstrated for PI(4,5)P$_2$-gated K$^+$ channels (8, 9). Additionally, Ci-VSP may be well suited to define processes that are sensitive to PI(3,4)P$_2$ and PI(3,4,5)P$_3$, generating, however, very different downstream products.

**DISCUSSION**

During the last decade, the diversity and number of cellular processes recognized as being controlled by PIs have been growing rapidly. The introduction of fluorescent probes to monitor PI dynamics has contributed substantially to recent success in understanding PI signaling, yet complementary methods for the precise manipulation of [PI] are still not well developed. In this respect, a voltage-switched PI-converting enzyme seems close to being an ideal tool. However, understanding the enzymatic activity of Ci-VSP in the living cell is the essential prerequisite for using Ci-VSP in this fashion.

Here, we used a set of specific PI-binding domains to determine the stereospecificity of CI-VSP activity reports depletion of PI(3,4,5)P$_3$ and generation of PI(3,4)P$_2$. A, cells coexpressing mRFP-Ci-VSP, PI3K(K227E), and one of the PI(3,4,5)P$_3$-binding PH domains indicated were subjected to the depolarizing voltage step indicated. Membrane association of Btk-PH-GFP (black; relative change of $-0.34 \pm 0.07; n = 6$ cells) and ARNO-PH (gray; relative change of $-0.15 \pm 0.03; n = 6$ cells) decreased upon activation of CI-VSP. B, mutation of the catalytic motif of CI-VSP, C363S, abolished depolarization-induced changes in PI(3,4,5)P$_3$ ($n = 4$ cells coexpressing mRFP-Ci-VSP(C363S), PI3K(K227E), and Btk-PH-GFP). Data from wild-type (wt) CI-VSP are replotted from A for comparison. C, the experiment was performed as described for A and B, with the PI(3,4,5)P$_3$-/PI(3,4)P$_2$ binding-specific Akt1-PH-GFP and the PI(3,4)P$_2$-specific TAPP1-PH-YFP probes. On average, Akt1-PH-GFP displayed an increase in membrane association ($n = 21$) upon activation of CI-VSP by depolarization. TAPP1-PH showed a strong increase in membrane association (relative change of $+0.68 \pm 0.21; n = 6$), indicating the generation of PI(3,4)P$_2$. The Btk-PH-GFP trace is replotted from A for comparison.

**FIGURE 5. Redistribution of Akt1-PH, ARNO-PH, Btk-PH, and TAPP1-PH caused by CI-VSP activity reports depletion of PI(3,4,5)P$_3$ and generation of PI(3,4)P$_2$.**

A, cells coexpressing mRFP-Ci-VSP, PI3K(K227E), and one of the PI(3,4,5)P$_3$-binding PH domains indicated were subjected to the depolarizing voltage step indicated. Membrane association of Btk-PH-GFP (black; relative change of $-0.34 \pm 0.07; n = 6$ cells) and ARNO-PH (gray; relative change of $-0.15 \pm 0.03; n = 6$ cells) decreased upon activation of CI-VSP. **B**, mutation of the catalytic motif of CI-VSP, C363S, abolished depolarization-induced changes in PI(3,4,5)P$_3$ ($n = 4$ cells coexpressing mRFP-Ci-VSP(C363S), PI3K(K227E), and Btk-PH-GFP). Data from wild-type (wt) CI-VSP are replotted from A for comparison. C, the experiment was performed as described for A and B, with the PI(3,4,5)P$_3$-/PI(3,4)P$_2$ binding-specific Akt1-PH-GFP and the PI(3,4)P$_2$-specific TAPP1-PH-YFP probes. On average, Akt1-PH-GFP displayed an increase in membrane association ($n = 21$) upon activation of CI-VSP by depolarization. TAPP1-PH showed a strong increase in membrane association (relative change of $+0.68 \pm 0.21; n = 6$), indicating the generation of PI(3,4)P$_2$. The Btk-PH-GFP trace is replotted from A for comparison.
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and the graded alteration of [PI] as a function of voltage. First, all concentration changes upon activation of Ci-VSP occurred within seconds. Such speed may permit us to directly follow the time course of processes that depend on the respective PI isoforms, which otherwise, e.g. by using constitutive overexpression or knockdown of PI kinases/phosphatases (e.g. Refs. 4 and 5), has not been possible. Moreover, rapid switching of PI concentrations will help to distinguish processes directly affected by PI binding from downstream effects.

Graded alteration of [PI] by Ci-VSP provides a so far unique assay for quantitative analysis of PI sensitivity of proteins or cellular processes in the native cellular environment. Here, we have shown the feasibility of this approach by demonstrating the differential PI(4,5)P₂ affinity of PLCδ₁-PH versus tubby-Cterm (Fig. 2B).

Recently, rapamycin-induced recruitment of PI-metabolizing enzymes to the plasma membrane has been invented as a powerful and elegant tool for selective and rapid manipulation of PI levels (6, 7). Although this method is versatile because different enzymes can be used (7) and it is nearly as fast in depleting PI(4,5)P₂ as Ci-VSP, it lacks reversibility and the potential to set [PI] to various intermediate levels. Therefore, the rapamycin system and Ci-VSP may be used as complementary tools for defining the cellular roles of PIs.

Additionally, the rapid switch off of Ci-VSP activity allows for investigation of cellular PI metabolism. Here, we have analyzed the resynthesis of PI(4,5)P₂ after depletion. Using kinase inhibitors and reduced availability of the substrate ATP, we have shown that replenishment of PI(4,5)P₂ occurs via rephosphorylation of PI(4)P by PIP5Ks. The slowed time course of recovery at reduced cytoplasmic ATP clearly shows that altered enzymatic activity can be resolved in this way. We anticipate that this approach will be highly useful for analyzing the mechanisms that regulate PIP5Ks. More generally, recovery after rapid disturbance of [PI] provides a unique quantitative assay for the activity of PI-converting enzymes in the living cell.

In addition to demonstrating the feasibility of this approach, our experiments deliver two interesting pieces of information. First, resynthesis of PI(4,5)P₂ is surprisingly rapid, indicating high endogenous activity of PIP5Ks. This is in line with the idea of a “futile” turnover cycle, where basal PIP5K activity is required for maintaining PI(4,5)P₂ levels (1). Second, although greatly slowed, residual resynthesis was evident even after prolonged dialysis of cells with ATP-free solution, when levels should have dropped to low micromolar values (24). This suggests a high ATP affinity of plasma membrane PIP5Ks in vivo, possibly even higher than the reported in vitro affinities (25–40 μM) (40, 41). For comparison, replenishment of PI(4,5)P₂ after breakdown by PLC, which requires successive phosphorylation at the 4’- and 5’-positions, is completely abolished by washout of ATP (21). Our results thus provide evidence that the ATP affinity of PIP5Ks is substantially higher than that of PI 4-kinases.

The biological function of Ci-VSP is not understood at present (8, 9). Because Ci-VSP affects several PI species, any of these changes may be the physiologically relevant signal in Ciona. However, it has been noted that the activation range of Ci-VSP is mostly beyond physiological membrane potentials (9); in other words, tonic depolarization to positive voltages is required to substantially change the high resting [PI(4,5)P₂] (Fig. 2). In contrast, even low enzymatic rates as obtained by moderate physiological depolarization may be sufficient to radically alter the abundance of a PI species of extremely low basal concentration such as PI(3,4)P₂ (42, 43). In fact, the most dramatic effect was observed with TAPP1-PH, which appears to be a biological sensor mediating cellular responses to PI(3,4)P₂ (34). Plasma membrane PI(3,4)P₂ levels are increased in response to activation of a variety of receptors, and it is thought that PI(3,4)P₂ has its own cellular signaling functions, with limited knowledge, however, about the relevant cellular responses (43). We therefore suggest that the biological function of Ci-VSP may be to generate a PI(3,4)P₂ signal rather than to deplete PI(4,5)P₂ or PI(3,4,5)P₃.

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