

# Kinetics of PIP<sub>2</sub> metabolism and KCNQ2/3 channel regulation studied with a voltage-sensitive phosphatase in living cells

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The signaling phosphoinositide phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) is synthesized in two steps from phosphatidylinositol by lipid kinases. It then interacts with KCNQ channels and with pleckstrin homology (PH) domains among many other physiological protein targets. We measured and developed a quantitative description of these metabolic and protein interaction steps by perturbing the PIP<sub>2</sub> pool with a voltage-sensitive phosphatase (VSP). VSP can remove the 5-phosphate of PIP<sub>2</sub> with a time constant of  $\tau < 300$  ms and fully inhibits KCNQ currents in a similar time. PIP<sub>2</sub> was then resynthesized from phosphatidylinositol 4-phosphate (PIP) quickly,  $\tau = 11$  s. In contrast, resynthesis of PIP<sub>2</sub> after activation of phospholipase C by muscarinic receptors took  $\sim 130$  s. These kinetic experiments showed that (1) PIP<sub>2</sub> activation of KCNQ channels obeys a cooperative square law, (2) the PIP<sub>2</sub> residence time on channels is  $< 10$  ms and the exchange time on PH domains is similarly fast, and (3) the step synthesizing PIP<sub>2</sub> by PIP 5-kinase is fast and limited primarily by a step(s) that replenishes the pool of plasma membrane PI(4)P. We extend the kinetic model for signaling from M<sub>1</sub> muscarinic receptors, presented in our companion paper in this issue (Falkenburger et al. 2010. *J. Gen. Physiol.* doi:10.1085/jgp.200910344), with this new information on PIP<sub>2</sub> synthesis and KCNQ interaction.

## INTRODUCTION

Phosphoinositides are minority phospholipids of biological membranes that are central in cellular signaling and as anchors for peripheral membrane proteins (e.g., De Matteis and Godi, 2004; Wenk and De Camilli, 2004). The phosphoinositide phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>), localized mainly in the cytoplasmic leaflet of the plasma membrane, is required for function of many ion channels and transporters (Hilgemann and Ball, 1996; Hilgemann et al., 2001; Suh and Hille, 2002, 2008). It is also important in exocytosis, endocytosis, cell adhesion, and motility (e.g., Di Paolo and De Camilli, 2006; Mao and Yin, 2007). Additionally, PIP<sub>2</sub> is the immediate precursor for three major second messengers, inositol 1,4,5-trisphosphate (IP<sub>3</sub>), diacylglycerol (DAG), and phosphatidylinositol 3,4,5-trisphosphate, which are generated by activation of plasma membrane receptors. Here, we determine the kinetics of production of PIP<sub>2</sub> and its regulation of KCNQ2/3 (Kv7.2/7.3) potassium channels.

We have previously investigated signaling to KCNQ channels by the M<sub>1</sub> muscarinic receptor (M<sub>1</sub>R). These studies established the requirements (Suh et al., 2004;

Horowitz et al., 2005), timing (Jensen et al., 2009), and rate constants (see Falkenburger et al. in this issue) of individual steps in the M<sub>1</sub>R signaling cascade: the binding of the muscarinic agonist oxotremorine-M (Oxo-M), the processing of G proteins on receptors, G protein dissociation/rearrangement, the binding of G<sub>α</sub><sub>q</sub> subunits to PLC, and PLC-mediated hydrolysis of PIP<sub>2</sub>. The PIP<sub>2</sub> depletion turns off KCNQ2/3 potassium channels (diagramed in Fig. 1, A and B).

Recovery of current after M<sub>1</sub>R activation requires regeneration of PIP<sub>2</sub>. Wortmannin sensitivity and an ATP requirement implicate a type III phosphatidylinositol (PI) 4-kinase in the recovery (Suh and Hille, 2002; Zhang et al., 2003). The PI 4-kinases phosphorylate PI at the inositol 4 position to produce PI(4)P, which is then phosphorylated by phosphatidylinositol 4-phosphate (PIP) 5-kinases at the 5 position to yield PIP<sub>2</sub> (Fig. 1 E). These two steps are required for the maintenance of the “hormone-sensitive” pool of PIP<sub>2</sub> at the plasma membrane (Nakanishi et al., 1995; Wang et al., 2004) and for its recovery after receptor activation.

Here, we seek a quantitative description of the lipid kinases and phosphatases that govern plasma membrane PIP<sub>2</sub>. Most interesting is PIP 5-kinase, the enzyme producing PIP<sub>2</sub>, which also mediates the effects of Rho family GTPases on actin organization (Chong et al., 1994;

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Abbreviations used in this paper: CFP, cyan fluorescent protein; Ci-VSP, voltage-sensitive phosphatase from *Ciona intestinalis*; DAG, diacylglycerol; Dr-VSP, voltage-sensitive phosphatase from *Danio rerio*; FRET, Förster resonance energy transfer; IP<sub>3</sub>, inositol 1,4,5-trisphosphate; IRES, internal ribosome entry site; M<sub>1</sub>R, M<sub>1</sub> muscarinic receptor; Oxo-M, oxotremorine-M; PH, pleckstrin homology; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP<sub>2</sub>, phosphatidylinositol 4,5-bisphosphate; VSP, voltage-sensitive phosphatase; YFP, yellow fluorescent protein.

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Oude Weernink et al., 2004). To perturb the system, we characterized and exploited a PIP<sub>2</sub> 5-phosphatase that can be activated by depolarization of the membrane potential, the voltage-sensitive phosphatase (VSP). VSP contains a voltage sensor domain homologous to those of voltage-gated ion channels and a phosphatase domain homologous to PTEN, a polyphosphoinositide phosphatase (Fig. 1, C and D) (Okamura et al., 2009). VSP can dephosphorylate PI(4,5)P<sub>2</sub> to PI(4)P (Iwasaki et al., 2008; Halaszovich et al., 2009). Recovery after VSP activation is then mediated by the endogenous PIP 5-kinases at the plasma membrane. As VSP-induced changes in PIP<sub>2</sub> were not accompanied by the generation of other second messengers that might modulate KCNQ2/3 current, this intervention also provided an opportunity to learn more about the interaction of PIP<sub>2</sub> with KCNQ2/3 channels, and about PIP<sub>2</sub> reporting by the pleckstrin homology (PH) domain probe we use.

## MATERIALS AND METHODS

### Cell culture and plasmids

Cells (tsA201) cultured in DMEM (Invitrogen) with 10% serum and 2% penicillin/streptomycin were passaged once a week. Cells were transfected at 75% confluence, plated on polylysine-coated glass chips 24 h after transfection, and used for experiments the next day. Cells were transfected with Lipofectamine 2000 (10  $\mu$ l for a 3-cm dish; Invitrogen) and 0.5–1.2  $\mu$ g DNA per plasmid: mouse M<sub>1</sub>R (provided by N. Nathanson, University of Washington, Seattle, WA); human eCFP-PH(PLC $\delta$ 1) and eYFP-PH(PLC $\delta$ 1; provided by K. Jalink, The Netherlands Cancer Institute, Amsterdam, Netherlands); human KCNQ2 and rat KCNQ3 (provided by D. McKinnon, State University of New York, Stony Brook, NY); PIP 5-kinase type I $\gamma$  (provided by Y. Aikawa and T.F. Martin, University of Wisconsin, Madison, WI); and Ci-VSP-IRES-GFP (Ci-VSP, VSP from *Ciona intestinalis*; internal ribosome reentry site [IRES]) and Dr-VSP-IRES-GFP (Dr-VSP) from zebrafish (*Danio rerio*; both provided by Y. Okamura, Osaka University, Osaka, Japan). “Dark” Dr-VSP (without IRES-GFP) was generated by subcloning the Dr-VSP cassette into pcDNA3.0 using HindIII and XhoI.

### Electrophysiology

Cells were continuously superfused with Ringer’s solution containing (in mM): 160 NaCl, 2.5 KCl, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 HEPES, and 8 glucose, pH 7.4 (NaOH). 10  $\mu$ M Oxo-M was applied via a two-barrel theta tube. Cells were recorded by whole cell gigaseal voltage clamp using borosilicate glass pipettes with a resistance of 1.6–2.2 M $\Omega$ . Internal solution was (in mM): 175 KCl, 5 MgCl<sub>2</sub>, 5 HEPES, 0.1 K<sub>4</sub>BAPTA, 3 Na<sub>2</sub>ATP, and 0.1 Na<sub>3</sub>GTP, pH 7.4 (KOH). Recordings used an EPC9 amplifier with either Patchmaster 2.35 or Pulse 8.53 software (HEKA). Holding potential was −60 mV. Voltage protocols are given in the figures and legends. Currents were filtered at 2.9 kHz. Sample intervals ranged from 0.1 ms for tail currents to 50 ms for 30-s records at −20 mV. Series resistance was compensated by 70% after compensation of fast and slow capacitance. Except where stated, leak was not subtracted. For measuring VSP “sensing currents,” we changed three conditions: the internal solution was (in mM) 100 HEPES, 65 NMDG, 3 MgCl<sub>2</sub>, and 1 EGTA; the external solution was (in mM) 180 HEPES, 75 NMDG, 1 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, and 10 glucose, pH 7.4

(as in Hossain et al., 2008); and linear leak and capacitive transients were subtracted by a p/5 procedure (five repetitions of 0.2 of the test pulse amplitude from a holding potential of −120 mV after the test pulse).

KCNQ2/3 current was measured in two ways. One was to hold the membrane potential at −20 mV continuously. Endogenous currents in tsA201 cells are small at −20 mV (−26 pA ± 5 pA;  $n = 16$  cells; see Fig. S2, A and C). The second method used tail currents. Every 0.5 or 1 s, the membrane potential was depolarized to −20 mV for 200 or 300 ms and repolarized to −60 mV. KCNQ2/3 current activates slowly upon depolarization and deactivates slowly upon repolarization (see Fig. S2 B). In contrast, endogenous currents deactivate too fast to be seen at the sampling frequency used. KCNQ2/3 tail currents were measured by comparing current at 20 and 200 ms after repolarization to −60 mV.

### Photometric measurement of PH domain Förster resonance energy transfer (FRET)

We used epifluorescence photometry to measure the FRET of PH domains simultaneously with measurement of KCNQ2/3 current. The photometry setup was different from that used previously (Jensen et al., 2009; Falkenburger et al., 2010). A monochromator (Polychrome IV; TILL Photonics) provided excitation light, and a three-color dichroic mirror in the microscope reflected at 440, 500, and 580 nm (CFP, YFP, and mCherry; 89006bs; Chroma Technology Corp.). The dichroic mirror is transparent at 460–480 and 520–560 nm. Fluorescence was detected by a photometry system consisting of a ViewFinderIII with two photodiodes connected to an FDU-2 detection unit (TILL Photonics). Light was split between the two photodiodes by a dichroic mirror (DCLP505). In addition, the short-wavelength channel contained a D480/40 emission filter, and the long-wavelength channel contained an ET535/30 filter (Chroma Technology Corp.). For near-simultaneous acquisition of CFP<sub>C</sub> (440-nm excitation and 480-nm emission), YFP<sub>C</sub> (440-nm excitation and 535-nm emission), and YFP<sub>Y</sub> (500-nm excitation and 535-nm emission), the excitation wavelength was scanned from 300 to 500 nm in 200 ms. Both photodiodes were sampled every 0.1 ms. A shutter was opened 10 ms before scanning and closed 100 ms after scanning. To measure KCNQ2/3 current at the same time, the membrane potential was depolarized to −20 mV for 300 ms and held at −60 mV for the remainder of the time. This protocol was repeated every 500 ms. After recording from each cell, an area of the coverslip without cells was measured as background. This background fluorescence was small and depended little on the excitation wavelength. The light intensity in the illuminated 139 × 158.5  $\mu$ m<sup>2</sup> area was 45  $\mu$ W at 430 nm and 35  $\mu$ W at 500 nm (~0.2 W/cm<sup>2</sup>).

Data were analyzed offline in IGOR Pro 6.0 (WaveMetrics). To calculate FRET, we extracted three values from each wavelength scan, similar to a three-cube FRET approach. We denote them CFP<sub>C</sub>, raw YFP<sub>C</sub>, and YFP<sub>Y</sub>, with the first part referring to the emission wavelength and the subscript referring to the excitation wavelength. For the CFP<sub>C</sub> value, emission in the short-wavelength channel (460–480-nm emission) was integrated over the time when excitation was 360–460 nm. For the raw YFP<sub>C</sub> value, the long-wavelength channel (535/30-nm emission) was integrated over the same time. For the YFP<sub>Y</sub> value, the long-wavelength channel was integrated over the time where excitation was 490–500 nm. The units for all three values were set as arbitrary fluorescence units (AFU). Background was subtracted from each. The raw YFP<sub>C</sub> value had to be corrected for cyan fluorescent protein (CFP) emission collected in the long-wavelength channel and for direct excitation of yellow fluorescent protein (YFP) by 440-nm light by subtracting 0.834\*CFP<sub>C</sub> and 0.065\*YFP<sub>Y</sub>. The corrected value is referred to as YFP<sub>C</sub> from now on. The correction factors were determined by measuring cells expressing only CFP or YFP. The spectral window for collection of CFP emission was smaller than

in our previous work (Jensen et al., 2009). Therefore CFP<sub>C</sub> had to be multiplied by a larger factor in correcting the long-wavelength channel for bleedthrough of CFP emission. The lower values for CFP<sub>C</sub> also affected the values of the FRET ratio, FRET<sub>r</sub> (see below). FRET was expressed as the ratio FRET<sub>r</sub> = YFP<sub>C</sub>/CFP<sub>C</sub>. This ratio is related to FRET efficiency, with two differences. A FRET efficiency of 20% means that 20% of CFP excitation is reemitted by YFP instead of CFP, thus CFP emission is reduced to 80%. If the short-wavelength detector (CFP<sub>C</sub>) and the long-wavelength detector (YFP<sub>C</sub> and YFP<sub>Y</sub>) had the same photon sensitivity, a FRET efficiency of 20% would correspond to a FRET<sub>r</sub> of 20/80 = 0.25. However, in the photometry setup used here, the absolute changes in YFP<sub>C</sub> were approximately threefold larger than the accompanying changes in CFP<sub>C</sub> (both in ΔAFU; compare Fig. 2, D with E). A true FRET efficiency of 20% would therefore correspond to a FRET<sub>r</sub> of 3\*20/80 = 0.75. We report FRET<sub>r</sub> in arbitrary units because the absolute values will differ between setups.

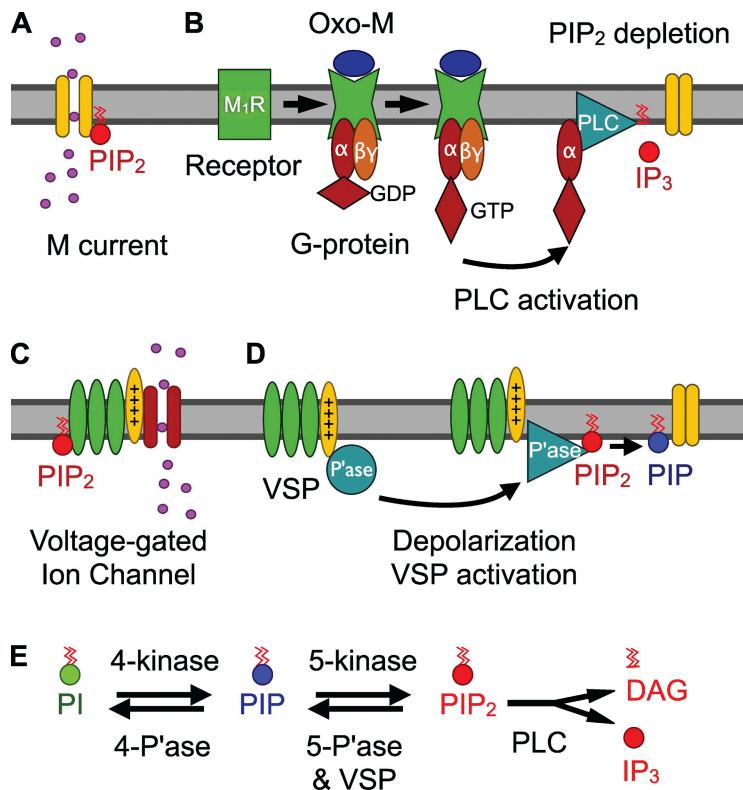
The YFP<sub>Y</sub> value should be unaffected by FRET. It showed a gradual decline over time, reflecting dialysis of the PH probes from the cell into the much larger patch pipette. The time constant of decline (~5 min) was similar to that for CFP protein diffusing from the patch pipette into the cell. The same decline was seen in the CFP<sub>C</sub> channel. Finally, as a consequence of the increasing distance between donors and acceptors, their FRET interaction also showed some decline.

#### Modeling

A kinetic model of phosphoinositide metabolism was formulated as a compartmental model in the Virtual Cell framework (University of Connecticut). The Virtual Cell Model “Falkenburger JGP2010” is publicly available at <http://www.vcell.org/> under shared models/hillelab.

#### Statistics

Summarized data include one data point per cell. Bars and markers represent mean ± SEM.



#### Online supplemental material

Fig. S1 shows VSP-sensing currents in tsA201 cells. Fig. S2 illustrates endogenous ion currents in tsA cells compared with currents in KCNQ2/3-transfected cells. Fig. S3 shows the theoretical prediction of the density of PH probes at the membrane for different amounts of PIP<sub>2</sub>, and a description of the dependence of FRET efficiency and FRET<sub>r</sub> for CFP/YFP-labeled PH probes on plasma membrane PIP<sub>2</sub>. Table S1 lists the model differential equations. The online supplemental material is available at <http://www.jgp.org/cgi/content/full/jgp.200910345/DC1>.

## RESULTS

Our laboratory has already formulated a preliminary kinetic description of the signaling steps from muscarinic receptor excitation to PIP<sub>2</sub> turnover and channel modulation (Suh et al., 2004). It was based on plausible estimates knowing the final modulation of KCNQ2/3 channels. Then, a second-generation model for the steps from receptor activation to turning on PLC was formulated in our companion paper (Falkenburger et al., 2010) based on our recent more complete set of kinetic measurements with FRET (Jensen et al., 2009). In this paper, to extend our model to include the steps of PIP<sub>2</sub> metabolism and the action of PIP<sub>2</sub> on channels, we have made extensive use of PH domains as indicators of PIP<sub>2</sub>, of a VSP to deplete PIP<sub>2</sub> rapidly, and of KCNQ2/3 channels to report their own activation by PIP<sub>2</sub>. We start by characterizing VSP.

#### The voltage sensor of VSP responds rapidly

As in voltage-gated ion channels, positive charges in the S4 segment of the voltage sensor of VSP (Fig. 1, C and D,

**Figure 1.** M<sub>1</sub>R signaling and phosphoinositide metabolism. (A) Neuronal M current is mediated by KCNQ2/3 potassium channels (yellow), which require membrane depolarization and PIP<sub>2</sub> to open. (B) Binding of the M<sub>1</sub>R agonist Oxo-M facilitates binding of G proteins to the receptor. This binding induces “activation” of G proteins, i.e., nucleotide exchange at the G<sub>α</sub> subunit from GDP to GTP, and dissociation of G<sub>α</sub> from G<sub>β</sub>γ. G<sub>α</sub>-GTP activates PLC, which cleaves PIP<sub>2</sub> into DAG and IP<sub>3</sub>. The absence of PIP<sub>2</sub> prevents KCNQ2/3 channels from opening. (C) Voltage-gated ion channels are tetramers. Each subunit consists of a four-segment (S1-S4) voltage sensor domain (green and yellow) and a pore-forming domain (dark red). The S4 segment (yellow) contains positive charges, which move upon depolarization. (D) VSPs are monomers. They contain a four-segment voltage sensor and a phosphatase domain. The phosphatase is activated by depolarization and dephosphorylates PIP<sub>2</sub> to PI(4)P. (E) Phosphoinositide metabolism. PI is phosphorylated first by a PI 4-kinase and then by a PIP 5-kinase to yield PI(4,5)P<sub>2</sub>. A 4-phosphatase and a 5-phosphatase mediate the reverse reactions. VSP is a 5-phosphatase.

yellow) move outward upon depolarization, making a transient outward current, termed “gating current” for ion channels and “sensing current” for this nonchannel enzyme. The charge movement leads to activation of the phosphatase activity (Murata et al., 2005; Murata and Okamura, 2007). We determined the voltage dependence of the charge movement and its time course for VSP from zebrafish (Dr-VSP) and *Ciona* (Ci-VSP) expressed in tsA201 cells (Fig. S1). Depolarization for 100 ms elicited an outward sensing current, and repolarization elicited an inward current. The integrals over both segments were equal and represent the total charge moved during voltage sensing. At +100 mV with Dr-VSP, for example, the exponential time constant of decay of the major sensing current was  $\tau = 39 \pm 4$  ms ( $n = 4$  cells), and on return to  $-60$  mV, the return charge flowed with time constant  $\tau = 8.6 \pm 1.1$  ms ( $n = 5$  cells), in full agreement with Hossain et al. (2008). We did not attempt to resolve additional slow time constants reported by others (Villalba-Galea et al., 2008). For Dr-VSP, the voltage dependence of this sensing charge (the Q-V curve) followed a Boltzmann relation, with a midpoint at +100 mV and a slope factor of 1.5 elementary charges. From this, Dr-VSP would be inactive at  $-20$  mV, where we measure KCNQ current. A depolarization to +100 mV moves 50% of VSP-sensing charge and is well tolerated by our cells. By comparison, the voltage dependence of Ci-VSP was left-shifted and less steep. These findings agree with previous measurements of VSP-sensing currents (Hossain et al., 2008). To ensure that VSP remained inactive at  $-20$  mV in our experiments, we subsequently used only Dr-VSP.

Total sensing charge also provides an estimate of the density of VSP molecules in transfected cells. The VSP construct we used included GFP after an IRES, so transfected cells would likely express more copies of VSP than of GFP. We chose cells by their visible GFP fluorescence, but unlike the previous papers (Jensen et al., 2009; Falkenburger et al., 2010), we did not quantitate this fluorescence by photon counting. In the group of cells chosen, the saturating charge movement was  $4.9 \pm 1.9$  pC for Dr-VSP– and  $2.1 \pm 0.8$  pC for Ci-VSP–transfected cells (five and three cells). These numbers are similar to those obtained by the Okamura group in the same cell type (4.6 pC for Dr-VSP and 5.2 pC for Ci-VSP; Hossain et al., 2008). This charge corresponds to around 20,000 and 9,000 elementary charges moved per  $\mu\text{m}^2$  of plasma membrane (assuming 15 pF of cell capacitance and 1,500  $\mu\text{m}^2$  of cell surface; Falkenburger et al., 2010). The slope of the voltage dependence suggests that 1.5 charges move per voltage sensor, indicating around 13,000 expressed Dr-VSP or 6,000 Ci-VSP molecules per  $\mu\text{m}^2$ . The density of Dr-VSP is thus two- to fourfold higher than that of fluorescent signaling proteins determined in our companion paper (Falkenburger et al., 2010). Of note, the VSP density is also a little higher

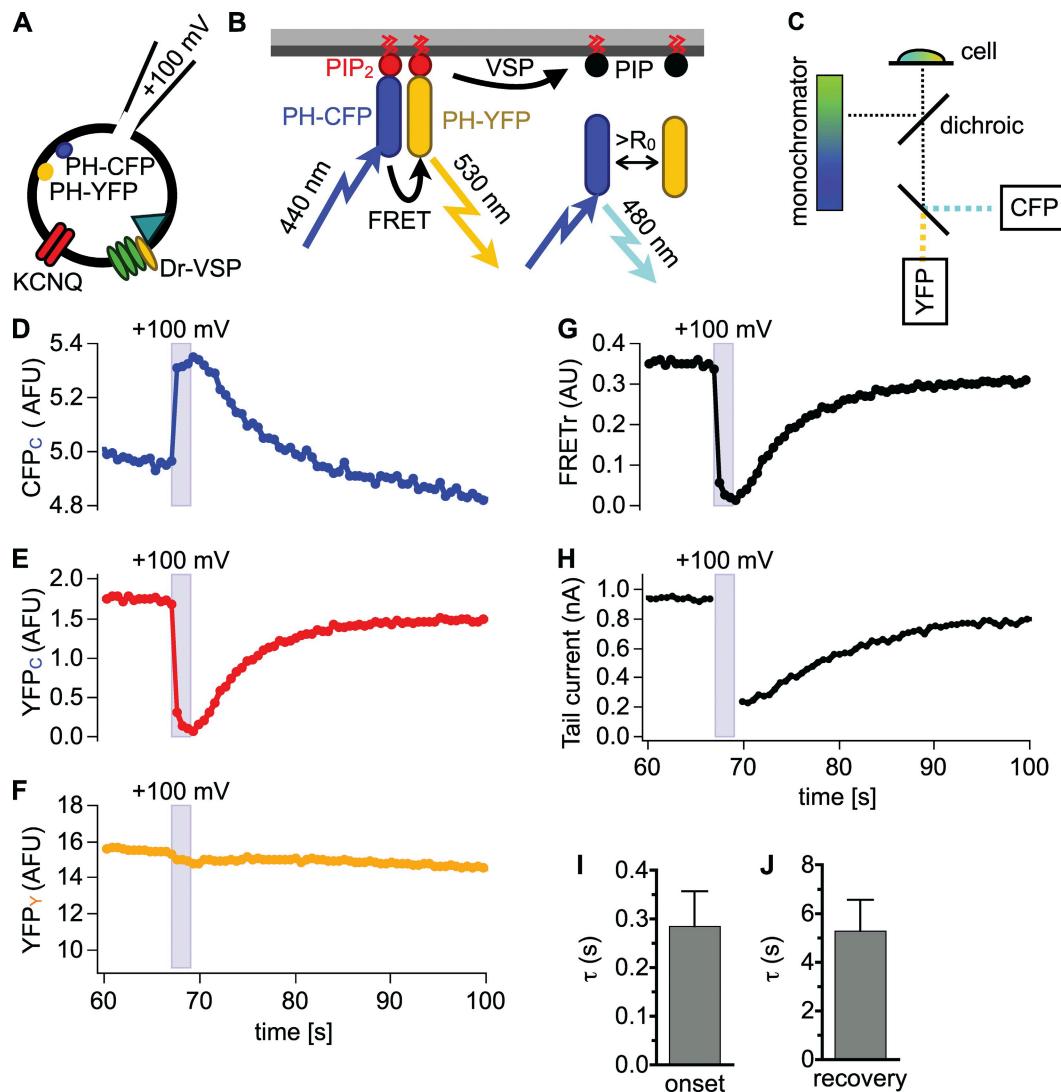
than the concentration that we chose for its substrate PIP<sub>2</sub> in our model.

Others have made kinetic models of the conformational changes of VSP, combining observations of charge movement and of signals from various attached fluorescent labels (Villalba-Galea et al., 2008; Akemann et al., 2009). They include fast conformational changes and sometimes several slower steps. The constructs they studied had the phosphatase enzyme mutated to be inactive or entirely deleted and sometimes replaced by one or two fluorescent proteins. None of these models gives direct information about how soon the phosphatase activity is turned on after the initial major charge movement occurs. However, our experiments below suggest that the enzyme activity is nearly synchronous with or follows charge movements within tens of milliseconds. For simplicity, we refer to our standard +100-mV depolarizations as “VSP activation.”

#### Activation of VSP reduces PIP<sub>2</sub> and KCNQ2/3 current

We now use VSP activation to perturb the endogenous plasma membrane PIP<sub>2</sub> pool. To measure PIP<sub>2</sub> depletion during VSP activation, we used FRET between two fluorescently labeled PH domains from PLC $\delta$ 1 (PH-CFP and PH-YFP) as described previously (van der Wal et al., 2001; Jensen et al., 2009). PH probe FRET reports PIP<sub>2</sub> surface density because binding to PIP<sub>2</sub> at the plasma membrane brings the CFP and YFP close enough together for FRET to occur. FRET between these probes decreases as PIP<sub>2</sub> is depleted and increases as PIP<sub>2</sub> recovers (Fig. 2, A–C, and Materials and methods). To calculate FRET, we measured three values (CFP<sub>C</sub>, raw YFP<sub>C</sub>, and YFP<sub>Y</sub>) during VSP activation, made corrections, and calculated the FRET ratio FRET<sub>r</sub> = YFP<sub>C</sub>/CFP<sub>C</sub> as described in Materials and methods. Upon activation of VSP by depolarization to +100 mV for 2 s, PIP<sub>2</sub> was depleted, and we observed (1) an increase in CFP<sub>C</sub> fluorescence (Fig. 2 D), (2) a decrease in YFP<sub>C</sub> fluorescence (Fig. 2 E), (3) little change in YFP<sub>Y</sub> fluorescence (Fig. 2 F), and (4) a strong reversible drop in FRET<sub>r</sub> (Fig. 2 G). FRET<sub>r</sub> fell almost to zero during VSP activation, suggesting that much of PIP<sub>2</sub> was lost in that time. The mean ON time constant of the VSP-induced depletion was 421 ms at +100 mV, and the mean recovery time constant after repolarization was 6.5 s at  $-60$  mV (Fig. 2, I and J). Thus, in a few hundred milliseconds we could convert most of the membrane PIP<sub>2</sub> to PI(4)P, and in 10–20 s the PIP<sub>2</sub> was restored.

The relationship between membrane PIP<sub>2</sub> and PIP<sub>2</sub>-sensitive KCNQ current was revealed by coexpressing KCNQ2/3 potassium channels with VSP. Initially, KCNQ2/3 current was measured by the tail current amplitude (see Materials and methods). The fluorescence and current traces in Fig. 2 (D–H) were recorded simultaneously during depletion of PIP<sub>2</sub>. Activation of VSP decreased the KCNQ2/3 tail current quickly (Fig. 2 H),

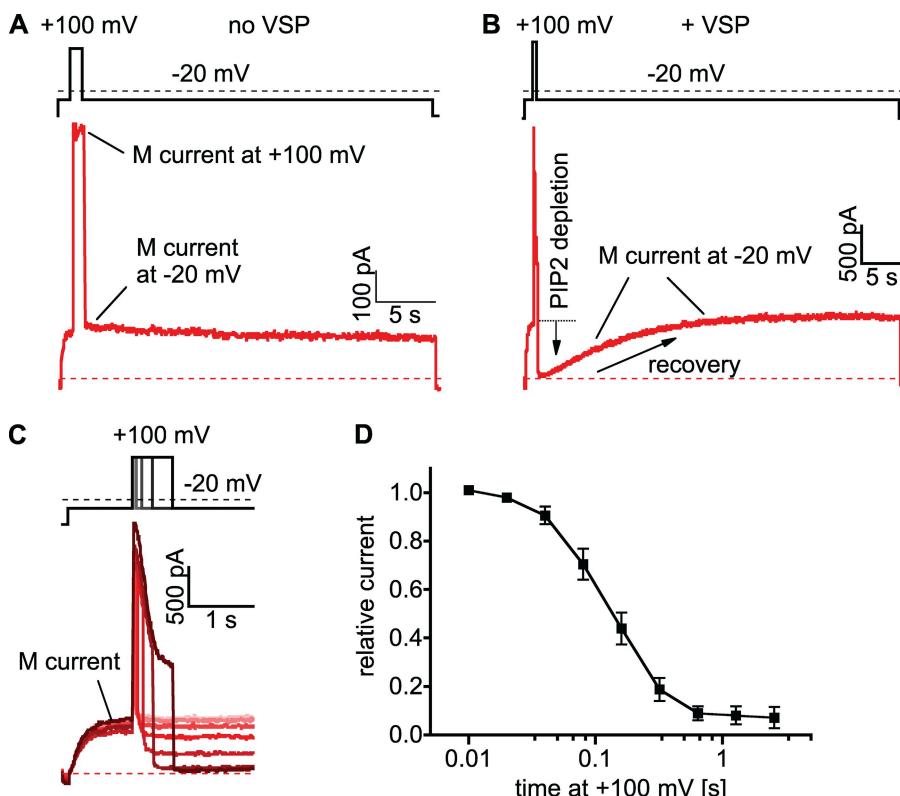


**Figure 2.** Activation of VSP (Dr-VSP) reduces PH probe FRET. (A) Cells were transfected with PIP<sub>2</sub>-binding PH probes (PH-PLC $\delta$ 1) fused to CFP or YFP, Dr-VSP, and KCNQ2 and KCNQ3 channel subunits and recorded in whole cell voltage clamp. (B) Principle of PIP<sub>2</sub> measurement by PH probe FRET (see Results and Fig. S3). (C) Photometry setup. Excitation light was scanned from 300 to 500 nm in 200 ms, every 500 ms, and reflected by a dichroic mirror around 440 and 500 nm. Emission light was separated into channels for CFP emission (480/40 nm) and YFP emission (535/30 nm). Time courses (D, E, F, and H) were acquired simultaneously. (D) CFP emission with 440-nm excitation (CFP<sub>c</sub>). (E) YFP emission with 440-nm excitation, corrected for CFP emission at 535/30 nm and for direct excitation of YFP by 440-nm excitation light (YFP<sub>c</sub>). (F) YFP emission with 500-nm excitation (YFP<sub>y</sub>). (G) FRET<sub>r</sub> = YFP<sub>c</sub>/CFP<sub>c</sub>. (H) Tail current amplitude. Membrane was held at  $-60$  mV and depolarized to  $-20$  mV for 300 ms every 500 ms, except for shaded area where membrane was held at  $+100$  mV for 2 s. Tail currents were measured during slow channel deactivation at  $-60$  mV. (I) Time constants of single-exponential fits to FRET<sub>r</sub> while membrane was held at  $+100$  mV (onset of VSP effect). A summary of 14 cells is shown. (J) Time constant of single-exponential fits to recovery of FRET<sub>r</sub> after 2 s at  $+100$  mV. A summary of 12 cells is shown.

as reported previously (Murata and Okamura, 2007). When VSP was turned off again, the recovery of current was slower than recovery of PH probe FRET<sub>r</sub> (compare Fig. 2, H with G).

To quantitate the kinetics of VSP actions on KCNQ2/3 current, we switched to measuring current by holding continuously at  $-20$  mV, where noninactivating KCNQ2/3 current can be maintained for a long time and other endogenous K<sup>+</sup> currents in tsA201 cells are minimally activated (Fig. S2, A and D). PH probes were not expressed in these experiments. VSP was activated by brief

steps to  $+100$  mV, a perturbation that also increased the driving force for K<sup>+</sup> and, in cells without VSP, increased current through KCNQ2/3 and endogenous channels (Fig. 3 A). When Dr-VSP was coexpressed (Fig. 3 B), KCNQ2/3 current decayed during the  $+100$ -mV depolarization. Current was much reduced upon return to  $-20$  mV and recovered thereafter. We compared currents at  $-20$  mV before and after varying lengths of VSP activation to track the onset of the VSP effect (Fig. 3, C and D). The effect on KCNQ2/3 was maximal after a 1-s activation pulse. A half-maximal effect required



**Figure 3.** Activation of VSP (Dr-VSP) inhibits KCNQ2/3 current. (A) Currents recorded in cells transfected with KCNQ2/3 alone. (B) Currents in cells transfected with KCNQ2/3 and Dr-VSP. Note the reduction of current at  $-20\text{ mV}$  after depolarization to  $+100\text{ mV}$ . (C) Responses in the same cell as in B to a family of stimuli with increasing duration at  $+100\text{ mV}$ . Magnified time scale as compared with B also shows reduction of current at  $+100\text{ mV}$ . (D) Summary of normalized outward current at  $-20\text{ mV}$  (after/before step to  $+100\text{ mV}$ ) for different durations at  $+100\text{ mV}$  (note log scale of x axis) for 5–12 cells.

$\sim 120\text{ ms}$ , and significant effect was already evident with only 40 ms of VSP activation, just enough time for 63% movement of the voltage sensor charge (Fig. S1 A). Thus, the coupling of the VSP voltage sensor to the phosphatase activity and the reporting of PIP<sub>2</sub> depletion by KCNQ2/3 current are both fast (<40 ms). Fitting exponentials to the decline of whole cell current at  $+100\text{ mV}$  (Fig. 4 A) gave a time course for the action of VSP consistent with that of Fig. 3 D, even though this measure would be contaminated by the slow activation of KCNQ2/3 current and the inactivation of endogenous currents at  $+100\text{ mV}$  (Fig. S2, A and B).

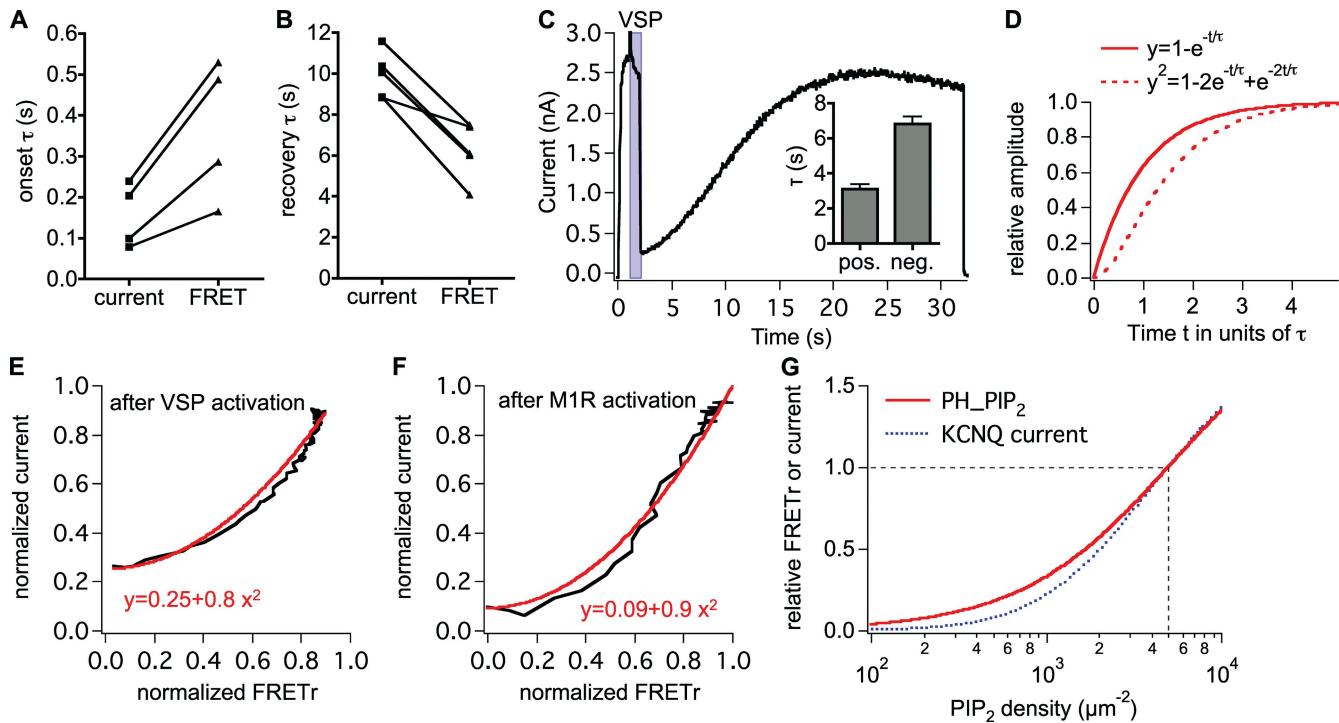
#### KCNQ2/3 current is proportional to the square of PH domain FRET

Now we can consider the relationship between PIP<sub>2</sub> and current. During and after VSP activation, the changes of KCNQ2/3 current and those of the PH domain FRET were not linearly related to each other. First, their exponential time constants differed systematically. For the onset of inhibition, current decayed faster than FRET, whereas for the recovery, current returned more slowly than FRET (Fig. 4, A and B). The recovery of current followed an S-shaped time course (Figs. 2 H, 3 B, and 4 C), and when current was plotted against FRET, the relationship was fitted better by a square law than by a straight line or cubic curve (Fig. 4 E). The same relationship was seen during recovery after M<sub>1</sub>R activation (Fig. 4 F, data taken from Jensen et al., 2009). This dependence of KCNQ2/3 current on the square of PH probe FRET

explains why the onset of VSP action on current is faster than that on FRET. If the decay of FRET followed an exponential time course with time constant  $\tau$ , the square of this function would decay exponentially with a time constant of  $\tau/2$ . It also explains why recovery of current is S shaped and slower than FRET when fitted with a single exponential (Fig. 4 D). If FRET recovery followed  $y = 1 - \exp(-t/\tau)$ , current recovery would follow  $y^2 = 1 - 2\exp(-t/\tau) + \exp(-2t/\tau)$ , which is S shaped. As predicted by that equation, our recovery data fitted with the double-exponential equation gave mean time constants of 3.1 s for the positive term and 6.8 s for the negative term (Fig. 4 C, inset; 30 cells). This relationship allows us to predict the dependence of KCNQ2/3 current on PIP<sub>2</sub> density from the known PIP<sub>2</sub> affinity of PH probes (Fig. 4 G; for details see Fig. S3), and suggests that more than one PIP<sub>2</sub> molecule binds to activate a KCNQ2/3 channel.

Interestingly, in a few cases (7/31 cells) KCNQ2/3 current recovered after VSP activation to values  $\sim 10\%$  higher than the steady-state value before VSP activation. Such over-recovery was observed both with holding at  $-20\text{ mV}$  and with tail currents. We have so far not investigated what underlies this phenomenon.

**Recovery after VSP activation reflects PIP 5-kinase activity**  
The observations so far might be interpreted in two ways. The straightforward model would be that while VSP is converting PIP<sub>2</sub> into PIP, the depletion of PIP<sub>2</sub> turns off the KCNQ2/3 channels, with a corollary that



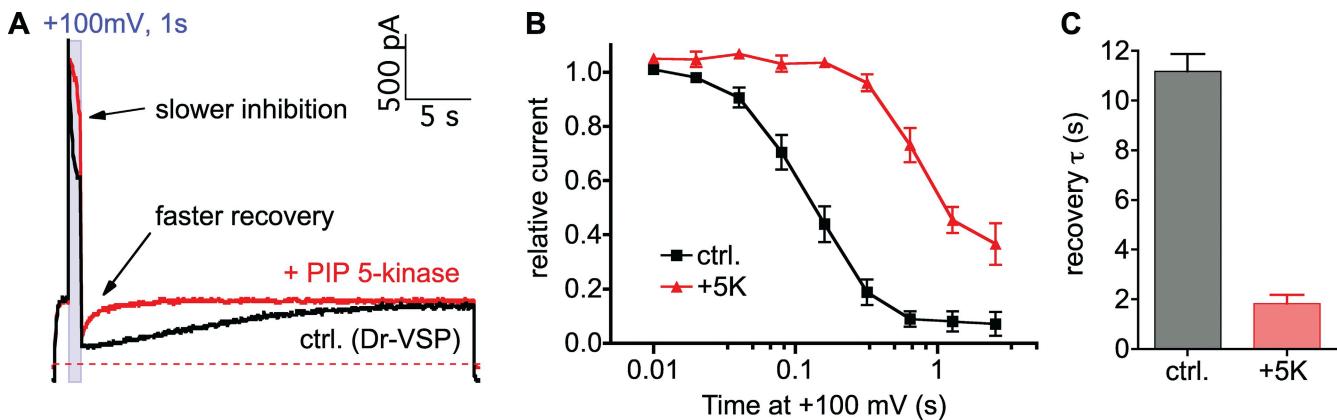
**Figure 4.** KCNQ2/3 current behaves like the square of PH probe FRET. (A) In four cells, single exponentials were fitted to simultaneously acquired KCNQ2/3 current and PH probe FRET<sub>r</sub> during VSP activation (2 s of +100 mV). Measurements from the same cell are connected by a line. (B) In five cells, single exponentials were fitted to simultaneously acquired KCNQ2/3 current and PH probe FRET<sub>r</sub> during recovery after VSP activation. KCNQ2/3 current was measured as tail current amplitude. (C) VSP effect on KCNQ current with voltage protocol as in Fig. 3 B. Recovery of KCNQ2/3 current at −20 mV was fitted with a double exponential:  $y = a - b \cdot \exp(-c^* t) + d \cdot \exp(-f^* t)$ . (Inset) Summary of time constants from 31 cells. Time constant of the positive term (pos.) is 1/f, and that of the negative term (neg.) is 1/c. (D) Illustration of the consequence of squaring an exponential of the form  $y = 1 - \exp(-t/\tau)$ . (E) Plot of KCNQ2/3 current at −20 mV (black) versus PH probe FRET<sub>r</sub> at the same time during recovery after VSP activation in the cell depicted in Fig. 2. Similar observations were made for three other cells. Red curve corresponds to the equation given. (F) Averaged KCNQ2/3 current at −20 mV versus averaged FRET<sub>r</sub> at the same time after M<sub>1</sub>R activation, measured in separate cells (data from Figs. 5 D and 7 B in Jensen et al., 2009). (G) Illustration of the dependence of FRET<sub>r</sub> (approximated by PH\_PIP<sub>2</sub>; see Fig. S3) and KCNQ current on PIP<sub>2</sub> concentration as predicted by the model outlined in Fig. 7 and Tables I and II:  $K_d$  of PH probe is 2,000  $\mu\text{m}^{-2}$  for PIP<sub>2</sub> and 0.1  $\mu\text{M}$  for IP<sub>3</sub> (0.16  $\mu\text{M}$  IP<sub>3</sub>);  $K_d$  of KCNQ is 2,000  $\mu\text{m}^{-2}$  for PIP<sub>2</sub>. KCNQ current =  $(\text{KCNQ}_\text{PIP}_2)^2$ .

PI(4)P is unable to support activity of the channels. An alternative model would be that channels are directly inhibited by the accumulating pool of PI(4)P rather than by depletion of PIP<sub>2</sub>. These possibilities might be distinguished by overexpressing an exogenous PIP 5-kinase to increase tonic PIP<sub>2</sub> levels. In the first model, the increased PIP<sub>2</sub> would make it harder for VSP to deplete PIP<sub>2</sub> quickly and to turn off channels. In the second model, increased PIP<sub>2</sub> might even result in intensified channel inhibition by providing a larger precursor pool for production of inhibitory PI(4)P. As predicted in the first model, we found that the transfected 5-kinase made activation of VSP much less effective at suppressing KCNQ2/3 current (Fig. 5). The relation between the duration of VSP activation and current inhibition was slowed eightfold (Fig. 5, A and B), whereas recovery after VSP activation was speeded fivefold (Fig. 5, A and C). PH probes were not expressed in these experiments. These findings support the concepts that PIP<sub>2</sub> is essential, PI(4)P does not inhibit or permit channel activity,

and PI(4)P and the activity of cellular PIP 5-kinase(s) are needed after VSP activation to restore PIP<sub>2</sub> and full channel activity.

#### Phosphorylation of PI(4)P by PIP 5-kinase is faster than PI(4)P supply by PI 4-kinase

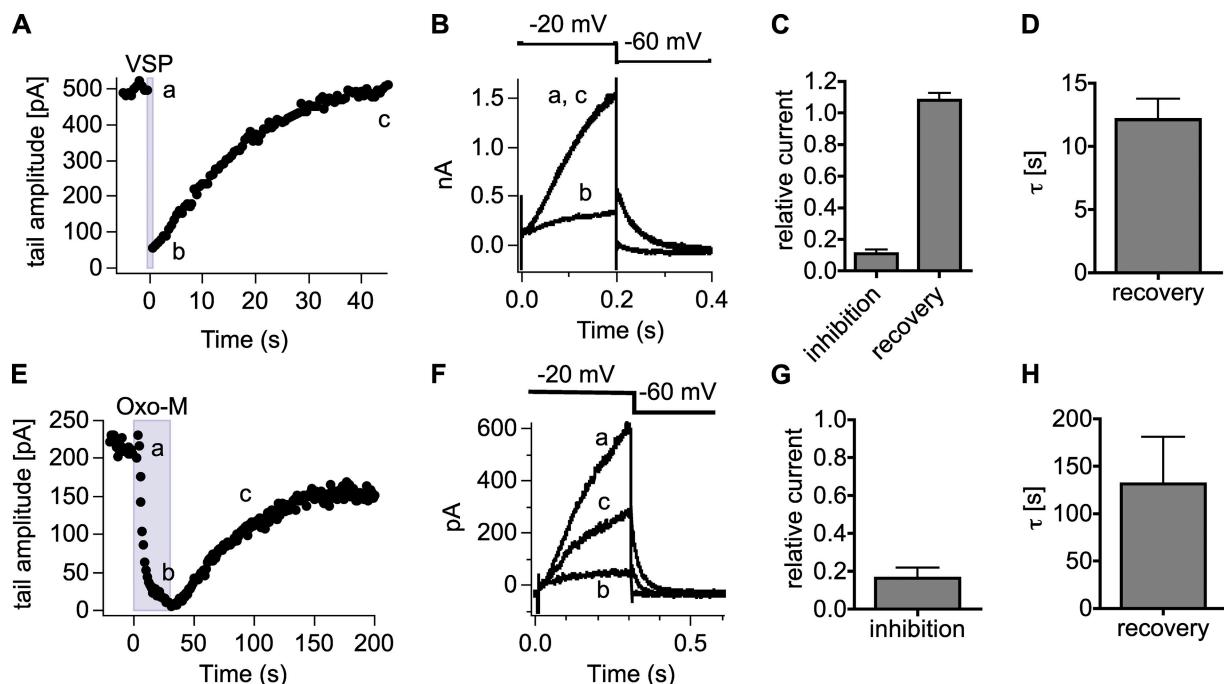
In contrast to VSP, PLC degrades PIP<sub>2</sub> to DAG and IP<sub>3</sub> and also leads to quick PI(4)P depletion (Willars et al., 1998; Horowitz et al., 2005). Recovery after PLC activation needs two steps for PIP<sub>2</sub> resynthesis: first, phosphorylation of PI by a PI 4-kinase, and then phosphorylation of PI(4)P by a PIP 5-kinase. We find that recovery of KCNQ2/3 current after VSP activation (Fig. 6 A) is 5–10-fold faster than recovery after M<sub>1</sub>R activation (Fig. 6 E), even when the extent of KCNQ2/3 current inhibition was similar (Fig. 6, C and G). Mean recovery time constants for current were 11 s after VSP activation and 130 s after M<sub>1</sub>R activation (Fig. 6, D and H). PH probes were not expressed in either case. The slower recovery after receptor activation cannot be attributed to slow turn



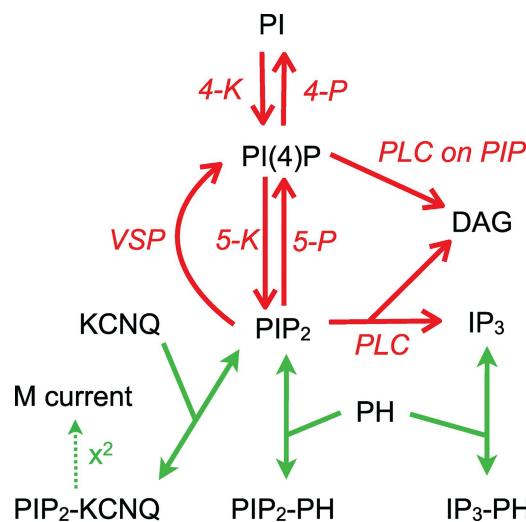
**Figure 5.** PIP 5-kinase overexpression antagonizes VSP effects. (A) Traces from two cells with similar current amplitudes transfected with Dr-VSP and KCNQ2/3 (ctrl., black trace) or with Dr-VSP, KCNQ2/3, and PIP 5-kinase Iy (+5K, red trace). (B) Dependence of current inhibition on the duration of VSP activation. Baseline-normalized currents at  $-20$  mV immediately after VSP activation are plotted for control (from Fig. 3 D) and +5K (three cells). (C) Time constants of single-exponential fits to current recovery after VSP activation. Summary of 16 cells for control and 4 cells for +5K.

off of PLC after agonist wash off because the measured interaction of  $G_q$  with PLC falls by 95% in only 2 s (Jensen et al., 2009; Falkenburger et al., 2010). Apparently, the production of PI(4)P by PI 4-kinase is rate limiting for recovery after  $M_1R$  stimulation, and the endogenous PIP 5-kinase is many-fold faster than the PI 4-kinase.

**Modeling of phosphoinositide metabolism, VSP, and PLC**  
We now return to a quantitative description of the signaling kinetics. For our model of phosphoinositide metabolism (Fig. 7, Tables I and II, and Table S1), we assumed that all phosphoinositide reactions take place in one compartment, and that the relevant kinases and phosphatases obey nonsaturating linear kinetics. For



**Figure 6.** PIP 5-kinase is faster than PI 4-kinase. Tail current amplitudes were used to measure current inhibition by Dr-VSP or  $M_1R$  activation and its recovery. (A) Time course of tail current amplitude in a cell transfected with Dr-VSP and KCNQ2/3 (2 points  $s^{-1}$ ). (B) Superimposed currents at time points before VSP activation (a), after VSP activation (b), and during recovery (c). (C) Summary of tail current amplitudes relative to baseline after VSP activation (b/a) and after recovery (c/a). (D) Time constant of a single-exponential fit to the recovery time course (time b to c). (E) Time course of tail current amplitudes in a cell transfected with  $M_1R$  and KCNQ2/3 (1 point  $s^{-1}$ ). (F) Superimposed currents at time points a, b, and c indicated in E. (G) Tail current inhibition by  $M_1R$  activation (summary of 10 cells). (H) Time constant of an exponential fit of recovery (summary of seven cells).



**Figure 7.** Schematic of the kinetic model. Model species are denoted by Roman letters, and reactions are in italics. Initial conditions and rate constants are listed in Tables I and II, and differential equations are in Table S1. For **PIP<sub>2</sub>** and **IP<sub>3</sub>** binding to **KCNQ** and **PH**, the association reactions are referred to as forward reactions, and dissociation reactions are referred to as reverse reactions. **KCNQ2/3** current depends on the square of **PIP<sub>2</sub>**-bound **KCNQ2/3** subunits (**PIP<sub>2</sub>-KCNQ2/3**).

phosphoinositide kinases and phosphatases, we do not know the number of enzyme molecules and so assign a bulk activity rather than a molecular-specific activity. For regulation of **KCNQ2/3** current by **PIP<sub>2</sub>**, we implemented a square law as suggested by Fig. 4: **KCNQ2/3** subunits bind **PIP<sub>2</sub>** with the same dissociation constant  $K_d$  as **PH** probes, and **KCNQ2/3** current is proportional to the square of the number of **PIP<sub>2</sub>**-bound channel subunits (Fig. 4 G). This implementation closely resembles our earlier model (Suh et al., 2004), where we had a  $K_d$  for **PIP<sub>2</sub>** binding of 1,000 **PIP<sub>2</sub>** per  $\mu\text{m}^2$  (instead of 2,000) and an exponent of 1.8 (instead of 2). Activation of **PLC**

by **M<sub>1</sub>R**s was determined by the model described in our companion paper (Falkenburger et al., 2010), which among other things reproduces time course and Oxo-M concentration–response curves for the interaction of  $G_{\alpha_q}$  with **PLC** as measured by FRET (Jensen et al., 2009). As before, our model does not include the well-known contribution of  $\text{Ca}^{2+}$  as a necessary cofactor in **PLC** stimulation by  $G_q$  (Horowitz et al., 2005).

We began with **VSP**. It was simple to pick a rate constant for **VSP** activity that reproduced the rapid time course of **KCNQ** current inhibition during **VSP** activation (Fig. 8 A). Then, it was straightforward to set the rate of the endogenous **PIP** 5-kinase to reproduce current recovery from **VSP**, which takes  $\sim 11$  s (Fig. 8 B). This gave us two rate constants and good agreement with the **VSP** experiments. Fig. 8 C illustrates the decline in **PIP<sub>2</sub>** and parallel rise in **PI(4)P** during **VSP** activation and their recovery hereafter. The choice of **PIP** 5-kinase rate constant did depend on the uncertain size of the resting **PI(4)P** pool relative to the **PIP<sub>2</sub>** pool. If the resting **PI(4)P** pool was relatively small, the **PIP** 5-kinase rate constant would have to be a little faster than if the pool was of comparable size to **PIP<sub>2</sub>** (see Discussion). The new experiments (Fig. 3) also showed that the interaction of **PIP<sub>2</sub>** with **KCNQ** channels must be more rapid than we assumed previously.

We then considered strong activation of **PLC** by **M<sub>1</sub>R** activation. Again, we could easily pick a rate constant for **PLC** acting on **PIP<sub>2</sub>** to match the rate of current inhibition by 10  $\mu\text{M}$  Oxo-M (Fig. 9 A, red solid line). Given a target pool size for **PI(4)P** and the rate constant for the **PIP** 5-kinase, we could also pick a rate constant for the **PI** 4-kinase to match the slow recovery from Oxo-M (Fig. 9 B, red solid line). This gave us two more rate constants and a reasonable fit.

Next, we turned to weaker activation of **PLC** by **M<sub>1</sub>R** activation with subsaturating agonist concentrations.

TABLE I  
Initial conditions

Species	Amount	Rationale
R	500 $\mu\text{m}^{-2}$	From fluorescence <sup>a</sup>
G	40 $\mu\text{m}^{-2}$	To fit concentration–response curve of current <sup>a</sup>
PLC	10 $\mu\text{m}^{-2}$	To fit concentration–response curve of current <sup>a</sup>
PI	140,000 $\mu\text{m}^{-2}$ <sup>b</sup>	Xu et al. (2003); Suh et al. (2004)
PIP	4,000 $\mu\text{m}^{-2}$	Suh et al. (2004); ratio to <b>PIP<sub>2</sub></b> as in Willars et al. (1998); Winks et al. (2005)
PIP <sub>2</sub>	5,000 $\mu\text{m}^{-2}$	To have 50% <b>PH</b> probes at the membrane, as in Horowitz et al. (2005), similar to Xu et al. (2003)
PH domains <sup>c</sup> (membrane)	3,000 $\mu\text{m}^{-2}$	From fluorescence <sup>a</sup> , similar to Xu et al. (2003)
PH domains <sup>c</sup> (cytosol)	3 $\mu\text{M}$	1 $\mu\text{M}$ free, 2 $\mu\text{M}$ <b>PH-IP<sub>3</sub></b> ; see Fig. S3, similar to Xu et al. (2003)
KCNQ2/3 channels	4 $\mu\text{m}^{-2}$	From whole cell current, open probability, and single-channel conductance; consistent with Zaika et al. (2008)
IP <sub>3</sub>	0.16 $\mu\text{M}$	Fink et al. (1999); Xu et al. (2003); Winks et al. (2005)

<sup>a</sup>See Falkenburger et al. (2010).

<sup>b</sup>Amount of **PI** is clamped at 140,000  $\mu\text{m}^{-2}$ .

<sup>c</sup>Not present in all experiments.

TABLE II  
Rate constants for phosphoinositide metabolism

Parameter	Value	Units	Rationale
k_PL	0.1	$\mu\text{m}^2 \text{s}^{-1}$	Oxo-M onset of current inhibition
k_PLConPIP	$0.14 * k_{PL}$	$\mu\text{m}^2 \text{s}^{-1}$	See Horowitz et al. (2005)
k_PLbasal	0.0025	$\text{s}^{-1}$	To keep resting IP <sub>3</sub> at 0.16 $\mu\text{M}$
k_4K	$2.6 \times 10^{-4}$	$\text{s}^{-1}$	Current recovery after Oxo-M
k_4P	0.006	$\text{s}^{-1}$	To keep PI(4)P levels stable at rest
k_5K	0.02	$\text{s}^{-1}$	Current recovery after VSP
k_5P	0.014	$\text{s}^{-1}$	To keep PIP <sub>2</sub> levels stable at rest
k_VSP <sup>a</sup>	$11.3 * f(V)$	$\text{s}^{-1}$	Fit to VSP onset, see Fig. S1
speed_KCNQ_PIP <sub>2</sub>	0.05	$\mu\text{m}^2 \text{s}^{-1}$	Rate limiting if <0.05
KD_KCNQ_PIP <sub>2</sub>	2,000	$\mu\text{m}^{-2}$	As for PH probes
I_KCNQ <sup>b</sup>	$a * (\text{PIP}_2 - \text{KCNQ})^2$	pA	See Fig. 4
speed_PH_PIP <sub>2</sub>	1	$\mu\text{M}^{-1} \text{s}^{-1}$	Affects timing if <1
KD_PH_PIP <sub>2</sub>	2	$\mu\text{M}$	Lemmon et al. (1995); Hirose et al. (1999); as in Xu et al. (2003); Winks et al. (2005)
speed_PH_IP <sub>3</sub>	10	$\mu\text{M}^{-1} \text{s}^{-1}$	To not be rate limiting, from Xu et al. (2003)
KD_PH_IP <sub>3</sub>	0.1	$\mu\text{M}$	Hirose et al. (1999); Lemmon et al. (1995), as in Winks et al. (2005); Xu et al. (2003)
k_IP <sub>3</sub> ase	0.08	$\text{s}^{-1}$	From Xu et al. (2003)

Forward reactions of PIP<sub>2</sub> binding are speed<sub>+</sub>, and reverse reactions are speed<sub>-</sub> \* KD<sub>-</sub>; see Table S1.

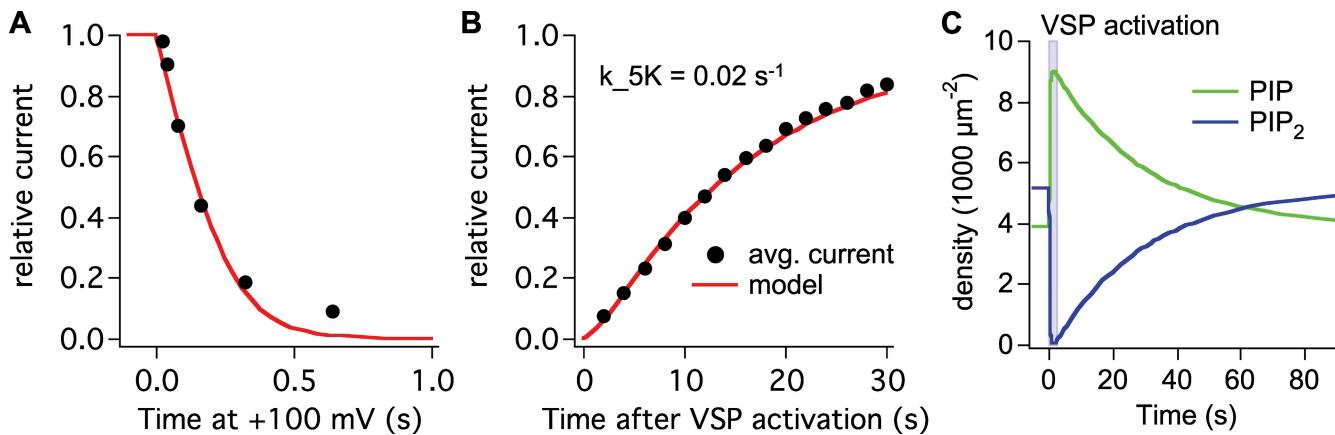
<sup>a</sup>f(V) = 1/(1+exp((-1.5\*q<sub>e</sub>/k<sub>B</sub>T\*(V-0.1))) with q<sub>e</sub>/k<sub>B</sub>T = 25 mV.

<sup>b</sup>a = channel number \* open probability \* single-channel current.

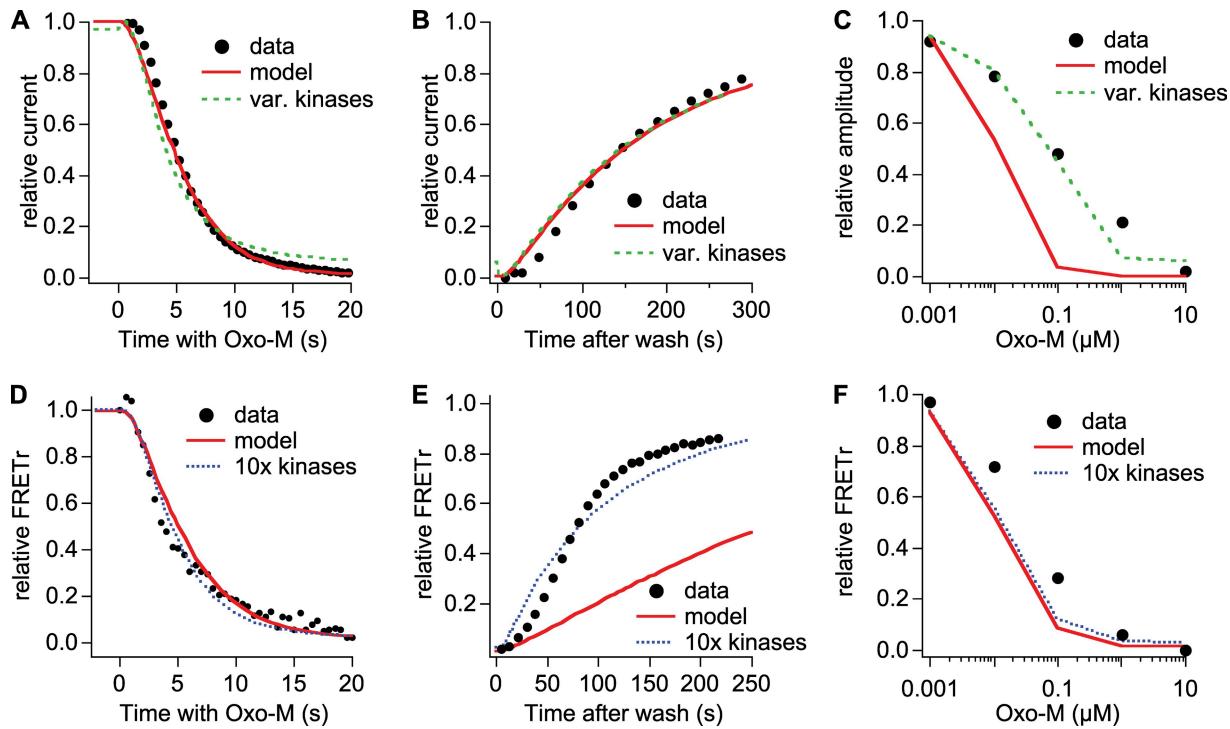
This revealed difficulties. Consider the concentration–response relations for Oxo-M inhibition of KCNQ current (data points in Fig. 9 C). The model for G protein activation by receptors and for G protein interaction with PLC is already known to give appropriate concentration–response relations for the early signaling steps (Falkenburger et al., 2010). Hence, it was unexpected that the model did very poorly with the low-concentration responses of the later signaling steps. For KCNQ current, it predicted too much suppression at low Oxo-M concentrations (0.001–1  $\mu\text{M}$ ; Fig. 9 C, red solid line). We discuss possible reasons for this discrepancy in the Discussion and show one possible solution here as the

green dashed line in Fig. 9 (A–C). In this simulation, the synthesis of new PIP<sub>2</sub> is accelerated several-fold during the Oxo-M application as several authors have already suggested (see Discussion and legend to Fig. 9). Accelerated synthesis counters the PIP<sub>2</sub> depletion catalyzed by weak activation of PLC.

Finally, we considered the actions of Oxo-M on FRET from PH domain probes. The unchanged model (red solid lines) simulates the decrease of FRETr with 10  $\mu\text{M}$  Oxo-M well (Fig. 9 D), and the concentration–response curve moderately well (Fig. 9 F), but it predicts much slower FRETr recovery than is actually seen (Fig. 9 E). Note that these calculations include the effects of 6,000  $\mu\text{m}^{-2}$



**Figure 8.** Modeling related to PIP 5-kinase and VSP. Traces are model predictions, and symbols are data. (A) Model current during VSP activation superimposed with the time course of current inhibition from Fig. 3 D. (B) Model current recovery superimposed with averaged time courses as in Fig. 6 A ( $n = 11$  cells). (C) Model predictions for PI(4)P and PIP<sub>2</sub> during VSP activation and recovery.



**Figure 9.** Modeling of  $\text{PIP}_2$  depletion by  $M_1\text{R}$  activation. Symbols are data from Jensen et al. (2009), and lines are model predictions. (A–C) KCNQ2/3 current. (D–F) PH probe FRET. (A and D) Time course of current/FRET inhibition by  $10 \mu\text{M}$  Oxo-M. (B and E) Time course of recovery after  $M_1\text{R}$  activation. (C and F) Concentration–response curves. Parameters for the red, solid curves are as listed in Tables I and II. For the blue, dotted curves, PI kinases and phosphatases were sped up by 10-fold ( $k_{-4K}$ ,  $2.6 \times 10^{-3} \text{ s}^{-1}$ ;  $k_{-4P}$ ,  $0.08 \text{ s}^{-1}$ ;  $k_{-5K}$ ,  $0.2 \text{ s}^{-1}$ ;  $k_{-5P}$ ,  $0.14 \text{ s}^{-1}$ ). For the green, dashed curves, the PI 4-kinase was sped up during Oxo-M (but not during recovery) in a manner depending on Oxo-M concentration ( $2.6 \times 10^{-4} \text{ s}^{-1}$  for  $0.001 \mu\text{M}$ ,  $5 \times 10^{-4} \text{ s}^{-1}$  for  $0.01 \mu\text{M}$ , and  $2.6 \times 10^{-3} \text{ s}^{-1}$  for  $0.1 \mu\text{M}$  and above).  $k_{-5K}$  was  $0.2 \text{ s}^{-1}$  during Oxo-M and  $0.02 \text{ s}^{-1}$  during recovery.  $k_{-4P}$  and  $k_{-5P}$  were not accelerated.  $k_{\text{PLC}}$  was  $0.2 \mu\text{m}^2 \text{ s}^{-1}$  to fit onset. PLConPIP was 0.

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of PH domains (Falkenburger et al., 2010) that are buffering the  $\text{PIP}_2$  as fast as it is made. It is our working hypothesis that in these cells that have been overexpressing PH domain probes as  $\text{PIP}_2$  buffers for 2 d, the activity of phosphoinositide-metabolizing enzymes has become augmented by compensatory gene expression. The blue dotted lines show much improvement in fitting the data from assuming that the 4- and 5-kinases and -phosphatases of these cells are augmented 10-fold. Such elevated rates are not appropriate for cells without PH domain expression and, for example, would predict recovery of current after Oxo-M in <20 s.

## DISCUSSION

We have completed a kinetic model of  $M_1\text{R}$  signaling, spanning from the binding of agonist to receptor through G proteins and PLC to  $\text{PIP}_2$  depletion and resynthesis. The steps involving receptor and G proteins are described in our companion paper (Falkenburger et al., 2010), and this work adds phosphoinositide metabolism and the gating of KCNQ2/3 current by  $\text{PIP}_2$ , with aspects of kinase regulation still unresolved. Our experiments lead to several model-independent conclusions. Princi-

pal among them are: (1)  $\text{PIP}_2$  interaction with KCNQ channels occurs in the millisecond timescale, (2) more than one bound  $\text{PIP}_2$  is needed for optimal activation of KCNQ channels, and (3)  $\text{PIP}_2$  recovery after VSP activation is much faster than after PLC activation.

### Gating of KCNQ2/3 current by $\text{PIP}_2$

Activation of Dr-VSP reduces  $\text{PIP}_2$  rapidly. KCNQ2/3 current responds to VSP activations as short as 40 ms, even before the sensing charge movement has finished. The rapidity of the inhibition means that  $\text{PIP}_2$  exchange at KCNQ2/3 channels is fast. In our earlier models (e.g., Suh et al., 2004), the residence time of  $\text{PIP}_2$  on a channel subunit was set at 4 s, but we had little evidence to go on. The VSP result here shows that 4 s is much too long because channel inhibition reaches a new steady level within tens of milliseconds after each VSP activation (Fig. 3 C). In the revised model, the residence time of  $\text{PIP}_2$  is 10 ms. It could be made shorter but not much longer. Apparently, the turnover time of  $\text{PIP}_2$  lipids on KCNQ subunits is shorter than the macroscopic gating times of the channel and is more on a timescale appropriate to underlie open–close transitions at the single-channel level, as was assumed by Park et al. (2005). This conclusion is predicated on the assumption that  $\text{PIP}_2$

must dissociate from channel subunits before VSP can cleave the 5-phosphate. Given that the PIP<sub>2</sub> binding site at VSP is buried in the enzyme molecule (Okamura et al., 2009), this assumption appears reasonable.

Recovery of PIP<sub>2</sub> and current after VSP activation was slow enough (10–20 s) to assume that KCNQ2/3 channels remain near equilibrium with PIP<sub>2</sub> throughout as the PIP<sub>2</sub> is restored gradually. The nonlinear relationship of KCNQ2/3 current to PH probe FRET indicates that current behaves like the square of the PH probe FRET and explains why KCNQ2/3 current turns off faster than PH probe FRET during PIP<sub>2</sub> depletion. Such an accelerated turn-off might be biologically relevant as a way to speed the loss of KCNQ2/3 current and the increase of neuronal excitability in response to receptor activation. The cooperativity in PIP<sub>2</sub> activation of KCNQ2/3 channels seen here in intact cells is consistent with Hill coefficients in the range of 1.35 to 1.9 for current activation obtained by application of (short-chain) diC<sub>8</sub>-PIP<sub>2</sub> to excised inside-out membrane patches (Zhang et al., 2003; Li et al., 2005).

Very few KCNQ2/3 channels are needed to measure PIP<sub>2</sub>—only around four channels  $\mu\text{m}^{-2}$  in our cells, based on whole cell current, open probability, and single-channel conductance (see also Zaika et al. 2008). That small number would not perturb the cellular PIP<sub>2</sub> pools; however, Zaika et al. (2008) report that for every electrophysiologically functional KCNQ channel, there can be many additional channels in the plasma membrane that do not contribute to current. Quite likely they would also bind PIP<sub>2</sub>. In contrast, PH probes require a high density to work as FRET reporters (Fig. S3 B): 1,700–3,000  $\mu\text{m}^{-2}$  for each PH probe (PH-CFP and PH-YFP) as determined by FRET efficiency and fluorescence intensity (Falkenburger et al., 2010). The pool of PIP<sub>2</sub> bound to the two PH probes is thus significant as compared with the pool of free PIP<sub>2</sub>, which we take to be 5,000  $\mu\text{m}^{-2}$ . This can alter phosphoinositide dynamics, as we and others find (Holz et al., 2000; Raucher et al., 2000; Lei et al., 2001; Várnai et al., 2002; Gamper et al., 2004; Szentpetery et al., 2009). The inhibition of KCNQ2/3 current by M<sub>1</sub>R activation was slower, and the recovery faster, in cells with PH probe expression (Fig. 7 in Jensen et al., 2009). The slower onset of current inhibition with PH probe expression is reproduced by the model (not depicted). It is explained by buffering of PIP<sub>2</sub> by PH probes providing a reserve of PIP<sub>2</sub> to be hydrolyzed; however, our model does not predict a faster recovery of KCNQ2/3 current with PH probe expression unless the rates of some steps are modified. It also does not reproduce a relatively fast recovery of PH probe FRET seen after M<sub>1</sub>R activation (Jensen et al., 2009). Such deviations from our simple predictions suggest that chronic expression of PH probes induces compensatory changes in phosphoinositide metabolism (see below).

## Phosphoinositide pools

Full interpretation of phosphoinositide kinetics is limited by uncertainty about the absolute endogenous levels of different phosphoinositide lipids and the enzymes that act on them at the plasma membrane and in other membranes. We begin with the lipids.

Even for PIP<sub>2</sub>, the density at the plasma membrane remains uncertain. McLaughlin et al. (2002) and Golebiewska et al. (2008) suggest an effective free concentration of 10  $\mu\text{M}$  referred to total cell volume, which is equivalent to  $\sim$ 10,000  $\mu\text{m}^{-2}$  at the membrane for the 10- $\mu\text{m}$  diameter cell they had in mind. Xu et al. (2003) give 4,000  $\mu\text{m}^{-2}$ , and Hilgemann (2007) suggests values of 20,000–60,000  $\mu\text{m}^{-2}$ . These estimates include potential errors in determining the total lipid content of a sample, the count of cells in the sample, and the plasma membrane area of each cell. According to measurements of PIP<sub>2</sub> diffusion by fluorescence correlation spectroscopy (Golebiewska et al., 2008), only one third of all PIP<sub>2</sub> (the 10  $\mu\text{M}$  above) is free, and two thirds is reversibly bound to membrane proteins with an exchange time of  $\sim$ 10 ms. In all our calculations, we assume that reactions of probes and enzymes like PIP<sub>2</sub> 5-phosphatase and PLC are restricted to free PIP<sub>2</sub> molecules. Here and before (Suh et al., 2004), we have assumed 5,000 free PIP<sub>2</sub>  $\mu\text{m}^{-2}$  in our modeling. This number would be compatible with the observation that  $\sim$ 50% of PH domains are bound to the plasma membrane and 50% are in the cytosol (Stauffer et al., 1998; van der Wal et al., 2001; Xu et al., 2003; Horowitz et al., 2005; Winks et al., 2005), with the following two assumptions: the *in vitro* dissociation constants for the binding of PIP<sub>2</sub> and of IP<sub>3</sub> to PH domains are valid, and the resting IP<sub>3</sub> concentration in the cell is 0.16  $\mu\text{M}$  (Fink et al., 1999; Xu et al., 2003; Winks et al., 2005; see Fig. S3 A). Had we assumed a high PIP<sub>2</sub> density (20,000–60,000  $\mu\text{m}^{-2}$ ), the expression of a pair of PH probes at 1,700–3,000  $\mu\text{m}^{-2}$  each would have had little impact on PIP<sub>2</sub> dynamics, so we consider this discrepancy as an argument against such high densities.

Biochemical measurements of PI and PI(4)P are even more difficult to interpret. Where are the pools of these PIP<sub>2</sub> precursors, how big are they, and where are the enzymes that act on them? The total cellular content of PI(4)P is similar to that of PIP<sub>2</sub> (Willars et al., 1998; Nasuhoglu et al., 2002; Horowitz et al., 2005). We have suggested before that the pool of PI(4)P that is accessible to PLC during a 60-s M<sub>1</sub>R activation (88% of the total) might all be at the plasma membrane (Horowitz et al., 2005). However, lipid trafficking from cytosolic vesicles to the plasma membrane might be fast enough ( $t_{1/2} = 2$  min; Maxfield and McGraw, 2004) to confound this concept. Some studies support the assumption that the majority of PI(4)P is at the plasma membrane (Hammond et al., 2009), but this could contradict the notion that PI(4)P is the characteristic phosphoinositide

of the Golgi complex and secretory vesicles. Endogenous PI 4-kinase activity is primarily associated with Golgi membranes, and overexpressed, fluorescently tagged type III PI 4-kinases localize primarily to ER and Golgi, but not to the plasma membrane (Cockcroft et al., 1985; Zhao et al., 2001; Wenk and De Camilli, 2004; Balla, 2007). Further, phosphoinositide transport proteins have been found necessary for sustained IP<sub>3</sub> generation in HL60 cells (Cunningham et al., 1995). These findings suggest that levels of PI(4)P at the plasma membrane might be low (<<88% of total PI(4)P), and that transfer of PI(4)P between membrane compartments might be rapid. However, no phosphoinositide transport proteins have been reported for PI(4)P so far. (Phosphoinositide transport proteins might also act as cofactors for lipid kinases at the plasma membrane; Wirtz, 1997.)

Our kinetic model contains a single pool of PI(4)P in the same kinetic compartment as PIP<sub>2</sub>. We have determined relative reaction fluxes from our time courses but have to remain skeptical about the absolute rate constants and absolute fluxes as long as the size and distribution of the PI(4)P pool(s) are unknown. Perhaps it is more realistic to represent the PI(4)P pool as several compartments connected by transport steps and to reinterpret the “synthesis” of PI(4)P by step 4-K in the model as including influx of PI(4)P from other compartments.

#### Metabolism of phosphoinositides

Ci-VSP has been shown to act as a PIP 5-phosphatase, both by biochemical assays and by monitoring PIP<sub>2</sub> and PI(4)P with fluorescent probes (Iwasaki et al., 2008; Halaszovich et al., 2009), implying that Dr-VSP also would act as a PIP 5-phosphatase. This is consistent with our observations that overexpression of PIP 5-kinase makes VSP activation less effective in suppressing current, requiring stronger depolarization to produce the same effect, and that recovery after VSP activation is speeded by overexpression of the PIP 5-kinase. VSP-sensing currents suggest that 13,000 VSP molecules are expressed per  $\mu\text{m}^2$  of membrane, 50% of which are activated at +100 mV. As the number of activated VSP molecules ( $6,500 \mu\text{m}^{-2}$ ) may surpass that of PIP<sub>2</sub> molecules, VSP might “deplete” all PIP<sub>2</sub> simply by binding them before completing a full cycle of enzymatic activity. Thus, the rapid burst of current inhibition by VSP might overestimate the maximum velocity of steady-state VSP action. In our model, the initial PIP<sub>2</sub> consumption rate for VSP (at +100 mV) is  $28,000 \text{ s}^{-1} \mu\text{m}^{-2}$ . For comparison, the cleavage rate with endogenous PLC during M<sub>1</sub>R activation is 35-fold slower in the model, around  $800 \text{ s}^{-1} \mu\text{m}^{-2}$ . The few PLC molecules ( $10 \mu\text{m}^{-2}$ ) would have to undergo many turnover cycles to deplete PIP<sub>2</sub> over the course of a few seconds.

Because VSP is a 5-phosphatase, recovery of PIP<sub>2</sub> and KCNQ current is governed by cellular PIP 5-kinases. All

of the depolarization-induced 5-phosphatase activity has to be from the plasma membrane VSP that the patch clamp voltage controls. Therefore, at least the PI(4)P generated by VSP activity and the PIP 5-kinase needed for rapid (few seconds) rephosphorylation should be in the plasma membrane. The chosen 5-kinase rate constant ( $0.02 \text{ s}^{-1}$ ), which depends on our assumption of initial plasma membrane PI(4)P, is in a similar range as in previous models ( $0.045 \text{ s}^{-1}$  in Horowitz et al., 2005;  $0.048 \text{ s}^{-1}$  in Xu et al., 2003).

The 5-kinase could be regulated. M<sub>1</sub>R activation can activate Rho kinase (Dutt et al., 2002), and several studies have shown stimulation of PIP 5-kinases by Rho family kinases and other signaling events (for review see Oude Weernink et al., 2004; Santarius et al., 2006; Mao and Yin, 2007). Measuring lipid and IP<sub>3</sub> turnover in response to muscarinic agonists, Willars et al. (1998) found: (1) both PIP<sub>2</sub> and PI(4)P are depleted quickly (as we also found; Horowitz et al., 2005), (2) yet IP<sub>3</sub> continues to be made in long stimulations, and (3), surprisingly, PIP<sub>2</sub> recovers before PIP. The more rapid recovery of PIP<sub>2</sub> might be explained by strong transient stimulation of PIP 5-kinase (and not the 5-phosphatase) during the recovery period. The extensive depletion of PI(4)P during receptor activation has at least two possible explanations. Accelerated 5-kinase could be converting the PI(4)P pool to PIP<sub>2</sub> that is then cleaved by PLC, or PLC might accept PI(4)P as a substrate in addition to PIP<sub>2</sub> (Wilson et al., 1984). Here, as before (Horowitz et al., 2005), our model assumes that PLC is able to cleave PI(4)P slowly (Table II), but we also suggest that speeding the 5-kinase during receptor activation helps explain the concentration–response relation for current (Fig. 9 C). Nevertheless, our measurements and modeling do not provide a clear preference for assuming that PLC does or does not cleave PI(4)P significantly. Removing the assumption forced changes in other rate constants but did not improve the fitting of, for example, the concentration–response curves.

Recovery after M<sub>1</sub>R activation requires a wortmannin-sensitive (type III) PI 4-kinase (Suh and Hille, 2002; Zhang et al., 2003; Winks et al., 2005); therefore, we called the step that supplies PI(4)P at the membrane “4-K.” This step may also be accelerated by receptor activation. Stimulation of PI 4-kinase by receptor activation has been invoked in studies estimating IP<sub>3</sub> production (e.g., Cunningham et al., 1995; Willars et al., 1998; Xu et al., 2003; Brown et al., 2008) and by the failure of bradykinin and purinergic agonists to deplete PIP<sub>2</sub> despite activating PLC in neurons (Gamper et al., 2004; Zaika et al., 2007). We also suggest a faster rate for this step during Oxo-M than during recovery to reproduce our data with subsaturating Oxo-M (Fig. 9, A–C, green dashed lines; see also details in the legend). It would prevent low agonist concentrations from depleting PIP<sub>2</sub> excessively. If PI 4-kinase and PIP 5-kinase are stimulated

by receptor activation, we do not know how long that effect lasts after agonist is removed. Hence, our estimates of their rates after agonist removal may be imperfect. (We expect no acceleration of kinases by VSP activation.) Current recovery from agonist was faster after overexpression of PLC or PH probes (Jensen et al., 2009). In these cases, however, the speeding might represent compensatory gene expression.

What is the mechanism of receptor-induced stimulation of PI(4)P synthesis? The speeding of PI 4-kinase by bradykinin requires IP<sub>3</sub>-mediated calcium release and neuronal calcium sensor 1 (Gamper et al., 2004; Zaika et al., 2007). Calmodulin-like neuronal calcium sensor 1 binds to PI 4-kinase β (in the Golgi) and stimulates the 4-kinase activity when calcium is increased by receptor activation (Zhao et al., 2001; Koizumi et al., 2002; Pan et al., 2002; Winks et al., 2005). The stimulation can be so strong that PIP<sub>2</sub> is not depleted despite PLC activation. It is possible that M<sub>1</sub>R activation, which makes a Ca<sup>2+</sup> transient in tsA201 cells, stimulates PI 4-kinase by a similar mechanism, but to a lesser extent. There might also be other messengers. If type III PI 4-kinases are located in the Golgi complex, it is possible that not the PI 4-kinase but the PI(4)P transport to the plasma membrane might be rate limiting and stimulated by receptor activation. Our model would not discriminate between the stimulation of PI 4-kinases and any other event that transiently increases plasma membrane PI(4)P. One such possibility is exocytosis. Secretory vesicles are thought to bear PI(4)P formed by PI 4-kinases in Golgi and by 5-phosphatases during endocytosis (De Matteis and Godi, 2004; Wenk and De Camilli, 2004; Balla et al., 2005). Exocytosis can increase upon stimulation of Gα<sub>q</sub>-coupled receptors both by Ca<sup>2+</sup> release and by activating PKC (e.g., Hille et al., 1999), and vesicle fusion with the plasma membrane would deliver PI(4)P to the plasma membrane.

The time course of Oxo-M effects on PH probe FRET was well reproduced with increased rates for PIP<sub>2</sub> synthesis (Fig. 9, D–F, dotted traces). Interestingly, the acceleration had little effect on the depression of PH probe FRET by Oxo-M (Fig. 9, D and F, dotted line vs. solid line). The reason is that PH probe FRET also responds to production of a second ligand, IP<sub>3</sub>, whereas current depends only on PIP<sub>2</sub>.

We proposed receptor-induced acceleration of PIP<sub>2</sub> synthesis in part to correct a discrepancy in the predicted agonist concentration–response curve and in part because it is frequently invoked in the literature. The discrepancy was that low concentrations of agonist suppressed currents too much in the model as if PLC were activated too strongly there. Now we suggest a second factor that is likely to contribute to this discrepancy as well. Strong stimulation of M<sub>1</sub>Rs in tsA cells invokes a Ca<sup>2+</sup> transient (via IP<sub>3</sub>) that accelerates PLC strongly in a positive feedback loop (Horowitz et al., 2005). In our model, PLC activity depends on Gα<sub>q</sub>, but the Ca<sup>2+</sup> de-

pendence is omitted. At low agonist concentrations, the IP<sub>3</sub> and Ca<sup>2+</sup> positive feedback should be much less, so our model will overestimate the stimulation of PLC there. This defect cannot be modeled until we study how IP<sub>3</sub> production, Ca<sup>2+</sup> elevation, and feedback to PLC depend on receptor activation. At high agonist concentrations, the time it takes to produce a full Ca<sup>2+</sup> elevation might also contribute to a small delay we see in the onset of current suppression after agonist application (Table I in Jensen et al., 2009).

### Conclusions

We have constructed a kinetic model of phosphoinositide metabolism informed by new kinetic studies after rapid dephosphorylation of PIP<sub>2</sub> by Dr-VSP. Although this model represents a substantial advance from the previous one, it also shows clearly where we have to learn more. We need better estimates of phosphoinositide amounts in different membrane compartments and to determine the extent to which compartments other than the plasma membrane are involved in M<sub>1</sub>R signaling and lipid dynamics. In particular, the step supplying PI(4)P at the plasma membrane remains elusive. It is possible that events other than phosphorylation of PI at the plasma membrane contribute to the timing. We have shown that the step synthesizing PIP<sub>2</sub> from PIP is much faster than the preceding step that supplies PIP, and we have advanced understanding of how PIP<sub>2</sub> binding allows KCNQ2/3 channels to open. More than one PIP<sub>2</sub> is needed per channel, and the residence time on a channel subunit is <10 ms. This insight is valuable for using KCNQ2/3 current to monitor cellular PIP<sub>2</sub> levels and might generalize to other PIP<sub>2</sub>-regulated ion channels. Finally, we have shown that the voltage regulation of VSP activity is fast and makes VSP a powerful tool for investigating and perturbing phosphoinositide physiology and metabolism.

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