

Regulation of voltage-gated potassium channels by PI(4,5)P₂

Martin Kruse,¹ Gerald R.V. Hammond,² and Bertil Hille¹

¹Department of Physiology and Biophysics, University of Washington, Seattle, WA 98195

²Department of Pharmacology, University of Cambridge, Cambridge CB2 1PD, England, UK

Phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂) regulates activities of numerous ion channels including inwardly rectifying potassium (K_{ir}) channels, KCNQ, TRP, and voltage-gated calcium channels. Several studies suggest that voltage-gated potassium (K_v) channels might be regulated by PI(4,5)P₂. Wide expression of K_v channels in different cells suggests that such regulation could have broad physiological consequences. To study regulation of K_v channels by PI(4,5)P₂, we have coexpressed several of them in tsA-201 cells with a G protein-coupled receptor (M₁R), a voltage-sensitive lipid 5-phosphatase (Dr-VSP), or an engineered fusion protein carrying both lipid 4-phosphatase and 5-phosphatase activity (pseudojanin). These tools deplete PI(4,5)P₂ with application of muscarinic agonists, depolarization, or rapamycin, respectively. PI(4,5)P₂ at the plasma membrane was monitored by Förster resonance energy transfer (FRET) from PH probes of PLCδ1 simultaneously with whole-cell recordings. Activation of Dr-VSP or recruitment of pseudojanin inhibited K_v7.1, K_v7.2/7.3, and K_{ir}2.1 channel current by 90–95%. Activation of M₁R inhibited K_v7.2/7.3 current similarly. With these tools, we tested for potential PI(4,5)P₂ regulation of activity of K_v1.1/K_vβ1.1, K_v1.3, K_v1.4, and K_v1.5/K_vβ1.3, K_v2.1, K_v3.4, K_v4.2, K_v4.3 (with different KChIPs and DPP6-s), and hERG/KCNE2. Interestingly, we found a substantial removal of inactivation for K_v1.1/K_vβ1.1 and K_v3.4, resulting in up-regulation of current density upon activation of M₁R but no changes in activity upon activating only VSP or pseudojanin. The other channels tested except possibly hERG showed no alteration in activity in any of the assays we used. In conclusion, a depletion of PI(4,5)P₂ at the plasma membrane by enzymes does not seem to influence activity of most tested K_v channels, whereas it does strongly inhibit members of the K_v7 and K_{ir} families.

INTRODUCTION

Voltage-gated potassium (K_v) channels are essential for repolarization of action potentials in neurons and cardiac, skeletal, and smooth muscle (Hille, 2001; Oliver et al., 2004; Pongs and Schwarz, 2010). Dysfunction of K_v channels can lead to severe disease phenotypes ranging from forms of epilepsy to cardiac arrhythmias (Peters et al., 2005; Brown and Passmore, 2009; Charpentier et al., 2010). Because of their important role in governing cell excitability, K_v channel activities are tightly controlled. Several modulatory mechanisms have been described. They include phosphorylation and dephosphorylation (Covarrubias et al., 1994; Martens et al., 1999), binding of calcium ions or of calcium-binding proteins like calmodulin (Gamper et al., 2005), binding of ATP (Seino, 1999), and translocation of channels into different cellular compartments by removal from the cell surface (Hicke, 1999). Over the last years, phospholipids have emerged as additional modulators of ion channels including K_v channels, especially the low-abundance plasma membrane phosphoinositide, phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂; Hilgemann and Ball,

1996; Hilgemann et al., 2001; Suh and Hille, 2002, 2008; Oliver et al., 2004; Falkenburger et al., 2010a,b; Logothetis et al., 2010; Suh et al., 2010). Here we investigate the PI(4,5)P₂ sensitivity of K_v channels.

PI(4,5)P₂ is localized to the cytoplasmic leaflet of the plasma membrane where it regulates ion channel and transporter activity and plays a role in cellular processes like exo- and endocytosis (Czech, 2000; Hille, 2001; Oliver et al., 2004; Di Paolo and De Camilli, 2006; Pongs and Schwarz, 2010). PI(4,5)P₂ can regulate ion channels by binding directly within the channel structure and modulating their gating (Peters et al., 2005; Brown and Passmore, 2009; Charpentier et al., 2010; Hansen et al., 2011; Whorton and MacKinnon, 2011), and it also is the precursor for the generation of second messengers like diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP₃) through the cleavage of PI(4,5)P₂ by phospholipase C (PLC; Covarrubias et al., 1994; Martens et al., 1999; Rhee, 2001). DAG and IP₃ in turn activate enzymes like PKC and increase intracellular Ca²⁺ levels, both known modulators of ion channel activity (Covarrubias et al., 1994; Martens et al., 1999; Gamper et al., 2005; Nilius et al., 2005). Therefore, regulation

Correspondence to Bertil Hille: hille@u.washington.edu

Abbreviations used in this paper: CHO, Chinese hamster ovary; DAG, diacylglycerol; FRET, Förster resonance energy transfer; IP₃, inositol 1,4,5-trisphosphate; K_v, voltage-gated potassium; Oxo-M, oxotremorine methiodide; PI(4,5)P₂, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C.

© 2012 Kruse et al. This article is distributed under the terms of an Attribution–Noncommercial–Share Alike–No Mirror Sites license for the first six months after the publication date (see <http://www.rupress.org/terms>). After six months it is available under a Creative Commons License (Attribution–Noncommercial–Share Alike 3.0 Unported license, as described at <http://creativecommons.org/licenses/by-nc-sa/3.0/>).

of ion channel activity by $\text{PI}(4,5)\text{P}_2$ breakdown can be through direct loss of a cofactor for channel gating or through secondary modifications.

Direct regulation of K_V channels by $\text{PI}(4,5)\text{P}_2$ has so far been reported for some $\text{K}_\text{V}1$, $\text{K}_\text{V}3$, $\text{K}_\text{V}7$, and $\text{K}_\text{V}11$ family members (Seino, 1999; Bian et al., 2001, 2004; Suh and Hille, 2002; Zhang et al., 2003; Oliver et al., 2004; Winks et al., 2005; Li et al., 2005; Decher et al., 2008). For example, quite dramatic changes of gating kinetics of exogenously expressed $\text{K}_\text{V}1.1/\text{K}_\text{V}\beta 1.1$, $\text{K}_\text{V}1.5/\text{K}_\text{V}\beta 1.3$, and $\text{K}_\text{V}3.4$ channels by $\text{PI}(4,5)\text{P}_2$ were seen in membrane patches excised from oocytes of *Xenopus laevis*. Addition of $\text{PI}(4,5)\text{P}_2$ to the cytoplasmic face led to a strong increase in current amplitudes through a near elimination of the normal rapid channel inactivation gating (Hicke, 1999; Oliver et al., 2004; Decher et al., 2008). Similar experiments with excised patches from oocytes or Chinese hamster ovary (CHO) cells expressing $\text{K}_\text{V}7$ (KCNQ) channels showed a $\text{PI}(4,5)\text{P}_2$ requirement for channel opening (Hilgemann and Ball, 1996; Hilgemann et al., 2001; Suh and Hille, 2002, 2008; Zhang et al., 2003; Oliver et al., 2004; Li et al., 2005; Falkenburger et al., 2010a,b; Logothetis et al., 2010; Suh et al., 2010), but for $\text{K}_\text{V}7$ channels, much additional evidence comes from another approach, namely by manipulating enzymes that make or destroy $\text{PI}(4,5)\text{P}_2$ in intact cells. The initial experiments on $\text{K}_\text{V}7$ channels used PLC and inhibitors of lipid kinases (Suh and Hille, 2002), and subsequent work exploited $\text{PI}(4,5)\text{P}_2$ phosphatases either by dimerization to a membrane anchor (Suh et al., 2006) or by voltage activation of a voltage-sensitive phosphatase (Murata and Okamura, 2007; Falkenburger et al., 2010b). We consider that by preserving the cellular environment and manipulating enzymes, such experiments explore a more physiological distribution and concentration range of $\text{PI}(4,5)\text{P}_2$ levels. Of the K_V channels, only $\text{K}_\text{V}7.2$ and $\text{K}_\text{V}7.3$ have been studied by this style of experiment. In this study, we express members of other families of K_V channels together with enzymes in tsA-201 cells, allowing us to manipulate $\text{PI}(4,5)\text{P}_2$ levels either by a change in membrane potential or by application of agonists like oxotremorine methiodide (Oxo-M) and rapamycin as we recorded K_V channel activity using whole-cell recording.

MATERIALS AND METHODS

Cell culture and plasmids

tsA-201 cells were cultured at 37°C and 5% CO_2 in DMEM (Invitrogen) supplemented with 10% FBS (PAA) and 0.2% penicillin/streptomycin (Invitrogen). Cells were plated at a density of 50–70% in 35-mm dishes 1 d before transfection. Transfection was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's specifications with 0.2–1.0 μg DNA per 35-mm dish. Cells were plated onto poly-L-lysine-coated glass chips 14–18 h before experiments. In brief, cells were treated with 400 μl 0.05% trypsin (Invitrogen) for 40 s before adding 1 ml DMEM

with FBS. The cell suspension was spun down for 1 min at 250 g and resuspended in 1 ml FBS-supplemented DMEM. 200- μl cell suspension was added to a 35-mm plastic dish with coated glass chips and incubated for 14–18 h at 37°C and 5% CO_2 .

The following plasmids were given to us: human eCFP-PH(PLC δ 1) and eYFP-PH(PLC δ 1) from K. Jalink (The Netherlands Cancer Institute, Amsterdam, Netherlands); Dr-VSP-IRES-GFP (Dr-VSP) of zebrafish (*Danio rerio*) from Y. Okamura (Osaka University, Osaka, Japan); LDR (Lyn $_{11}$ -targeted FRB)-CFP from T. Balla (National Institutes of Health, Bethesda, MD); the phosphatase construct FKBP (FK506-binding protein)-Inp54p from T. Inoue (Johns Hopkins University School of Medicine, Baltimore, MD); M $_1$ R (M $_1$ muscarinic receptor)-YFP from N. Nathanson (University of Washington, Seattle, WA); rat $\text{K}_\text{V}1.1$, rat $\text{K}_\text{V}1.4$, and rat $\text{K}_\text{V}\beta 1.1$ from J. Trimmer (University of California, Davis, CA); human $\text{K}_\text{V}1.3$, human $\text{K}_\text{V}1.5$, human $\text{K}_\text{V}\beta 1.3$, and human $\text{K}_\text{V}2.1$ as well as hERG from O. Pongs (University of Hamburg, Hamburg, Germany); human KCNE2 from G. Abbott (Weill Cornell Medical College, New York, NY); rat $\text{K}_\text{V}3.4$ from J. Surmeier (Northwestern University, Chicago, IL); human $\text{K}_\text{V}4.2$, $\text{K}_\text{V}4.3$, and human KChIP1, KChIP2, and DPP6-s from B. Rudy (New York University School of Medicine, New York, NY); hK $_7$ 1-GFP and hKCNE1 from M. Shapiro (University of Texas, San Antonio, TX); human $\text{K}_\text{V}7.2$ and human $\text{K}_\text{V}7.3$ from D. McKinnon (State University of New York, Stony Brook, NY) and T. Jentsch (Max Delbrück Center for Molecular Medicine Berlin-Buch, Berlin, Germany); and human K $_r$ 2.1 from D. Logothetis (Virginia Commonwealth University, Richmond, VA). We also used a new dual phosphatase, pseudojanin to deplete both $\text{PI}(4,5)\text{P}_2$ and $\text{PI}(4)\text{P}$ (Lindner et al., 2011; Hammond et al., 2012). In brief, the construct was based around a previously described expression construct (Varnai et al., 2006) consisting of mRFP and FKBP, to which the *Saccharomyces cerevisiae* Sac1p phosphatase (GenBank/EMBL/DBJ accession no. NM_001179777; residues 2–517) and the INPP5E 5-phosphatase domains (GenBank accession no. NM_019892; residues 214–644; with the C-terminal prenylation motif destroyed by mutagenesis) were inserted separated by a flexible linker (GGTARGAAA[GAG] $_2$ R). Pseudojanin-YFP was generated by replacing mRFP with YFP using NheI and NotI. “Dark” Dr-VSP (without IRES-GFP) was generated by subcloning the Dr-VSP cassette into pcDNA3.0 (Falkenburger et al., 2010b).

Electrophysiology

Whole-cell recordings were made with an EPC9 patch-clamp amplifier (HEKA) at a sampling rate of 10 kHz. Patch electrodes had a DC resistance between 1 and 3 M Ω when filled with intracellular solution for whole-cell recordings. Series resistance was compensated by 75% after compensation of fast and slow capacitance. For recordings of K_V channels, bath solution (Ringer's) consisted of 160 mM NaCl, 2.5 mM KCl, 2 mM CaCl_2 , 1 mM MgCl_2 , 8 mM glucose, and 10 mM HEPES at pH 7.4. Pipette solution contained 175 mM KCl, 5 mM MgCl_2 , 0.1 mM K_r -BAPTA, 3 mM Na_2ATP , 0.1 mM Na_3GTP , and 5 mM HEPES at pH 7.4. For recordings of $\text{K}_\text{r}2.1$ channels, bath solution consisted of 135 mM NaCl, 5 mM KCl, 1 mM MgCl_2 , 10 mM glucose, and 10 mM HEPES at pH 7.3. Pipette solution contained 140 mM KCl, 2 mM MgCl_2 , 0.1 mM K_r -BAPTA, 3 mM Na_2ATP , 0.1 mM Na_3GTP , and 10 mM HEPES at pH 7.3. PMA was dissolved in DMSO at a concentration of 10 mM. A final concentration of 100 nM in Ringer's solution was achieved by dilution of PMA stock solution in Ringer's solution. Voltage protocols are given in figures and legends. Recordings were performed at room temperature (22–25°C).

Photometric measurements of calcium and Förster resonance energy transfer (FRET)

FRET between CFP and YFP was measured as previously described (Falkenburger et al., 2010a) and reported as the corrected

fluorescence emission ratio FRET_r. For intracellular Ca²⁺ measurements, 100 μ M Fura-4F (Invitrogen) was added to the pipette solution and dialyzed into cells via the whole-cell pipette. Optical recordings were started 5 min after establishing whole-cell configuration to allow equilibration of the dye into the cells. Fura-4F was excited at 340 and 380 nm, and changes in Ca²⁺ concentrations are reported as the ratio F₃₄₀/F₃₈₀ of fluorescence emission intensities recorded with a 535/30-nm emission filter.

Confocal laser microscopy

All experiments were performed at room temperature on an LSM710 microscope (Carl Zeiss). Cells were superfused with Ringer's solution throughout the experiments.

Data analysis and statistics

Data analysis used Igor Pro (WaveMetrics) and Excel (Microsoft). Statistical data are given as mean \pm SEM unless otherwise stated. Student's *t* test was used to test for statistical significance. P-values of <0.05 were considered significant.

Online supplemental material

Figs. S1–S6 provide current traces of K_v7.2/K_v7.3 channels in response to activation of M₁R (Fig. S1), current traces of endogenous K_v channels in tsA-201 cells (Fig. S2), current traces of K_v2.1 and K_v7.2/K_v7.3 channels in response to activation of M₁R (Fig. S3), FRET_r traces of PH probes in response to an activation of Dr-VSP combined with simultaneous whole-cell recording of K_v1.5/K_vβ1.3 current (Fig. S4), line scan measurements of PH probe fluorescence in cells before and after activation of M₁R, Dr-VSP, and pseudonin (Fig. S5), and current traces of K_v4.3 channels alone or with coexpressed auxiliary subunits before and after activation of Dr-VSP (Fig. S6). Online supplemental material is available at <http://www.jgp.org/cgi/content/full/jgp.201210806/DC1>.

RESULTS

Modulation of K_v7 channels by PI(4,5)P₂ depletion at the plasma membrane

Unexpectedly, many of the K_v channels we studied proved to be insensitive to enzymatic depletion of PI(4,5)P₂. Because of such negative results, we begin with control experiments. They verify the effectiveness of our tools for manipulating phosphoinositides in tsA-201 cells using two families of potassium channels already known to be PI(4,5)P₂ sensitive. First, we and others have shown a strong PI(4,5)P₂ requirement of K_v7.2 (KCNQ2)/K_v7.3 (KCNQ3) channel currents. Decreases in PI(4,5)P₂ lead to a strong inhibition of K_v7.2/K_v7.3-mediated currents (Suh and Hille, 2002; Zhang et al., 2003; Li et al., 2005; Winks et al., 2005; Falkenburger et al., 2010b).

Fig. 1 A shows typical potassium outward current in K_v7.2/K_v7.3 channels. The channels activate during a test pulse to -20 mV from a holding potential of -60 mV and deactivate with a characteristic tail current upon stepping back to -60 mV. These channels were coexpressed with a voltage-sensitive lipid 5-phosphatase (Dr-VSP; Murata et al., 2005; Murata and Okamura, 2007; Okamura et al., 2009). The phosphatase dephosphorylates PI(4,5)P₂ on the 5 position to give PI(4)P during large depolarization. Activation of Dr-VSP by a 2-s test pulse to 100 mV led to an immediate $94 \pm 4\%$

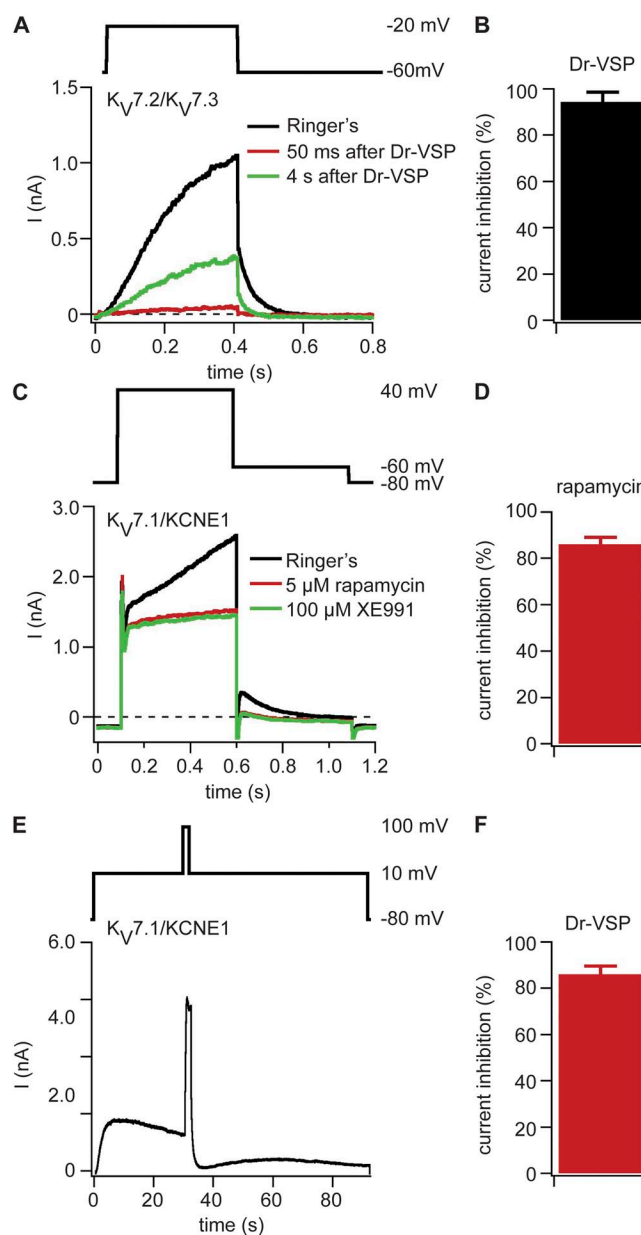


Figure 1. PI(4,5)P₂ depletion at the plasma membrane inhibits K_v7.x channels. (A) Currents with coexpression of K_v7.2, K_v7.3, and Dr-VSP in tsA-201 cells. The black current trace was recorded before Dr-VSP activation, the red trace was recorded 50 ms after Dr-VSP activation by a 2-s pulse to 100 mV, and the green trace was recorded 4 s after Dr-VSP activation. Test pulse protocol is shown above the traces. (B) Reduction of K_v7.2/K_v7.3-mediated tail currents by activation of Dr-VSP (*n* = 10). (C) Current traces recorded from a cell expressing K_v7.1, KCNE1, Ins-5-P-FKBP-CFP, and LDR-CFP. Traces are shown before application of 5 μ M rapamycin (black), after 60 s of rapamycin application (red), and after application of 100 μ M XE991 (green). (D) Rapamycin-induced inhibition of XE991-sensitive current (*n* = 5). (E) Current trace of a cell expressing K_v7.1, KCNE1, and Dr-VSP. Currents were recorded at 10 mV, Dr-VSP was activated after 30 s with a 2-s long test pulse to 100 mV, and then the membrane potential was returned to 10 mV. At the end of the recording, XE991 was applied to determine the amount of K_v7.1-mediated current (not depicted). (F) Inhibition of XE991-sensitive current by Dr-VSP activation (*n* = 6). (B, D, and F) Error bars represent \pm SEM.

($n = 10$) inhibition of the current (Fig. 1, A and B) followed by partial recovery at 4 s and typically a nearly complete recovery by 20 s as PI(4,5)P₂ levels returned to their original state. Fig. S1 shows a comparable inhibition of K_V7.2/K_V7.3 channels upon activation of coexpressed M₁ muscarinic receptors (M₁R), as in previous work (Shapiro et al., 2000). Thus, activation of Dr-VSP or of M₁R depletes PI(4,5)P₂ sufficiently to elicit a nearly full but reversible drop in K_V7.2/K_V7.3 channel activity.

After testing the familiar K_V7.2/K_V7.3 channel, we tried the closely related K_V7.1, which has not been studied previously by the voltage-sensitive phosphatase method, together with its β subunit KCNE1. Their co-expression reconstitutes most characteristics of the I_{Ks} current recorded in cardiac myocytes (Charpentier et al., 2010). Recent studies suggest regulation of this channel complex by PI(4,5)P₂ (Loussouarn et al., 2003; Park et al., 2005; Charpentier et al., 2010; Piron et al., 2010; Li et al., 2011). We expressed K_V7.1 and KCNE1 together with a rapamycin-translocatable lipid 5-phosphatase (FKBP-Inp54p) and the membrane anchor LDR-CFP. The FKBP and LDR domains become tightly (irreversibly) dimerized when membrane-permeable rapamycin is added; recruitment of the lipid phosphatase to the plasma membrane dephosphorylates PI(4,5)P₂ on the 5 position to PI(4)P (Inoue et al., 2005). The K_V7.1/KCNE1 channel activates very slowly and requires a large depolarization, so we used a 500-ms test pulse to 40 mV from a holding potential of -80 mV and measured outward tail currents at -60 mV (Fig. 1 C). Upon application of rapamycin, the slowly activating outward currents and the tail currents were

nearly eliminated (Fig. 1 C). We applied 100 μ M XE991, a K_V7 channel inhibitor, to block any residual K_V7.1/KCNE1-mediated current and found the remaining current to be almost all XE991 insensitive (Fig. 1 C). On average, rapamycin-induced depletion of PI(4,5)P₂ suppressed the K_V7.1/KCNE1-mediated currents by $86 \pm 3\%$ ($n = 5$; Fig. 1 D). We considered the XE991-insensitive current to represent endogenous K⁺ current of the tsA-201 cells. A similar XE991-insensitive current was present in mock-transfected cells (Fig. S2). It activates mainly with large depolarizations positive to 0 mV.

Does transient PI(4,5)P₂ depletion by activation of Dr-VSP also inhibit K_V7.1/KCNE1 channels? We coexpressed these proteins, activated channels by a long test pulse to 10 mV, and recorded the resulting potassium outward current. After 30 s, we activated Dr-VSP by a 2-s test pulse to 100 mV and stepped back to the 10 mV level for 60 s more (Fig. 1 E). The K_V7.1/KCNE1-mediated outward current was initially decreased by $86 \pm 4\%$ ($n = 6$; Fig. 1 F). At the end of the test pulse, XE991 was applied to correct for endogenous currents. The K_V7.1/KCNE1-mediated currents recovered modestly after the Dr-VSP-activating test pulse was turned off, presumably reflecting partial recovery of PI(4,5)P₂ levels after the transient depletion overlaid with slow accumulating channel inactivation at 10 mV (Fig. 1 E). Thus, both Inp54p recruitment to the plasma membrane and activation of Dr-VSP led to extensive inhibition of K_V7.1/KCNE1 channel activity, confirming direct and strong regulation of this channel by PI(4,5)P₂. We score the effects of PI(4,5)P₂ depletion on K_V7 channels as strong inhibition in summary Table 1.

TABLE 1
M₁ receptor and phosphatase effects on K_V channel

α subunit	Auxiliary subunit	M ₁ R	Dr-VSP	Pseudojanin or FKBP-Inp54p (in case of K _V 7.1)
K _V 1.1	K _V β 1.1	Slowing of inactivation ^a	No effect	No effect
K _V 1.3	None	No effect	ND	No effect
K _V 1.4	None	No effect	No effect	No effect
K _V 1.5	\pm K _V β 1.3	No effect	No effect	No effect
K _V 2.1	None	$\sim 22\%$ decrease	No effect	No effect
K _V 3.4	None	Slowing of inactivation ^a	No effect	No effect
K _V 4.2/K _V 4.3	None	No effect	No effect	ND
K _V 4.2/K _V 4.3	KChIP1	ND	No effect	ND
K _V 4.2/K _V 4.3	KChIP1 + DPP6-s	ND	No effect	ND
K _V 4.2/K _V 4.3	KChIP2	ND	No effect	ND
K _V 7.1 (KCNQ1)	KCNE1	ND	Strong inhibition	Strong inhibition
K _V 7.2/7.3 (KCNQ2/Q3)	None	Strong inhibition ^a	Strong inhibition ^a	Strong inhibition
K _V 11.1 (hERG)	None	ND	No effect	ND
K _V 11.1 (hERG)	KCNE2	$\sim 30\%$ decrease	No effect	No effect
K _V 12.1	None	No effect	$\sim 45\%$ decrease	Strong inhibition

^aThese effects duplicate published work, see Discussion.

$K_{ir}2.1$ channels are inhibited by activation of Dr-VSP or pseudojanin

The second potassium channel family whose $PI(4,5)P_2$ requirement is well established is the inwardly rectifying K_{ir} family. The Logothetis laboratory compared dose-response studies for restoration of channel activity by solutions of water-soluble $diC_8-PI(4,5)P_2$ applied to excised membrane patches containing $K_{V7.2}/K_{V7.3}$ (apparent $K_d = 87 \mu M$; Zhang et al., 2003; Li et al., 2005) or containing $K_{ir}2.1$ (apparent $K_d = 5 \mu M$; Rohács et al., 2003). Comparing the midpoints of these curves suggests that $K_{ir}2.1$ channels have a 17-fold higher $PI(4,5)P_2$ affinity. Can our methods deplete $PI(4,5)P_2$ enough to turn off such a high-affinity channel? We addressed this question by expressing $K_{ir}2.1$ together either with Dr-VSP, M_1R , or with the new translocatable dual phosphatase tool pseudojanin and LDR. Pseudojanin can be recruited to the plasma membrane by addition of rapamycin to

cells expressing LDR where it converts $PI(4,5)P_2$ in two steps all the way to PI , depleting both $PI(4,5)P_2$ and $PI(4)P$ (Hammond et al., 2012).

In the first experiments, we expressed $K_{ir}2.1$ together with Dr-VSP. Test pulses to -120 mV from a holding potential of -60 mV elicited inwardly rectifying potassium currents in this channel. Dr-VSP was activated by a 2-s depolarization to 100 mV. It reduced $K_{ir}2.1$ -mediated currents by $47 \pm 6\%$ ($n = 5$; Fig. 2, A and B). The amount of inhibition is less than it was for K_V7 channels, consistent with the concept that a higher $PI(4,5)P_2$ affinity would reduce the ability of our tools to inhibit the channel. Nevertheless, even for this channel with very high $PI(4,5)P_2$ affinity, a short depletion by Dr-VSP is able to inhibit half the current.

We hypothesized that pseudojanin's dual phosphatases might be able to decrease $K_{ir}2.1$ activity even further as it continuously dephosphorylates $PI(4,5)P_2$ and

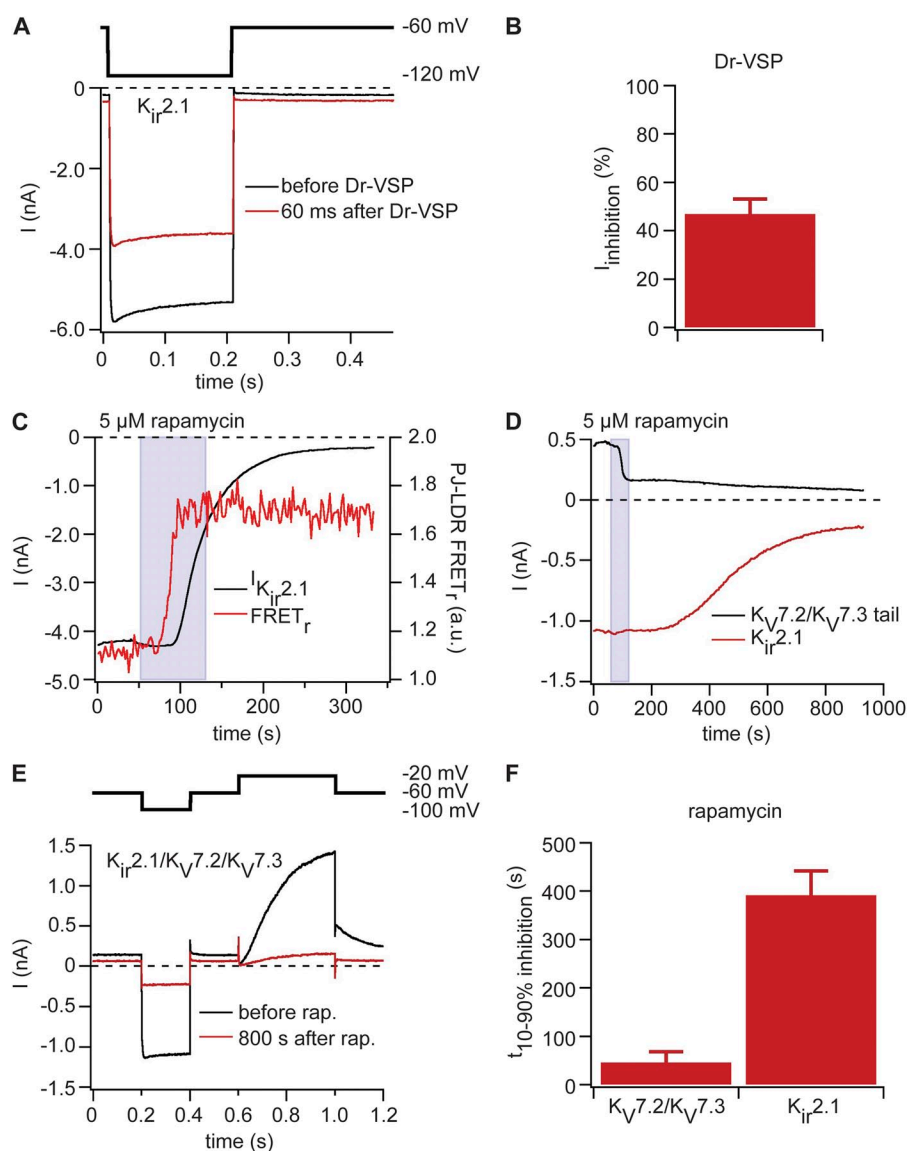


Figure 2. Dr-VSP and pseudojanin can inhibit $PI(4,5)P_2$ -sensitive $K_{ir}2.1$ channels. (A) Currents in $K_{ir}2.1$ channels coexpressed with Dr-VSP. Black indicates current trace before activation of Dr-VSP, and red indicates current trace 60 ms after activation of Dr-VSP by a 2-s pulse to 100 mV. Hyperpolarizing test pulse protocol is shown above the traces. (B) Inhibition of $K_{ir}2.1$ -mediated currents by activation of Dr-VSP ($n = 5$). (C) Currents with coexpression of $K_{ir}2.1$, pseudojanin (PJ)-YFP, and LDR-CFP recorded with the protocol shown in A. Black trace shows steady-state current over time, and the red trace is FRET ratio between pseudojanin-YFP and LDR-CFP. (D) Same experiment as in C but with expression of $K_V7.2$ and $K_V7.3$ as well as $K_{ir}2.1$. Black trace shows $K_V7.2/K_V7.3$ -mediated tail currents, and red trace shows $K_{ir}2.1$ -mediated steady-state current over time. (E) Current traces and pulse protocol for the recording shown in D. Black trace shows currents at start of rapamycin (rap.) application (60 s), and red trace is at 900 s (end of recording). (F) Time needed to decrease either $K_V7.2/K_V7.3$ or $K_{ir}2.1$ -mediated currents from 90 to 10% original amplitude ($n = 4$). (B and F) Error bars represent \pm SEM.

PI(4)P once rapamycin is added. Thus, we expressed $K_{ir}2.1$ together with pseudojanin-YFP and LDR-CFP and applied the same pulse protocol. The $K_{ir}2.1$ currents were stable before the addition of rapamycin. After addition of rapamycin, successful translocation of pseudojanin to the plasma membrane was signaled by an increase in FRET between YFP (on pseudojanin) and CFP (on the LDR anchor; Fig. 2 C, red line) within 25 s. After 50 s, the $K_{ir}2.1$ current began to decrease, and over several hundred seconds it gradually decayed nearly to zero (Fig. 2 C). Thus, tools like Dr-VSP and pseudojanin inhibit this potassium channel effectively despite its high apparent affinity for PI(4,5)P₂.

Finally, to monitor the K_{ir} and K_V channel activities simultaneously in the same cell, we coexpressed $K_{ir}2.1$ with $K_V7.2/K_V7.3$, pseudojanin-YFP, and LDR-CFP. The dual-pulse protocol comprised a first hyperpolarizing step to -100 mV to activate $K_{ir}2.1$ and a brief step to -60 mV followed by a depolarization to -20 mV to

activate $K_V7.2/K_V7.3$. As anticipated, the $K_V7.2/K_V7.3$ current was reduced quickly after addition of rapamycin, and $K_{ir}2.1$ current was reduced much more slowly (Fig. 2 D). Current traces before and after addition of rapamycin are shown in Fig. 2 E. The time for current inhibition from 10 to 90% was 46 ± 22 s for $K_V7.2/K_V7.3$ and 392 ± 50 s ($n = 4$) for $K_{ir}2.1$ (Fig. 2 F). This finding is consistent with different affinities of $K_V7.2/K_V7.3$ and $K_{ir}2.1$ channels for PI(4,5)P₂, resulting in different kinetics of channel inhibition as PI(4,5)P₂ at the plasma membrane is declining.

Having observed that Dr-VSP inhibits $K_V7.2/K_V7.3$ channels almost completely but $K_{ir}2.1$ channels only to about $\sim 47\%$, we asked whether activation of M_1R would inhibit $K_{ir}2.1$ channels. We followed the same strategy used for our pseudojanin experiments and expressed $K_{ir}2.1$ together with $K_V7.2$ and $K_V7.3$ in the same cell. Current recordings were performed as before. Interestingly, we observed an inhibition of $K_V7.2/K_V7.3$ activity

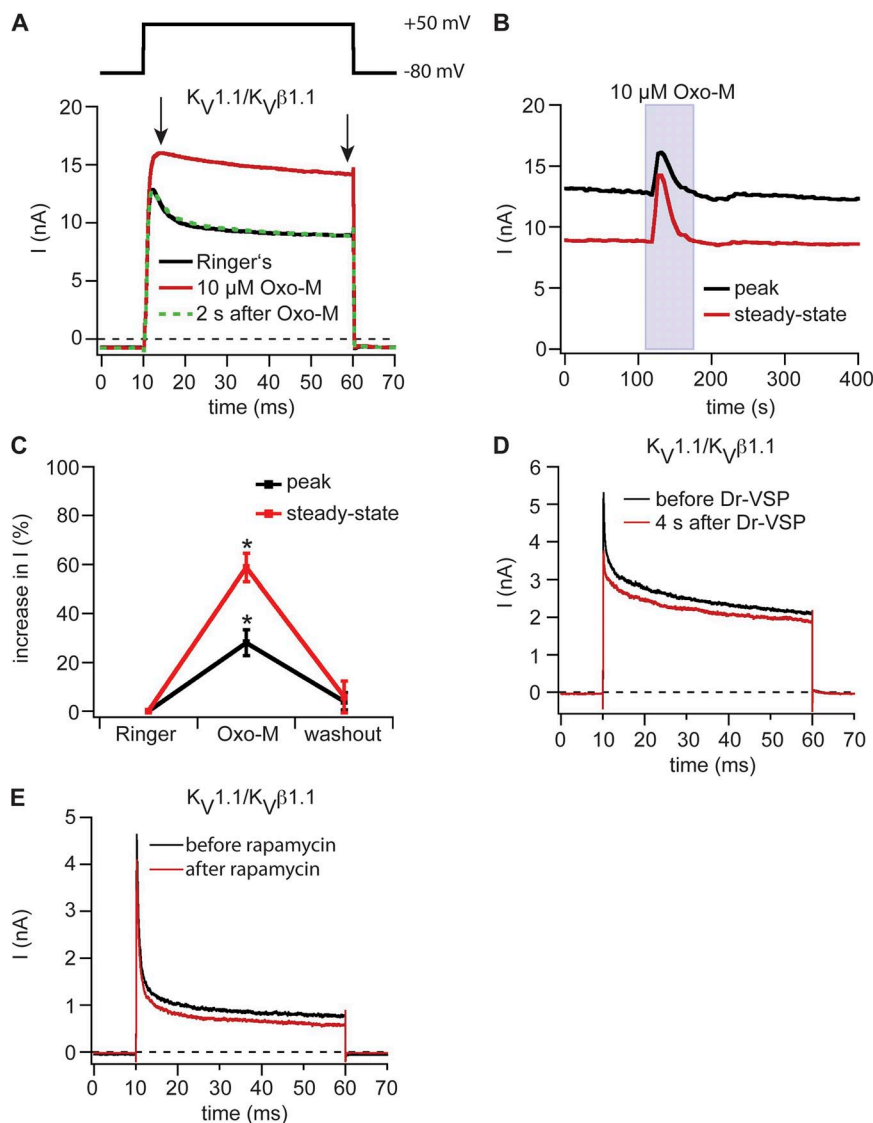


Figure 3. Modulation of Kv1 family channels by depletion of PI(4,5)P₂ at the plasma membrane. (A) Potassium outward currents in Kv1.1/Kvβ1.1 channel complexes coexpressed with M_1R . Black indicates control current trace in Ringer's solution, red indicates during application of 10 μM Oxo-M, and green indicates after washout of Oxo-M. Arrows indicate points at which peak and steady-state current amplitudes were measured. (B) Current amplitudes of peak and steady-state current of the cell shown in A over time. (C) Percent increase of peak and steady-state currents of Kv1.1/Kvβ1.1 channel complexes coexpressed with M_1R upon stimulation with Oxo-M ($n = 7$). Error bars represent \pm SEM. *, $P < 0.05$. (D) Current time course in a cell expressing Kv1.1/Kvβ1.1 channel complexes and Dr-VSP. Dr-VSP was activated by a 2-s pulse to 100 mV. Test pulse protocol as shown in A. (E) Same as D but with expression of pseudojanin-YFP and LDR-CFP instead of Dr-VSP and addition of 5 μM rapamycin.

by $61 \pm 6\%$ ($n = 5$) after activation of M_1R , but $K_{ir}2.1$ activity remained unaffected (Fig. S3). We conclude that the activation of PLC is not enough to reduce $PI(4,5)P_2$ levels below the level needed to keep $K_{ir}2.1$ channels active. Presumably lipid kinases are continually supplying new $PI(4,5)P_2$ molecules.

$K_V1.1/K_V\beta1.1$ channel complexes are sensitive to activation of M_1 muscarinic receptors but not to activation of VSP. With these controls in hand, we proceeded to additional K_V channels starting with the K_V1 family. $K_V1.1$ and the auxiliary $K_V\beta1.1$ subunit were coexpressed in tsA-201 cells together with M_1R . A 50-ms depolarization to 50 mV from a holding potential of -80 mV elicited

outward currents that inactivated rapidly, but only partially, to a sustained steady-state current (Fig. 3 A). Activation of M_1R by 10 μM Oxo-M increased the currents with almost complete removal of fast inactivation (Fig. 3, A and B). Peak currents increased $28 \pm 5\%$, and steady-state currents increased $59 \pm 6\%$ ($n = 7$; Fig. 3 C). Remarkably, the observed augmentation was transient, returning to the control amplitude even during continued Oxo-M application (Fig. 3, A and B).

Is the augmentation caused by depletion of $PI(4,5)P_2$? This seems less plausible because the augmentation did not persist during application of Oxo-M despite continued $PI(4,5)P_2$ depletion. Indeed two direct tests were negative. We observed no change in current

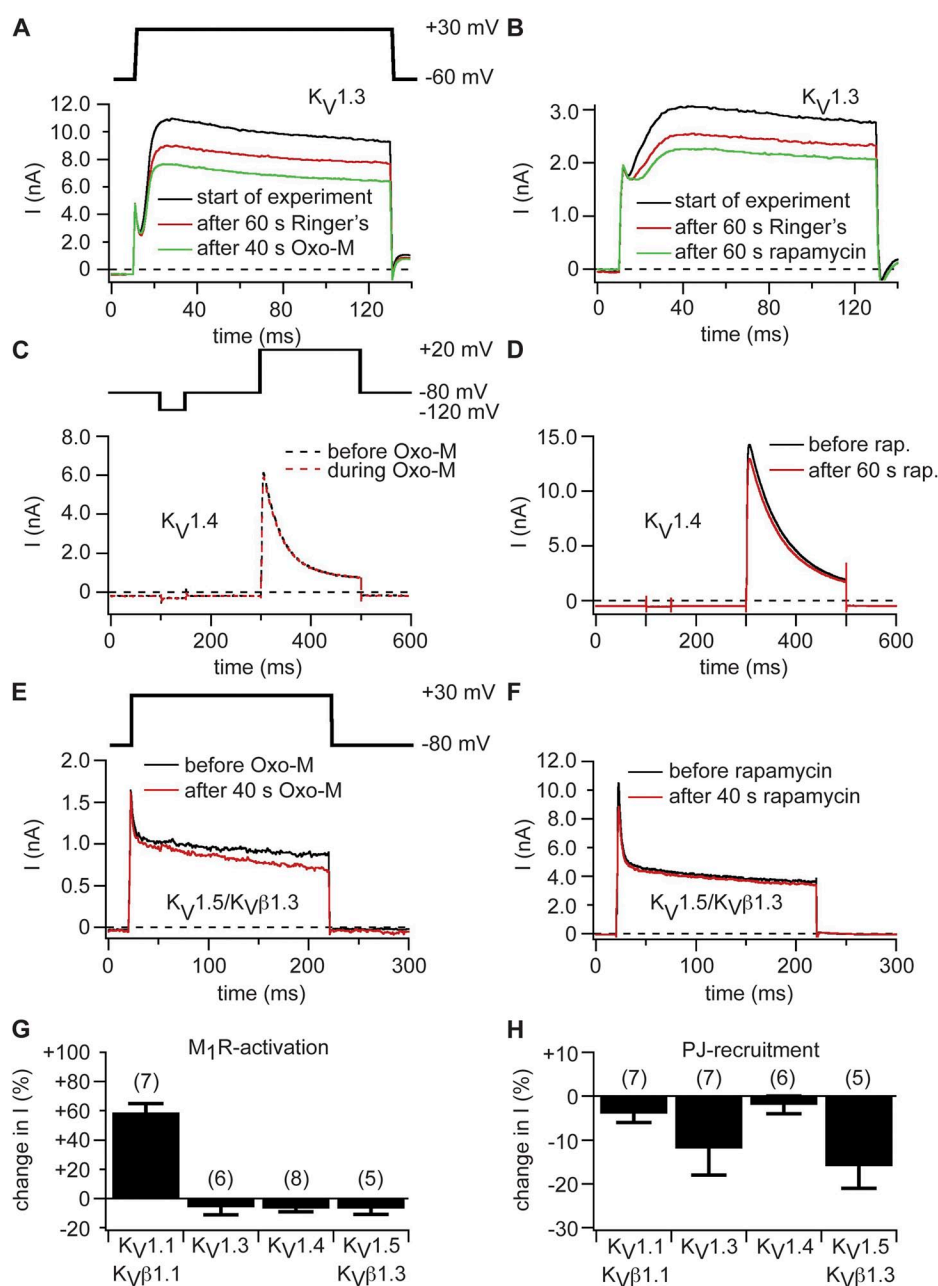


Figure 4. $K_V1.3$, $K_V1.4$, and $K_V1.5$ channels are insensitive to depletion of $PI(4,5)P_2$ at the plasma membrane. (A) Current traces of $K_V1.3$ channels coexpressed with M_1R . Black indicates traces at start of experiment, red indicates after 60 s in Ringer's, and green indicates traces after 40-s superfusion with 10 μM Oxo-M (100 s after start of experiment). (B) Currents in $K_V1.3$ channels coexpressed with pseudojanin-YFP and LDR-CFP. Black indicates traces at start of experiment, red indicates after 60 s in Ringer's, and green indicates traces after 60-s rapamycin application (120 s after start of experiment). Same pulse protocol as in A. (C) Currents in a cell expressing $K_V1.4$ and M_1R . Black indicates current trace in Ringer's solution, and red indicates current trace during application of 10 μM Oxo-M. (D) $K_V1.4$ channels coexpressed with pseudojanin-YFP and LDR-CFP. Black indicates current before application of rapamycin (rap.), and red indicates traces after 60 s of rapamycin application. (E and F) Currents in $K_V1.5$ channels coexpressed with $K_V\beta1.3$ and M_1R (E) or pseudojanin-YFP and LDR-CFP (F). Black indicates current before application of Oxo-M (E) or rapamycin (F), and red indicates current after 40-s application of Oxo-M (E) or rapamycin (F). (G and H) Percent changes in steady-state current amplitudes of $K_V1.x$ channels after activation of M_1R (G) or recruitment of pseudojanin (PJ) to the plasma membrane (H). Numbers in parentheses indicate n numbers for individual experiments. Error bars represent \pm SEM.

amplitudes or inactivation when $K_V1.1/K_V\beta1.1$ channel currents were tested by activation of Dr-VSP (Fig. 3 D). Similarly, there was no change of currents when pseudojanin was translocated to the plasma membrane in cells coexpressing $K_V1.1/K_V\beta1.1$, YFP-tagged pseudojanin, and LDR-CFP (Fig. 3 E). Thus, depletion of $PI(4,5)P_2$ is not likely to explain the strong augmentation of $K_V1.1/K_V\beta1.1$ channel current by M_1R activation; rather, one needs to consider other signaling pathways downstream of $PI(4,5)P_2$ hydrolysis (see Discussion).

To test other members of the K_V channel family, we expressed $K_V1.3$, $K_V1.4$, and $K_V1.5$ channels ($K_V1.5$ alone and with the β subunit $K_V\beta1.3$) together with Dr-VSP, pseudojanin, or M_1R but did not observe any changes in current amplitudes or channel kinetics with any of these tools (Fig. 4). For example, Fig. 4 A shows representative traces of $K_V1.3$ currents recorded with coexpressed M_1R . We observed no significant effect after M_1R activation. A certain amount of rundown of current was already present before activation of the receptor. Similarly, there was no response to translocation of pseudojanin (Fig. 4 B). Fig. 4 (C–F) shows

traces of $K_V1.4$ and $K_V1.5$ currents. Again there is no alteration in the potassium currents after activation of M_1R (Fig. 4, C and E) or translocation of pseudojanin (Fig. 4, D and F).

We performed simultaneous internal controls with FRET to show that $PI(4,5)P_2$ depletion was occurring in each experiment with no modulation of potassium currents. For example, in Fig. S4, we coexpressed eCFP-PH(PLC δ 1) and eYFP-PH(PLC δ 1) as FRET reporters to monitor $PI(4,5)P_2$ levels at the plasma membrane together with $K_V1.5/K_V\beta1.3$ channels and Dr-VSP. Decreases in $PI(4,5)P_2$ levels at the plasma membrane would be reported as a decrease in FRET interaction between CFP and YFP simultaneous with the patch-clamp measurements. Activation of Dr-VSP led to a substantial transient decrease in FRET ratio, indicating a transient decrease in $PI(4,5)P_2$ at the plasma membrane, without significant change in channel activity (Fig. S4). The validity of the FRET method is confirmed by parallel experiments of translocation of PH probes performed with confocal microscopy in cells that did not express exogenous channels. We coexpressed

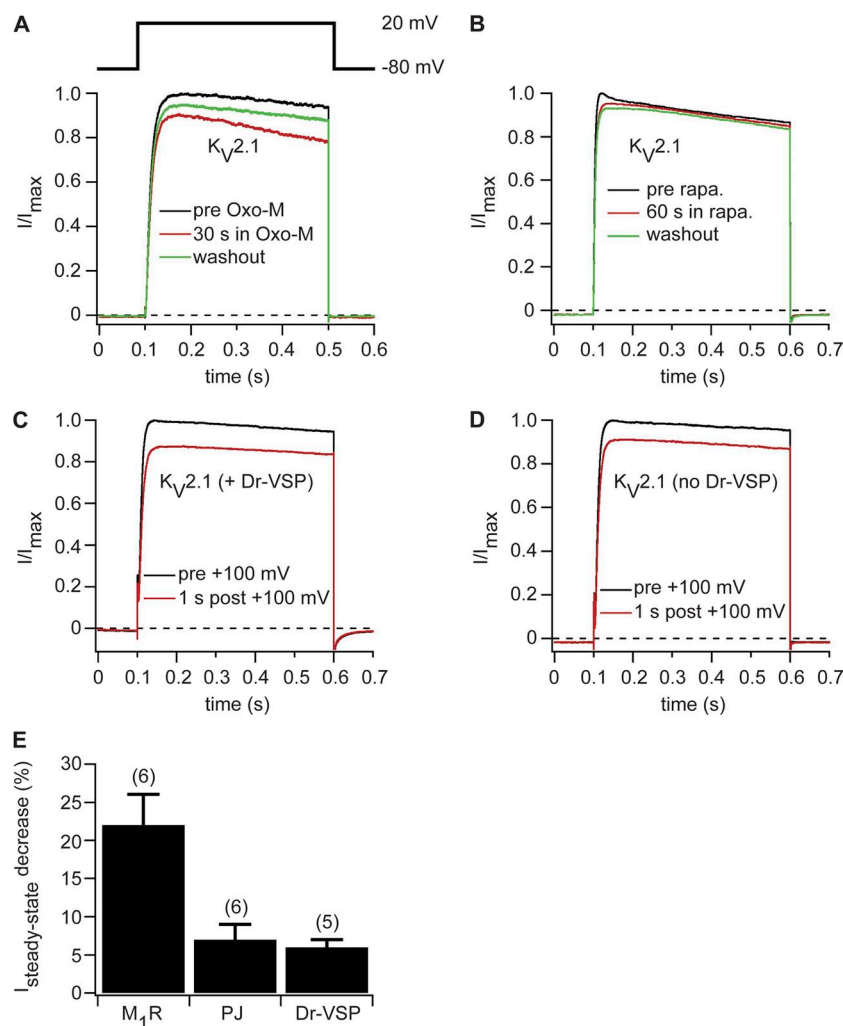


Figure 5. $K_V2.1$ channels are not sensitive to depletion of $PI(4,5)P_2$ by activation of pseudojanin or Dr-VSP. (A) Normalized current traces recorded before (black), during (red), and after (green) application of 10 μ M Oxo-M in a cell transiently transfected with $K_V2.1$ and M_1R . (B) Current traces before (black), after 60 s of application (red), and after washout of 5 μ M rapamycin (rapa.) to a cell expressing $K_V2.1$, pseudojanin-YFP, and LDR-CFP. (C) Normalized $K_V2.1$ -mediated current traces before (black) and 1 s after (red) a 2-s depolarizing pulse to 100 mV in cells coexpressing Dr-VSP. (D) Same as in C but without coexpression of Dr-VSP. (E) Decrease in $K_V2.1$ -mediated steady-state currents at 20 mV after activation of M_1R or Dr-VSP or recruitment of pseudojanin (PJ) to the plasma membrane. Numbers in parentheses indicate numbers of individual experiments. Error bars represent \pm SEM.

YFP-tagged PH probes with M₁R, Dr-VSP, or pseudojanin and performed line scan measurements with confocal laser microscopy before and after activation of these PI(4,5)P₂-depleting enzymes. As expected, we observed a strong localization of PH probes at the plasma membrane before PI(4,5)P₂ depletion and a reduction in YFP fluorescence at the plasma membrane of >70% after PI(4,5)P₂ depletion with any of the enzymes we used (Fig. S5). As first reported by van der Wal et al. (2001), these experiments show that FRET and translocation methods give a similar picture about PI(4,5)P₂ depletion. The confocal images (Fig. S5, A–F, insets) show that our three tools for PI(4,5)P₂ depletion act uniformly along the membrane. They do not leave patches of unaltered PI(4,5)P₂ behind. As a final internal control for PLC activation, we loaded cells with Fura-4F via the patch pipette while coexpressing K_V channels and M₁R to confirm that Ca²⁺ transients occurred during activation of M₁R (see Fig. 8 D for example). With these internal controls, we feel confident in scoring the tested K_V1 channels as not sensitive to PI(4,5)P₂ depletion (Table 1).

K_V2.1 channels are not sensitive to PI(4,5)P₂ depletion

Next, we asked whether K_V2.1 channels are sensitive to PI(4,5)P₂ depletion. First, we expressed K_V2.1 channels together with M₁R. Currents were elicited by a depolarizing test pulse to 20 mV from a holding potential of –80 mV. Upon activation of M₁R, we observed a 22 ± 4% (*n* = 6) decrease in steady-state current at the end of

the depolarizing test pulse (Fig. 5, A and E). Upon wash-out of the agonist Oxo-M, steady-state currents increased again (Fig. 5 A). To test whether the decrease in current amplitude is caused by a direct modulation of K_V2.1 channels by PI(4,5)P₂ or a result of downstream signaling pathways after PI(4,5)P₂ hydrolysis by PLC, we expressed K_V2.1 channels together with either pseudojanin or Dr-VSP, which deplete PI(4,5)P₂ without generating second messengers. Recruiting pseudojanin to the plasma membrane had no significant effect on K_V2.1-mediated currents (Fig. 5, B and E). In another approach, we activated Dr-VSP by a 2-s depolarizing pulse to 100 mV and measured currents before and after activation of Dr-VSP. We observed a reduction of ~25% in steady-state current amplitude when we tested for K_V2.1-mediated currents 1 s after the Dr-VSP-activating pulse (Fig. 5 C). However, in control experiments on cells that did not express Dr-VSP but were subjected to the same depolarizing test pulse, we observed an almost identical reduction of K_V2.1-mediated currents (Fig. 5 D). Thus, the reduction in current amplitude is likely caused by voltage-dependent inactivation of K_V2.1 channels from the depolarizing pulse used to activate Dr-VSP. In summary, neither recruitment of pseudojanin to the plasma membrane nor activation of Dr-VSP led to a significant change in K_V2.1-mediated currents (Fig. 5 E). We conclude that the observed small reduction of K_V2.1-mediated currents after M₁R activation is caused by signaling pathways downstream of PI(4,5)P₂ hydrolysis by activation of PLC or is a result of a direct loss of PI(4,5)P₂.

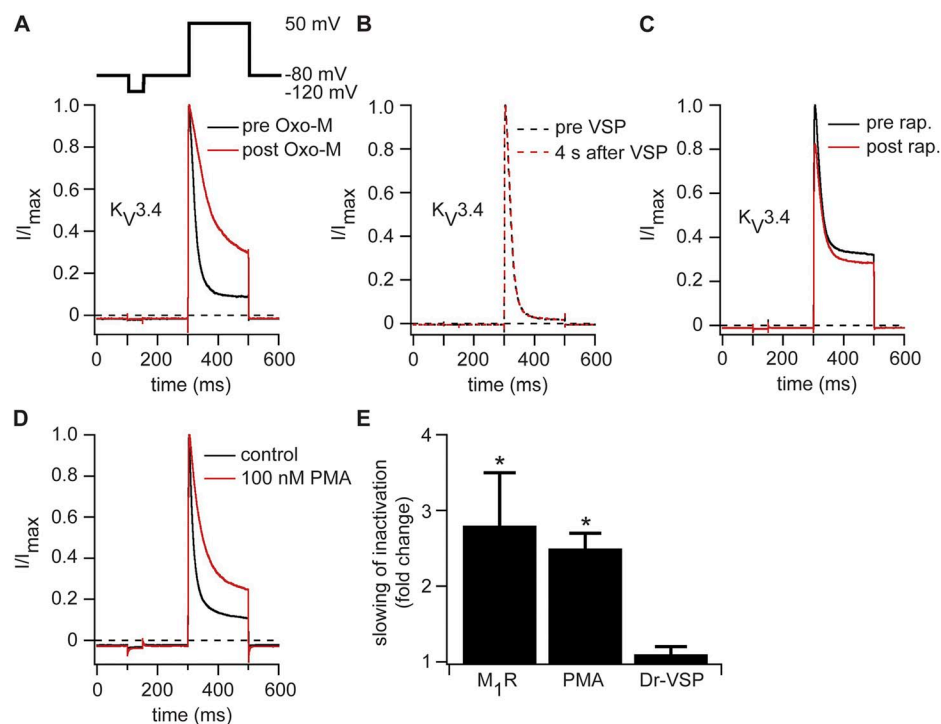


Figure 6. K_V3.4 channels are modulated by activation of M₁R but not by activation of Dr-VSP or pseudojanin. (A) Normalized current traces recorded before (black) and during (red) application of 10 μM Oxo-M in a cell transiently transfected with K_V3.4 and M₁R. (B) Same as in A but with expression of Dr-VSP and an activating pulse instead of M₁R. (C) Current traces before (black) and after (red) application of 5 μM rapamycin (rap.) to a cell expressing K_V3.4, pseudojanin-YFP, and LDR-CFP. (D) Representative normalized current traces of cells expressing K_V3.4 recorded under control conditions (black) or after 15-min treatment with 100 nM PMA in Ringer's solution (red). (E) Fold slowing of inactivation after activation of M₁R, application of 100 nM PMA, or activation of Dr-VSP (*n* = 4 [M₁R] or 5 [PMA and Dr-VSP]). Error bars represent ±SEM. *, *P* < 0.05.

K_V3.4 currents also can be increased and inactivation slowed upon M₁R activation

In a similar approach, we expressed K_V3.4 channels together with M₁R, Dr-VSP, or pseudojanin. Whole-cell recordings showed fast activating and inactivating outward potassium currents upon membrane depolarization as previously reported for these channels (Oliver et al., 2004). Activation of M₁R by Oxo-M decreased the amount of N-type inactivation, leading to an increase in current amplitude at the end of the depolarizing test pulse (Fig. 6 A). The time constant for N-type inactivation was 44 ± 18 ms under control conditions and 97 ± 30 ms ($n = 4$) after application of Oxo-M, a 2.8 ± 0.7 -fold slowing (Fig. 6 E). Despite the clear action of stimulating M₁R, activating Dr-VSP or recruiting pseudojanin to the plasma membrane had no detected effect on K_V3.4 current amplitudes (Fig. 6, B and C) or kinetics (Fig. 6 E). As was the case for K_V1.1/K_Vβ1.1 channels, these experiments find no direct regulation of K_V3.4 channels by depletion of PI(4,5)P₂, but they do find a large effect of M₁R, suggesting a possible role for the products of PI(4,5)P₂ hydrolysis. Reports in the literature describe a decrease of inactivation of K_V3.4 after phosphorylation of the N-terminal inactivation domain by PKC (Covarrubias et al., 1994; Ritter et al., 2012). Thus, the decrease of inactivation we observed after M₁R activation could be the result of an activation of PKC by the DAG produced during PI(4,5)P₂ hydrolysis by PLC. Indeed, incubating tsA-201 cells expressing K_V3.4 for 15 min with 100 nM PMA to activate PKC produced a decrease

in inactivation of K_V3.4 much as we saw with M₁R activation (Fig. 6 D). We measured a 2.5 ± 0.2 -fold slowing ($n = 5$; Fig. 6 E), consistent with the notion that phosphorylation of K_V3.4 by PKC underlies the effects of M₁R activation.

Complexes of K_V4.2 and K_V4.3 channels with KChIPs

and DPP6-s are not sensitive to PI(4,5)P₂ depletion

K_V4.2 and K_V4.3 channels play an important role in the brain and heart (Amarillo et al., 2008; Levy et al., 2010). In most cell types, they are associated with β subunits called KChIPs that are located on the cytoplasmic side of the plasma membrane and influence K_V4 channel current amplitude and inactivation kinetics (Pongs and Schwarz, 2010). They also can associate with another cofactor, DPP6-s, a protein that binds to K_V4 channels from the extracellular side of the plasma membrane and exerts similar effects as KChIPs on channel activity (Nadal et al., 2003; Kim et al., 2008; Kaulin et al., 2009). Because of their wide-spread physiological roles, we assessed whether these channel complexes can be regulated directly by PI(4,5)P₂. Accordingly, we expressed K_V4.2 or K_V4.3 channels together with Dr-VSP either alone or together with KChIPs and DPP6-s.

The pulse protocol was similar to that for recordings of K_V3.4 channels (Fig. 7 A). Without any β subunits, K_V4.2 and K_V4.3 channels gave fast-activating currents that displayed fast inactivation as reported before (Birnbaum et al., 2004). Fig. 7 shows K_V4.2 traces, and Fig. S6 shows K_V4.3 traces. Activation of Dr-VSP had no

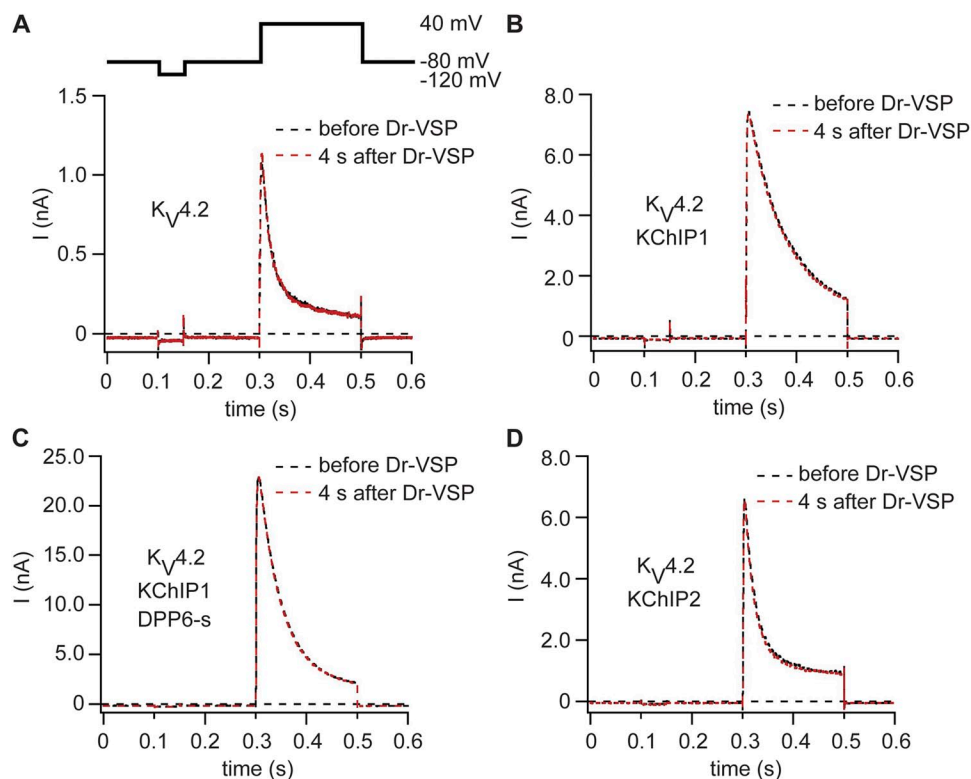


Figure 7. K_V4.2 channels are insensitive to depletion of PI(4,5)P₂ by Dr-VSP. (A) Current traces of a cell expressing K_V4.2 and Dr-VSP. Black indicates before activation of Dr-VSP, and red indicates 4 s after activation of Dr-VSP. (B–D) Same protocol as in A but with coexpression of KChIP1 (B), KChIP1 and DPP6-s (C), or KChIP2 (D).

detectable effect on current amplitudes or channel kinetics (Fig. 7 A). Coexpression with KChIP1 increased peak current amplitudes and slowed inactivation but did not introduce any sensitivity to Dr-VSP activation (Fig. 7 B). Biochemical experiments and recorded channel activities imply that under physiological conditions K_v4 channels are simultaneously associated with KChIP1 and DPP6-s as auxiliary subunits (Pongs and Schwarz, 2010). We mimicked this situation by expressing $K_v4.2$ together with KChIP1 and DPP6-s. This produced a dramatic increase in current amplitude as reported before for K_v4 channels (Fig. 7 C; Nadal et al., 2003; Pongs and Schwarz, 2010) but no sensitivity to $PI(4,5)P_2$ depletion. As a last configuration, we expressed K_v4 channels together with KChIP2 and Dr-VSP. Again, activation of Dr-VSP led to no change of channel activity (Fig. 7 D). To check for a successful decrease of $PI(4,5)P_2$ levels, all experiments included PH probes as FRET reporters to monitor $PI(4,5)P_2$ levels at the plasma membrane.

In all analyzed cells, activation of Dr-VSP led to a strong decrease in the FRET signal, indicative of a depletion of $PI(4,5)P_2$ at the plasma membrane. Evidently, K_v4 channels alone or together with their β subunits are not sensitive to a transient depletion of $PI(4,5)P_2$ at the plasma membrane (Table 1).

hERG channels are not sensitive to $PI(4,5)P_2$ depletion

As a final K_v family, we expressed hERG ($K_v11.1$) channels together with Dr-VSP and PH probes to monitor $PI(4,5)P_2$ levels. The hERG channel activity was assessed as tail currents at -40 mV after test pulses to 10 mV from a holding potential of -80 mV. Each activation of Dr-VSP led to a significant reduction in PH domain FRET_r, verifying reduction of $PI(4,5)P_2$ (not depicted), but there were no detectable changes in current amplitude or time course (Fig. 8 A). Repeating the experiment with coexpression of the β subunit KCNE2 did not change this negative result (Fig. 8 B). Next we tested

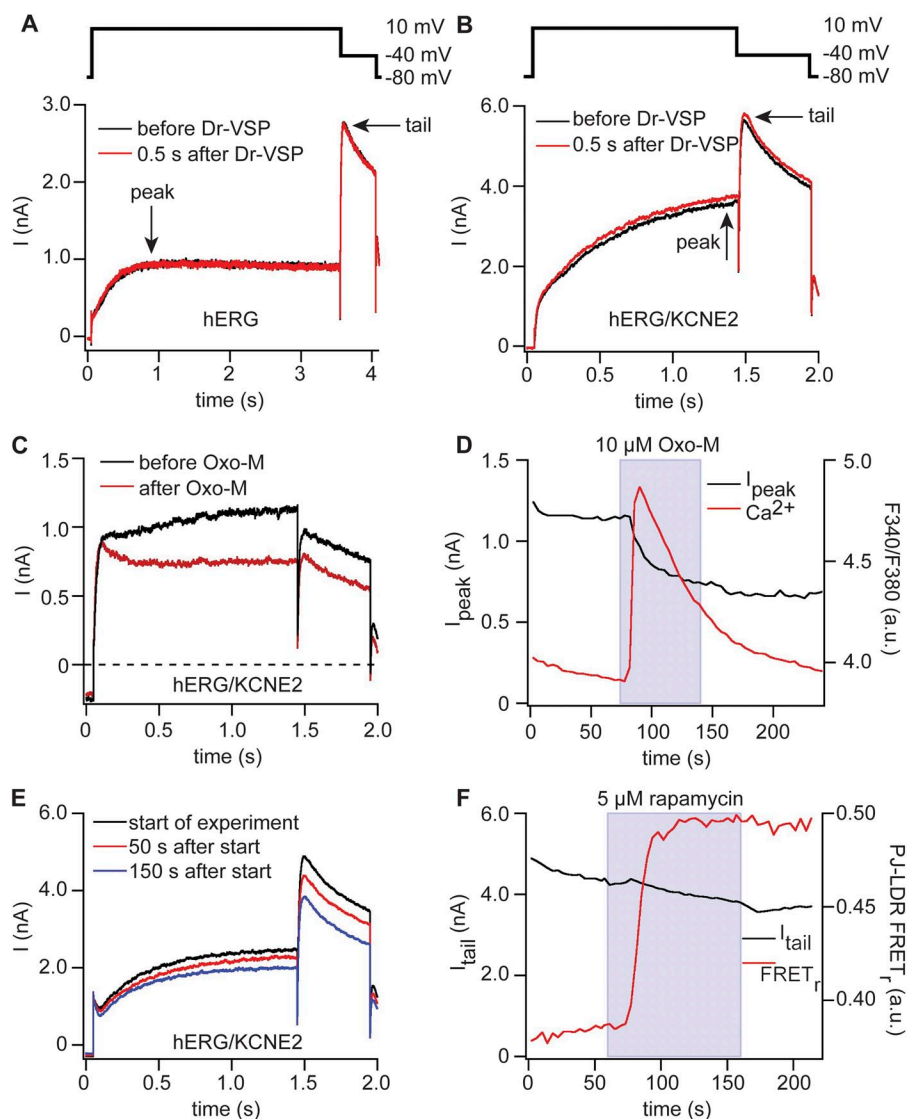


Figure 8. hERG channels are sensitive to M_1R activation but not to activation of Dr-VSP or pseudojanin. (A) Currents in hERG channels coexpressed with Dr-VSP. Black indicates before activation of Dr-VSP, and red indicates directly after activation of Dr-VSP. Arrows indicate points at which peak and tail current amplitudes were measured. (B) Same as in A but with additional coexpression of KCNE2 and modified pulse protocol. (C) Currents in hERG channels coexpressed with KCNE2 and M_1R . Black indicates before application of $10 \mu M$ Oxo-M, and red indicates after application of Oxo-M. Pulse protocol as in B. (D) Time course of current amplitudes at 10 mV and Fura-4F ratio of cell in C. (E) Currents in a cell expressing hERG, KCNE2, pseudojanin-YFP, and LDR-CFP. Currents are shown at the beginning of the recordings (black), directly before application of rapamycin (50 s after start of experiment; red), and at the end of rapamycin application (150 s after start of experiment; blue). Pulse protocol as in B. (F) Time course of current amplitudes of tail currents at -40 mV of the experiment in E (black) and FRET ratio between pseudojanin (PJ)-YFP and the anchor LDR-CFP (red).

whether activation of M₁R modulates channel activity. With hERG and KCNE2, M₁R activation led to a small decrease in both peak and tail currents (Fig. 8 C). The onset of the decrease in currents coincided with a rise of intracellular Ca²⁺ (Fig. 8 D), but, when the Ca²⁺ transient relaxed back to its original level, current amplitudes did not recover.

The depression of hERG current during M₁R activation developed slowly over tens of seconds (Fig. 8 D). This suggested that our VSP experiment (Fig. 8, A and B) may have missed the appropriate time frame because we had looked for an effect only 0.5 s after VSP activation. We switched to recruiting pseudojanin to the plasma membrane to make a lasting depletion. FRET between the membrane anchor LDR-CFP and pseudojanin-YFP increased sharply as rapamycin was perfused (Fig. 8 F), but there was no significant effect on hERG current amplitudes or kinetics (Fig. 8, E and F). In the pseudojanin-expressing cells there was some current rundown starting even before application of rapamycin, which was not changed by recruiting pseudojanin to the plasma membrane (Fig. 8 F). Collectively, these findings support a small indirect regulation of channel activity by M₁R activation but give no evidence for a direct modulation of hERG channels by PI(4,5)P₂ depletion.

DISCUSSION

We have studied the PI(4,5)P₂ sensitivity of nine K_V channels, K_V1.1, K_V1.3, K_V1.4, K_V1.5, K_V2.1, K_V3.4, K_V4.2, K_V4.3, and K_V11.1/KCNE (hERG), together with various additional auxiliary subunits (Table 1). When we depleted PI(4,5)P₂ by activating Dr-VSP or recruiting pseudojanin to the plasma membrane, the observed properties of these expressed channels did not change. This result was quite unexpected because published experiments describe PI(4,5)P₂ sensitivity of several of these channels, K_V1.1 with K_Vβ1.1, K_V1.4, K_V1.5 with K_Vβ1.3 and K_V3.4 (Oliver et al., 2004; Decher et al., 2008), as well as hERG (K_V11.1; Bian et al., 2001, 2004; Bian and McDonald, 2007), especially in experiments with direct application of PI(4,5)P₂ to large inside-out membrane patches from *Xenopus* oocytes. We must now consider possible sources of this apparent discrepancy. Is our use of “physiological” phosphatases in intact cells exploring the same range of PI(4,5)P₂ as that with PI(4,5)P₂ perfusion onto excised patches? Are different K⁺ channels localized to different plasma membrane subdomains where they experience different pools of PI(4,5)P₂? Could superfusion of PI(4,5)P₂ onto excised patches lead to unspecific side effects which might be an explanation for the differences between the published studies and our results? Could other lipids besides PI(4,5)P₂ and PI(4)P be keeping K_V channels active?

Depletion of PI(4,5)P₂

Our results with activation of phosphatases were unambiguously negative, so it was essential to show that our tools actually depleted plasma membrane PI(4,5)P₂. In many experiments, we recorded FRET from PH domains of PLCδ1 simultaneously with the current traces and found that PI(4,5)P₂ was being depleted. We verified these results in line scan measurements of cells transfected with a YFP-tagged PH probe. We found a uniform localization of PH probes at the plasma membrane before PI(4,5)P₂ depletion. Right after activation of Dr-VSP, M₁R, or pseudojanin, we detected a strong decrease of YFP fluorescence at the plasma membrane and an obvious translocation of the PH probes into the cytoplasm. The PI(4,5)P₂ depletion was uniform at the plasma membrane. It should be noted that because phosphatases do not generate IP₃, the translocation of PH domains during phosphatase activation really reflects loss of free membrane PI(4,5)P₂ rather than any gain of cytoplasmic IP₃. In some experiments when activating M₁R, we also validated activation of PLC by measuring the concomitant rises in Ca²⁺. The phosphatase tools, especially pseudojanin, were quite capable of inhibiting low-affinity K_V7.2/7.3 channels, as is already well known (Suh et al., 2006; Murata and Okamura, 2007; Falkenburger et al., 2010b), as well as K_V7.1/KCNE and high-affinity K_{ir}2.1 channels by at least 90%. Even a short activation of Dr-VSP inhibited K_{ir}2.1 by 45%. Activation of M₁R was not sufficient to inhibit high-affinity K_{ir}2.1 channels, whereas it strongly inhibited low-affinity K_V7.2/K_V7.3 channels. As both Dr-VSP and pseudojanin showed inhibition of K_{ir}2.1 channels, we concluded that we would be able to detect a PI(4,5)P₂ dependence of K_V channels even if they have a high affinity to PI(4,5)P₂ by using Dr-VSP and pseudojanin.

We can attempt to calibrate the membrane PI(4,5)P₂ levels in terms of equivalent diC₈-PI(4,5)P₂ concentrations in solution. In these terms, the equivalent diC₈-PI(4,5)P₂ concentration of a resting cell would be near the apparent low-affinity K_d of 40–87 μM for K_V7.2/7.3 channels (Zhang et al., 2003; Li et al., 2005; Telezhkin et al., 2012) so that these channels would be half activated. After pseudojanin activation, the equivalent concentration would be reduced considerably below the apparent K_d of 5 μM for K_{ir}2.1 channels so that only 10% of these channels would be active (Rohács et al., 2003). Indeed, <1 μM diC₈-PI(4,5)P₂ suffices to support 10% K_{ir}2.1 channel activity (Du et al., 2004). These parameters define the window of PI(4,5)P₂ levels we have studied in terms of an equivalent diC₈-PI(4,5)P₂ scale.

Are other investigators exploring a different range of available PI(4,5)P₂ than we are? It was when adding 10 μM brain PI(4,5)P₂ to patches from *Xenopus* oocytes with K_V1.1 with K_Vβ1.1, K_V1.4, and K_V1.5 with K_Vβ1.3, and K_V3.4 channels that Oliver et al. (2004) and Decher et al. (2008) saw an extensive loss of fast inactivation.

In inside-out patches excised into a medium without ATP, the PI(4,5)P₂ pools might well gradually fall through irreversible phosphatase activity below the lowest levels we reach with pseudojanin in intact cells. In these conditions, Oliver et al. (2004) and Decher et al. (2008) reported inactivating gating kinetics much like those we and others see in whole-cell experiments with normal endogenous PI(4,5)P₂. Consistent with our phosphatase results, they did not describe any rundown that accompanied gradual loss of endogenous PI(4,5)P₂. When these authors then applied PI(4,5)P₂, they used long-chain brain PI(4,5)P₂ for which we unfortunately have no equivalent concentration calibration. At the reported 10-μM concentration, the phospholipid would have been suspended as micelles rather than in free solution. It made the currents become much larger and longer lasting. They report that PI(4)P, PI(4,5)P₂, and PI(3,4,5)P₃ were equally effective.

Could superfusion of excised patches with long-chain PI(4,5)P₂ induce nonspecific effects? One possibility is that it leads to supraphysiological high concentrations of PI(4,5)P₂ in the patch, above the concentration range we studied with our tools. At these elevated concentrations, PI(4,5)P₂ might interact with domains in the channel proteins that would not normally couple to endogenous PI(4,5)P₂. Another possible explanation for the apparent discrepancy between our results and those of Oliver et al. (2004) and Decher et al. (2008) could be that the nonspecific large cytoplasmic polyphosphoinositide-micelle effects are not mediated by incorporation into the membrane. Perhaps the micelles interact with extramembranous domains of the channel proteins like the N-terminal inactivation domains, thereby preventing these domains from interacting with the gate and conferring inactivation to the K_V channels. This possibility seems plausible to us and would be an artifact.

Alternatively, can the differences be explained by postulating segregation of different channels into hypothetical lipid domains on the plasma membrane? One domain would contain K_V7 channels, K_{ir}2.1 channels, and PI(4,5)P₂. It would also contain the M₁Rs, PLC, PH domains, VSP, and the membrane anchor LDR-CFP so that activation of PLC or recruiting phosphatases to this domain would deplete PI(4,5)P₂, inhibiting K_V7 and K_{ir}2.1 channel function and freeing almost all PH domains. This lipid domain would contain the great majority of the plasma membrane PI(4,5)P₂ because, according to our chemical measurements in M₁R-expressing CHO cells, 93% of the total cell PI(4,5)P₂ can be cleaved in a 60-s treatment with carbachol (Horowitz et al., 2005). The other, presumably smaller, domain would harbor and segregate all the other K_V channels we have tested. It would lack PI(4,5)P₂ and not be influenced by the PI(4,5)P₂ depletion. However, like the other domain, it would accept applied PI(4,5)P₂, so that

the K_V channels would respond to that manipulation. We regard this, highly isolated, two-domain model as too contrived and implausible.

Our work did not reveal any lipid dependence of K_V channels outside the K_V7 family. Because our tests involved depleting only PI(4,5)P₂ and PI(4)P, the possibility remains that K_V channels can be kept active by lipids we did not deplete, although we know no reason to invoke a lipid requirement so far. There are examples in the literature. Thus, long-chain acyl-CoA esters, a metabolically active form of free fatty acids, activate K_{ATP} channels (Larsson et al., 1996; Bränström et al., 1997). Further, phosphatidic acid interacts with a variety of proteins (Kooijman and Burger, 2009) and activates, for instance, the bacterial mechanosensitive channel of large conductance (MscL; Powl et al., 2005a,b). Lastly, even K_V7.2/K_V7.3 channels can be kept active by phospholipids other than PI(4,5)P₂ that have at least an acyl chain and a phosphate head group (Telezhkin et al., 2012). However, PI(4,5)P₂ still has the highest affinity, and the natural abundance of any others is insufficient to keep the channels functioning after we recruit the 5-phosphatase to the plasma membrane.

Activation of G_q-coupled receptors and PKC

We also explored the sensitivity of some of the K_V channels to activation of M₁R, a G_q-coupled receptor. As expected, the K_V7.2/7.3 currents were strongly depressed. Some other K_V channels were not affected by M₁R activation (K_V1.3, K_V1.4, and K_V1.5 ± K_Vβ1.3), but the inactivation gating kinetics of two of the K_V channels (K_V1.1/K_Vβ1.1 and K_V3.4) were strongly slowed. From our observations, we conclude that slowing of inactivation for K_V1.1/K_Vβ1.1 and K_V3.4 during PLC activation is not caused by the resulting depletion of PI(4,5)P₂. Instead we consider it to be identical to slowing attributed by other authors to activation of PKC (Jonas and Kaczmarek, 1996; Levy et al., 1998; Winkhofer et al., 2003). For example, consider K_Vβ1.1, whose coexpression with K_V1.1 makes fast inactivation by contributing an inactivation ball. This inactivation is slowed if PKC is activated pharmacologically or through a G_q-coupled metabotropic glutamate receptor; the slowing uses an indirect mechanism that may remove a phosphate at a PKA consensus site on K_V1.1, Ser-446 (Jonas and Kaczmarek, 1996; Levy et al., 1998; Winkhofer et al., 2003). Although the mechanism is different for K_V3.4 channels, the net effect of PKC is quite similar, a loss of inactivation (Covarrubias et al., 1994; Beck et al., 1998). Serines in the inactivation gate become phosphorylated, stopping inactivation. The effect can be mimicked by phosphomimetic mutations. Such receptor-induced loss of inactivation from K_V3.4 channels has been seen in primary dorsal root ganglion neurons (Ritter et al., 2012). Similarly, we showed that stimulation of PKC with PMA closely mimics the decrease in inactivation we observed

after M₁R activation. This finding agrees with the published studies and reiterates the importance of DAG as a PKC-activating product downstream of PI(4,5)P₂ hydrolysis after activation of G_q-coupled receptors.

In contrast, in *Drosophila melanogaster*, the *shab* channel (K_V2 family) apparently is inhibited by PI(4,5)P₂ in photoreceptors and in a fly S2 cell expression system (Krause et al., 2008). Photostimulation relieves the inhibition in a manner that requires PLC but not IP₃ or a calcium rise and with the same rapid kinetics as photo-transduction. Direct application of 40 μM diC₈-PI(4,5)P₂ to patches excised from S2 cells reversibly inhibits currents. These channels lack N-type fast inactivation so the rapid effects are not likely to involve the inactivation process. Rather they include a leftward shift of the conductance-voltage relation by light.

hERG channels

We come finally to hERG K_V11.1 channels. Activating M₁R reduced hERG current amplitude slightly; however, activating Dr-VSP yielded no detectable change. With pseudo-janin, we did sometimes observe rundown of hERG current amplitude, but this rundown began before addition of rapamycin to recruit pseudojanin to the plasma membrane and was not accelerated by the rapamycin. The literature does suggest some PI(4,5)P₂ dependence. In CHO cells, hERG current amplitude increases upon dialysis of 10 μM brain PI(4,5)P₂ into cells via the patch pipette, and it decreases upon activation of G_q-coupled α_{1A}-adrenergic receptors in a manner that is not blocked by PKC inhibitors or calcium chelators (Bian et al., 2001, 2004; Bian and McDonald, 2007). The effects are small changes of current amplitude and kinetic parameters. The small effects of α_{1A}-adrenergic receptor stimulation resemble ours with M₁R, but our experiments did not find evidence for regulation by PI(4,5)P₂. We conclude that PI(4,5)P₂ modulates hERG channels very modestly if at all and is not essential for hERG channel function. The current regulation through G_q-coupled GPCRs could well have some physiological effect on the cardiac action potential.

Use of heterologous expression systems

We performed all of our experiments in tsA-201 cells. This conferred certain advantages: We could record the activity of the K_V channels of interest in isolation without interference from the many other ion channels that would be present in primary cells and might also be subject to modulation. In addition, we could readily express phosphoinositide-manipulating or reporting proteins like Dr-VSP and PH probes. There are also some disadvantages. Although they seem to have similar phosphoinositide metabolism, the tsA-201 cells may lack K_V-interacting proteins present in primary cells that might govern their responses to PI(4,5)P₂ depletion. In addition, the spatial organization of proteins in the plasma membrane might

be different in tsA-201 cells, again affecting signaling pathways. Nonetheless, we are encouraged by the similarity of KCNQ channel modulation by receptors in tsA-201 cells and primary sympathetic neurons.

Conclusions

In summary, we confirm that members of the K_V7 and K_{ir}2 channel families have a strong PI(4,5)P₂ requirement and that PI(4,5)P₂ depletion reduces currents conducted by these channels nearly completely. In vivo, a graded reduction of these currents should occur during activation of G_q protein-coupled receptors that activate PLC in the plasma membrane. This situation is known for K_V7.2/K_V7.3-mediated I_M (Shapiro et al., 1994; Suh and Hille, 2002; Brown et al., 2007; Hughes et al., 2007) and for N-type Ca²⁺ channels in sympathetic neurons (Shapiro et al., 1994; Suh et al., 2010) upon activation of endogenous M₁ muscarinic receptors. Some K_V channels, like K_V1.1/K_Vβ1.1, K_V3.4, and hERG, are modulated by apparently conventional second-messenger signaling pathways downstream of PI(4,5)P₂ hydrolysis. Unexpectedly, many K_V channels showed no change in properties when PI(4,5)P₂ was manipulated by lipid phosphatases. We feel we have explored the range of free PI(4,5)P₂ levels that might be experienced physiologically in the plasma membrane. Thus, any ability to modulate some of them with applications of brain PI(4,5)P₂ as micelles may be unphysiological.

We would like to thank all colleagues who have generously provided us with plasmids (see Materials and methods). Byung-Chang Suh obtained preliminary results with muscarinic modulation of K_V1.1/K_Vβ1.1, K_V1.4, and K_V3.4 that inspired this project. We are also thankful to Sharon E. Gordon, William N. Zagotta, Byung-Chang Suh, Eamonn J. Dickson, and Björn Falkenburger for comments on the manuscript, to all members of the Hille laboratory and many members of the Department of Physiology and Biophysics at the University of Washington for discussions and experimental advice, and to Lea M. Miller for technical help.

This study was supported by National Institutes of Health grant R01 NS08174 and the Alexander von Humboldt-Foundation (awards to M. Kruse).

Christopher Miller served as editor.

Submitted: 23 March 2012

Accepted: 9 July 2012

REFERENCES

- Amarillo, Y., J.A. De Santiago-Castillo, K. Dougherty, J. Maffie, E. Kwon, M. Covarrubias, and B. Rudy. 2008. Ternary K_{4.2} channels recapitulate voltage-dependent inactivation kinetics of A-type K⁺ channels in cerebellar granule neurons. *J. Physiol.* 586:2093–2106. <http://dx.doi.org/10.1113/jphysiol.2007.150540>
- Beck, E.J., R.G. Sorensen, S.J. Slater, and M. Covarrubias. 1998. Interactions between multiple phosphorylation sites in the inactivation particle of a K⁺ channel. Insights into the molecular mechanism of protein kinase C action. *J. Gen. Physiol.* 112:71–84. <http://dx.doi.org/10.1085/jgp.112.1.71>

- Bian, J.S., and T.V. McDonald. 2007. Phosphatidylinositol 4,5-bisphosphate interactions with the HERG K⁺ channel. *Pflugers Arch.* 455:105–113. <http://dx.doi.org/10.1007/s00424-007-0292-5>
- Bian, J., J. Cui, and T.V. McDonald. 2001. HERG K⁺ channel activity is regulated by changes in phosphatidyl inositol 4,5-bisphosphate. *Circ. Res.* 89:1168–1176. <http://dx.doi.org/10.1161/hh2401.101375>
- Bian, J.S., A. Kagan, and T.V. McDonald. 2004. Molecular analysis of PIP₂ regulation of HERG and I_{Kr}. *Am. J. Physiol. Heart Circ. Physiol.* 287:H2154–H2163. <http://dx.doi.org/10.1152/ajpheart.00120.2004>
- Birnbaum, S.G., A.W. Varga, L.-L. Yuan, A.E. Anderson, J.D. Sweatt, and L.A. Schrader. 2004. Structure and function of K_v4-family transient potassium channels. *Physiol. Rev.* 84:803–833. <http://dx.doi.org/10.1152/physrev.00039.2003>
- Bränström, R., B.E. Corkey, P.O. Berggren, and O. Larsson. 1997. Evidence for a unique long chain acyl-CoA ester binding site on the ATP-regulated potassium channel in mouse pancreatic beta cells. *J. Biol. Chem.* 272:17390–17394. <http://dx.doi.org/10.1074/jbc.272.28.17390>
- Brown, D.A., and G.M. Passmore. 2009. Neural KCNQ (K_v7) channels. *Br. J. Pharmacol.* 156:1185–1195. <http://dx.doi.org/10.1111/j.1476-5381.2009.00111.x>
- Brown, D.A., S.A. Hughes, S.J. Marsh, and A. Tinker. 2007. Regulation of M(K_v7.2/7.3) channels in neurons by PIP₂ and products of PIP₂ hydrolysis: significance for receptor-mediated inhibition. *J. Physiol.* 582:917–925. <http://dx.doi.org/10.1113/jphysiol.2007.132498>
- Charpentier, F., J. Mérot, G. Loussouarn, and I. Baró. 2010. Delayed rectifier K⁺ currents and cardiac repolarization. *J. Mol. Cell. Cardiol.* 48:37–44. <http://dx.doi.org/10.1016/j.yjmcc.2009.08.005>
- Covarrubias, M., A. Wei, L. Salkoff, and T.B. Vyas. 1994. Elimination of rapid potassium channel inactivation by phosphorylation of the inactivation gate. *Neuron.* 13:1403–1412. [http://dx.doi.org/10.1016/0896-6273\(94\)90425-1](http://dx.doi.org/10.1016/0896-6273(94)90425-1)
- Czech, M.P. 2000. PIP₂ and PIP₃: complex roles at the cell surface. *Cell.* 100:603–606. [http://dx.doi.org/10.1016/S0092-8674\(00\)80696-0](http://dx.doi.org/10.1016/S0092-8674(00)80696-0)
- Decher, N., T. Gonzalez, A.K. Streit, F.B. Sachse, V. Renigunta, M. Soom, S.H. Heinemann, J. Daut, and M.C. Sanguinetti. 2008. Structural determinants of K_vbeta1.3-induced channel inactivation: a hairpin modulated by PIP₂. *EMBO J.* 27:3164–3174. <http://dx.doi.org/10.1038/emboj.2008.231>
- Di Paolo, G., and P. De Camilli. 2006. Phosphoinositides in cell regulation and membrane dynamics. *Nature.* 443:651–657. <http://dx.doi.org/10.1038/nature05185>
- Du, X., H. Zhang, C. Lopes, T. Mirshahi, T. Rohács, and D.E. Logothetis. 2004. Characteristic interactions with phosphatidylinositol 4,5-bisphosphate determine regulation of K_v channels by diverse modulators. *J. Biol. Chem.* 279:37271–37281. <http://dx.doi.org/10.1074/jbc.M403413200>
- Falkenburger, B.H., J.B. Jensen, and B. Hille. 2010a. Kinetics of M₁ muscarinic receptor and G protein signaling to phospholipase C in living cells. *J. Gen. Physiol.* 135:81–97. <http://dx.doi.org/10.1085/jgp.200910344>
- Falkenburger, B.H., J.B. Jensen, and B. Hille. 2010b. Kinetics of PIP₂ metabolism and KCNQ2/3 channel regulation studied with a voltage-sensitive phosphatase in living cells. *J. Gen. Physiol.* 135:99–114. <http://dx.doi.org/10.1085/jgp.200910345>
- Gamper, N., Y. Li, and M.S. Shapiro. 2005. Structural requirements for differential sensitivity of KCNQ K⁺ channels to modulation by Ca²⁺/calmodulin. *Mol. Biol. Cell.* 16:3538–3551. <http://dx.doi.org/10.1091/mbc.E04-09-0849>
- Hammond, G.R.V., M.J. Fischer, K.E. Anderson, J. Holdich, A. Koteci, T. Balla, and R.F. Irvine. 2012. PI4P and PI(4,5)P₂ are essential but independent lipid determinants of membrane identity. *Science.* <http://dx.doi.org/10.1126/science.1222483>
- Hansen, S.B., X. Tao, and R. MacKinnon. 2011. Structural basis of PIP₂ activation of the classical inward rectifier K⁺ channel K_v2.2. *Nature.* 477:495–498. <http://dx.doi.org/10.1038/nature10370>
- Hicke, L. 1999. Gettin' down with ubiquitin: turning off cell-surface receptors, transporters and channels. *Trends Cell Biol.* 9:107–112. [http://dx.doi.org/10.1016/S0962-8924\(98\)01491-3](http://dx.doi.org/10.1016/S0962-8924(98)01491-3)
- Hilgemann, D.W., and R. Ball. 1996. Regulation of cardiac Na⁺, Ca²⁺ exchange and KATP potassium channels by PIP₂. *Science.* 273:956–959. <http://dx.doi.org/10.1126/science.273.5277.956>
- Hilgemann, D.W., S. Feng, and C. Nasuhoglu. 2001. The complex and intriguing lives of PIP₂ with ion channels and transporters. *Sci. STKE.* 2001:re19. <http://dx.doi.org/10.1126/stke.2001.111.re19>
- Hille, B. 2001. Ion Channels of Excitable Membranes. Third edition. Sinauer, Sunderland, MA. 814 pp.
- Horowitz, L.F., W. Hirdes, B.-C. Suh, D.W. Hilgemann, K. Mackie, and B. Hille. 2005. Phospholipase C in living cells: activation, inhibition, Ca²⁺ requirement, and regulation of M current. *J. Gen. Physiol.* 126:243–262. <http://dx.doi.org/10.1085/jgp.200509309>
- Hughes, S., S.J. Marsh, A. Tinker, and D.A. Brown. 2007. PIP₂-dependent inhibition of M-type (K_v7.2/7.3) potassium channels: direct on-line assessment of PIP₂ depletion by G_q-coupled receptors in single living neurons. *Pflugers Arch.* 455:115–124. <http://dx.doi.org/10.1007/s00424-007-0259-6>
- Inoue, T., W.D. Heo, J.S. Grimley, T.J. Wandless, and T. Meyer. 2005. An inducible translocation strategy to rapidly activate and inhibit small GTPase signaling pathways. *Nat. Methods.* 2:415–418. <http://dx.doi.org/10.1038/nmeth763>
- Jonas, E.A., and L.K. Kaczmarek. 1996. Regulation of potassium channels by protein kinases. *Curr. Opin. Neurobiol.* 6:318–323. [http://dx.doi.org/10.1016/S0959-4388\(96\)80114-0](http://dx.doi.org/10.1016/S0959-4388(96)80114-0)
- Kaulin, Y.A., J.A. De Santiago-Castillo, C.A. Rocha, M.S. Nadal, B. Rudy, and M. Covarrubias. 2009. The dipeptidyl-peptidase-like protein DPP6 determines the unitary conductance of neuronal K_v4.2 channels. *J. Neurosci.* 29:3242–3251. <http://dx.doi.org/10.1523/JNEUROSCI.4767-08.2009>
- Kim, J., M.S. Nadal, A.M. Clemens, M. Baron, S.C. Jung, Y. Misumi, B. Rudy, and D.A. Hoffman. 2008. K_v4 accessory protein DPPX (DPP6) is a critical regulator of membrane excitability in hippocampal CA1 pyramidal neurons. *J. Neurophysiol.* 100:1835–1847. <http://dx.doi.org/10.1152/jn.90261.2008>
- Kooijman, E.E., and K.N.J. Burger. 2009. Biophysics and function of phosphatidic acid: a molecular perspective. *Biochim. Biophys. Acta.* 1791:881–888.
- Krause, Y., S. Krause, J. Huang, C.H. Liu, R.C. Hardie, and M. Weckström. 2008. Light-dependent modulation of *Shab* channels via phosphoinositide depletion in *Drosophila* photoreceptors. *Neuron.* 59:596–607. <http://dx.doi.org/10.1016/j.neuron.2008.07.009>
- Larsson, O., J.T. Deeney, R. Bränström, P.O. Berggren, and B.E. Corkey. 1996. Activation of the ATP-sensitive K⁺ channel by long chain acyl-CoA. A role in modulation of pancreatic beta-cell glucose sensitivity. *J. Biol. Chem.* 271:10623–10626. <http://dx.doi.org/10.1074/jbc.271.18.10623>
- Levy, D.I., E. Cepaitis, S. Wanderling, P.T. Toth, S.L. Archer, and S.A.N. Goldstein. 2010. The membrane protein MiRP3 regulates K_v4.2 channels in a KChIP-dependent manner. *J. Physiol.* 588:2657–2668. <http://dx.doi.org/10.1113/jphysiol.2010.191395>
- Levy, M., J. Jing, D. Chikvashvili, W.B. Thornhill, and I. Lotan. 1998. Activation of a metabotropic glutamate receptor and protein kinase C reduce the extent of inactivation of the K⁺ channel K_v1.1/K_vbeta1.1 via dephosphorylation of K_v1.1. *J. Biol. Chem.* 273:6495–6502. <http://dx.doi.org/10.1074/jbc.273.11.6495>

- Li, Y., N. Gamper, D.W. Hilgemann, and M.S. Shapiro. 2005. Regulation of K_v7 (KCNQ) K^+ channel open probability by phosphatidylinositol 4,5-bisphosphate. *J. Neurosci.* 25:9825–9835. <http://dx.doi.org/10.1523/JNEUROSCI.2597-05.2005>
- Li, Y., M.A. Zaydman, D. Wu, J. Shi, M. Guan, B. Virgin-Downey, and J. Cui. 2011. KCNE1 enhances phosphatidylinositol 4,5-bisphosphate (PIP_2) sensitivity of I_{Ks} to modulate channel activity. *Proc. Natl. Acad. Sci. USA.* 108:9095–9100. <http://dx.doi.org/10.1073/pnas.1100872108>
- Lindner, M., M.G. Leitner, C.R. Halaszovich, G.R.V. Hammond, and D. Oliver. 2011. Probing the regulation of TASK potassium channels by $PI4,5P_2$ with switchable phosphoinositide phosphatases. *J. Physiol.* 589:3149–3162. <http://dx.doi.org/10.1113/jphysiol.2011.208983>
- Logothetis, D.E., V.I. Petrou, S.K. Adney, and R. Mahajan. 2010. Channelopathies linked to plasma membrane phosphoinositides. *Pflugers Arch.* 460:321–341. <http://dx.doi.org/10.1007/s00424-010-0828-y>
- Loussouarn, G., K.-H. Park, C. Bellocq, I. Baró, F. Charpentier, and D. Escande. 2003. Phosphatidylinositol-4,5-bisphosphate, PIP_2 , controls KCNQ1/KCNE1 voltage-gated potassium channels: a functional homology between voltage-gated and inward rectifier K^+ channels. *EMBO J.* 22:5412–5421. <http://dx.doi.org/10.1093/emboj/cdg526>
- Martens, J.R., Y.G. Kwak, and M.M. Tamkun. 1999. Modulation of K_v channel α/β subunit interactions. *Trends Cardiovasc. Med.* 9:253–258. [http://dx.doi.org/10.1016/S1050-1738\(00\)00037-2](http://dx.doi.org/10.1016/S1050-1738(00)00037-2)
- Murata, Y., and Y. Okamura. 2007. Depolarization activates the phosphoinositide phosphatase Ci-VSP, as detected in *Xenopus* oocytes coexpressing sensors of PIP_2 . *J. Physiol.* 583:875–889. <http://dx.doi.org/10.1113/jphysiol.2007.134775>
- Murata, Y., H. Iwasaki, M. Sasaki, K. Inaba, and Y. Okamura. 2005. Phosphoinositide phosphatase activity coupled to an intrinsic voltage sensor. *Nature.* 435:1239–1243. <http://dx.doi.org/10.1038/nature03650>
- Nadal, M.S., A. Ozaita, Y. Amarillo, E. Vega-Saenz de Miera, Y. Ma, W. Mo, E.M. Goldberg, Y. Misumi, Y. Ikehara, T.A. Neubert, and B. Rudy. 2003. The CD26-related dipeptidyl aminopeptidase-like protein DPPX is a critical component of neuronal A-type K^+ channels. *Neuron.* 37:449–461. [http://dx.doi.org/10.1016/S0896-6273\(02\)01185-6](http://dx.doi.org/10.1016/S0896-6273(02)01185-6)
- Nilius, B., J. Prenen, J. Tang, C. Wang, G. Owsianik, A. Janssens, T. Voets, and M.X. Zhu. 2005. Regulation of the Ca^{2+} sensitivity of the nonselective cation channel TRPM4. *J. Biol. Chem.* 280:6423–6433. <http://dx.doi.org/10.1074/jbc.M411089200>
- Okamura, Y., Y. Murata, and H. Iwasaki. 2009. Voltage-sensing phosphatase: actions and potentials. *J. Physiol.* 587:513–520. <http://dx.doi.org/10.1113/jphysiol.2008.163097>
- Oliver, D., C.C. Lien, M. Soom, T. Baukrowitz, P. Jonas, and B. Fakler. 2004. Functional conversion between A-type and delayed rectifier K^+ channels by membrane lipids. *Science.* 304:265–270. <http://dx.doi.org/10.1126/science.1094113>
- Park, K.-H., J. Piron, S. Dahimene, J. Mérot, I. Baró, D. Escande, and G. Loussouarn. 2005. Impaired KCNQ1-KCNE1 and phosphatidylinositol-4,5-bisphosphate interaction underlies the long QT syndrome. *Circ. Res.* 96:730–739. <http://dx.doi.org/10.1161/01.RES.0000161451.04649.a8>
- Peters, H.C., H. Hu, O. Pongs, J.F. Storm, and D. Isbrandt. 2005. Conditional transgenic suppression of M channels in mouse brain reveals functions in neuronal excitability, resonance and behavior. *Nat. Neurosci.* 8:51–60. <http://dx.doi.org/10.1038/nn1375>
- Piron, J., F.S. Choveau, M.Y. Amarouch, N. Rodriguez, F. Charpentier, J. Mérot, I. Baró, and G. Loussouarn. 2010. KCNE1-KCNQ1 osmoregulation by interaction of phosphatidylinositol-4,5-bisphosphate with Mg^{2+} and polyamines. *J. Physiol.* 588:3471–3483. <http://dx.doi.org/10.1113/jphysiol.2010.195313>
- Pongs, O., and J.R. Schwarz. 2010. Ancillary subunits associated with voltage-dependent K^+ channels. *Physiol. Rev.* 90:755–796. <http://dx.doi.org/10.1152/physrev.00020.2009>
- Powl, A.M., J. Carney, P. Marius, J.M. East, and A.G. Lee. 2005a. Lipid interactions with bacterial channels: fluorescence studies. *Biochem. Soc. Trans.* 33:905–909. <http://dx.doi.org/10.1042/BST20050905>
- Powl, A.M., J.M. East, and A.G. Lee. 2005b. Heterogeneity in the binding of lipid molecules to the surface of a membrane protein: hot spots for anionic lipids on the mechanosensitive channel of large conductance MscL and effects on conformation. *Biochemistry.* 44:5873–5883. <http://dx.doi.org/10.1021/bi047439e>
- Rhee, S.G. 2001. Regulation of phosphoinositide-specific phospholipase C. *Annu. Rev. Biochem.* 70:281–312. <http://dx.doi.org/10.1146/annurev.biochem.70.1.281>
- Ritter, D.M., C. Ho, M.E. O'Leary, and M. Covarrubias. 2012. Modulation of $K_v3.4$ channel N-type inactivation by protein kinase C shapes the action potential in dorsal root ganglion neurons. *J. Physiol.* 590:145–161.
- Rohács, T., C.M. Lopes, T. Jin, P.P. Ramdya, Z. Molnár, and D.E. Logothetis. 2003. Specificity of activation by phosphoinositides determines lipid regulation of K_v channels. *Proc. Natl. Acad. Sci. USA.* 100:745–750. <http://dx.doi.org/10.1073/pnas.0236364100>
- Seino, S. 1999. ATP-sensitive potassium channels: a model of heteromultimeric potassium channel/receptor assemblies. *Annu. Rev. Physiol.* 61:337–362. <http://dx.doi.org/10.1146/annurev.physiol.61.1.337>
- Shapiro, M.S., L.P. Wollmuth, and B. Hille. 1994. Angiotensin II inhibits calcium and M current channels in rat sympathetic neurons via G proteins. *Neuron.* 12:1319–1329. [http://dx.doi.org/10.1016/0896-6273\(94\)90447-2](http://dx.doi.org/10.1016/0896-6273(94)90447-2)
- Shapiro, M.S., J.P. Roche, E.J. Kaftan, H. Cruzblanca, K. Mackie, and B. Hille. 2000. Reconstitution of muscarinic modulation of the KCNQ2/KCNQ3 K^+ channels that underlie the neuronal M current. *J. Neurosci.* 20:1710–1721.
- Suh, B.C., and B. Hille. 2002. Recovery from muscarinic modulation of M current channels requires phosphatidylinositol 4,5-bisphosphate synthesis. *Neuron.* 35:507–520. [http://dx.doi.org/10.1016/S0896-6273\(02\)00790-0](http://dx.doi.org/10.1016/S0896-6273(02)00790-0)
- Suh, B.C., and B. Hille. 2008. PIP_2 is a necessary cofactor for ion channel function: how and why? *Annu. Rev. Biophys.* 37:175–195. <http://dx.doi.org/10.1146/annurev.biophys.37.032807.125859>
- Suh, B.-C., T. Inoue, T. Meyer, and B. Hille. 2006. Rapid chemically induced changes of $PtdIns(4,5)P_2$ gate KCNQ ion channels. *Science.* 314:1454–1457. <http://dx.doi.org/10.1126/science.1131163>
- Suh, B.C., K. Leal, and B. Hille. 2010. Modulation of high-voltage activated Ca^{2+} channels by membrane phosphatidylinositol 4,5-bisphosphate. *Neuron.* 67:224–238. <http://dx.doi.org/10.1016/j.neuron.2010.07.001>
- Telezhkin, V., J.M. Reilly, A.M. Thomas, A. Tinker, and D.A. Brown. 2012. Structural requirements of membrane phospholipids for M-type potassium channel activation and binding. *J. Biol. Chem.* 287:10001–10012. <http://dx.doi.org/10.1074/jbc.M111.322552>
- van der Wal, J., R. Habets, P. Várnai, T. Balla, and K. Jalink. 2001. Monitoring agonist-induced phospholipase C activation in live cells by fluorescence resonance energy transfer. *J. Biol. Chem.* 276:15337–15344. <http://dx.doi.org/10.1074/jbc.M007194200>
- Várnai, P., B. Thyagarajan, T. Rohács, and T. Balla. 2006. Rapidly inducible changes in phosphatidylinositol 4,5-bisphosphate levels influence multiple regulatory functions of the lipid in intact living cells. *J. Cell Biol.* 175:377–382. <http://dx.doi.org/10.1083/jcb.200607116>

- Whorton, M.R., and R. MacKinnon. 2011. Crystal structure of the mammalian GIRK2 K⁺ channel and gating regulation by G proteins, PIP₂, and sodium. *Cell*. 147:199–208. <http://dx.doi.org/10.1016/j.cell.2011.07.046>
- Winklhofer, M., K. Matthias, G. Seifert, M. Stocker, S. Sewing, T. Herget, C. Steinhäuser, and S. Saaler-Reinhardt. 2003. Analysis of phosphorylation-dependent modulation of Kv1.1 potassium channels. *Neuropharmacology*. 44:829–842. [http://dx.doi.org/10.1016/S0028-3908\(03\)00070-4](http://dx.doi.org/10.1016/S0028-3908(03)00070-4)
- Winks, J.S., S. Hughes, A.K. Filippov, L. Tatulian, F.C. Abogadie, D.A. Brown, and S.J. Marsh. 2005. Relationship between membrane phosphatidylinositol-4,5-bisphosphate and receptor-mediated inhibition of native neuronal M channels. *J. Neurosci*. 25:3400–3413. <http://dx.doi.org/10.1523/JNEUROSCI.3231-04.2005>
- Zhang, H., L.C. Craciun, T. Mirshahi, T. Rohács, C.M. Lopes, T. Jin, and D.E. Logothetis. 2003. PIP₂ activates KCNQ channels, and its hydrolysis underlies receptor-mediated inhibition of M currents. *Neuron*. 37:963–975. [http://dx.doi.org/10.1016/S0896-6273\(03\)00125-9](http://dx.doi.org/10.1016/S0896-6273(03)00125-9)