Regulation of voltage-gated potassium channels by PI(4,5)P_2

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Phosphatidylinositol 4,5-bisphosphate (PI(4,5)P_2) 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)P_2. 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)P_2, we have coexpressed several of them in tsA-201 cells with a G protein–coupled receptor (M_1R), 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)P_2 with application of muscarinic agonists, depolarization, or rapamycin, respectively. PI(4,5)P_2 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 M_1R inhibited K_V7.2/7.3 current similarly. With these tools, we tested for potential PI(4,5)P_2 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 M_1R 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)P_2 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.

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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)P_2; 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)P_2 sensitivity of K_V channels.

PI(4,5)P_2 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)P_2 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 (IP_3) through the cleavage of PI(4,5)P_2 by phospholipase C (PLC; Covarrubias et al., 1994; Martens et al., 1999; Rhee, 2001). DAG and IP_3 in turn activate enzymes like PKC and increase intracellular Ca^{2+} 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

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Abbreviations used in this paper: CHO, Chinese hamster ovary; DAG, diacylglycerol; FRET, Förster resonance energy transfer; IP_3, inositol 1,4,5-trisphosphate; K_V, voltage-gated potassium; Oxo-M, oxotremorine methiodide; PI(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C.

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of ion channel activity by $PI(4,5)P_2$ breakdown can be through direct loss of a cofactor for channel gating or through secondary modifications.

Direct regulation of K_V channels by $PI(4,5)P_2$ has so far been reported for some K_V1, K_V3, K_V7, and K_V11 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 $K_V1.1/K_V\beta1.1$, $K_V1.5/K_V\beta1.3$, and $K_V3.4$ channels by $PI(4,5)P_2$ were seen in membrane patches excised from oocytes of *Xenopus laevis*. Addition of $PI(4,5)P_2$ 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 K_V7 (KCNQ) channels showed a $PI(4,5)P_2$ 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 K_V7 channels, much additional evidence comes from another approach, namely by manipulating enzymes that make or destroy $PI(4,5)P_2$ in intact cells. The initial experiments on K_V7 channels used PLC and inhibitors of lipid kinases (Suh and Hille, 2002), and subsequent work exploited $PI(4,5)P_2$ 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 $PI(4,5)P_2$ levels. Of the K_V channels, only $K_V7.2$ and $K_V7.3$ 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 $PI(4,5)P_2$ 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(PLCδ1) and eYFP-PH(PLCδ1) 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_1R (M_1 muscarinic receptor)-YFP from N. Nathanson (University of Washington, Seattle, WA); rat $K_V1.1$, rat $K_V1.4$, and rat $K_V\beta1.1$ from J. Trimmer (University of California, Davis, Davis, CA); human $K_V1.3$, human $K_V1.5$, human $K_V\beta1.3$, and human $K_V2.1$ 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 $K_V3.4$ from J. Surmeier (Northwestern University, Chicago, IL); human $K_V4.2$, $K_V4.3$, and human KChIP1, KChIP2, and DPP6-s from B. Rudy (New York University School of Medicine, New York, NY); $hK_V7.1$-GFP and hKCNE1 from M. Shapiro (University of Texas, San Antonio, TX); human $K_V7.2$ and human $K_V7.3$ 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 $K_{ir}2.1$ from D. Logothetis (Virginia Commonwealth University, Richmond, VA). We also used a new dual phosphatase, pseudojanin to deplete both $PI(4,5)P_2$ and PI(4)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/DDBJ 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 MΩ 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_4-BAPTA, 3 mM Na_2ATP, 0.1 mM Na_3GTP, and 5 mM HEPES at pH 7.4. For recordings of $K_{ir}2.1$ 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_4-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 Ca^{2+} measurements, 100 µM 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 Ca^{2+} concentrations are reported as the ratio F_{340}/F_{380} 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 ± 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 M_1R (Fig. S1), current traces of endogenous K_V channels in tsA-201 cells (Fig. S2), current traces of $K_{ir}2.1$ and $K_V7.2/K_V7.3$ channels in response to activation of M_1R (Fig. S3), $FRET_r$ traces of PH probes in response to an activation of Dr-VSP combined with simultaneous whole-cell recording of $K_V1.5/K_V\beta1.3$ current (Fig. S4), line scan measurements of PH probe fluorescence in cells before and after activation of M_1R, Dr-VSP, and pseudojanin (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 $PI(4,5)P_2$ depletion at the plasma membrane

Unexpectedly, many of the K_V channels we studied proved to be insensitive to enzymatic depletion of $PI(4,5)P_2$. 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 $PI(4,5)P_2$ sensitive. First, we and others have shown a strong $PI(4,5)P_2$ requirement of $K_V7.2$ (KCNQ2)/$K_V7.3$ (KCNQ3) channel currents. Decreases in $PI(4,5)P_2$ 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 $PI(4,5)P_2$ 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 ± 4%

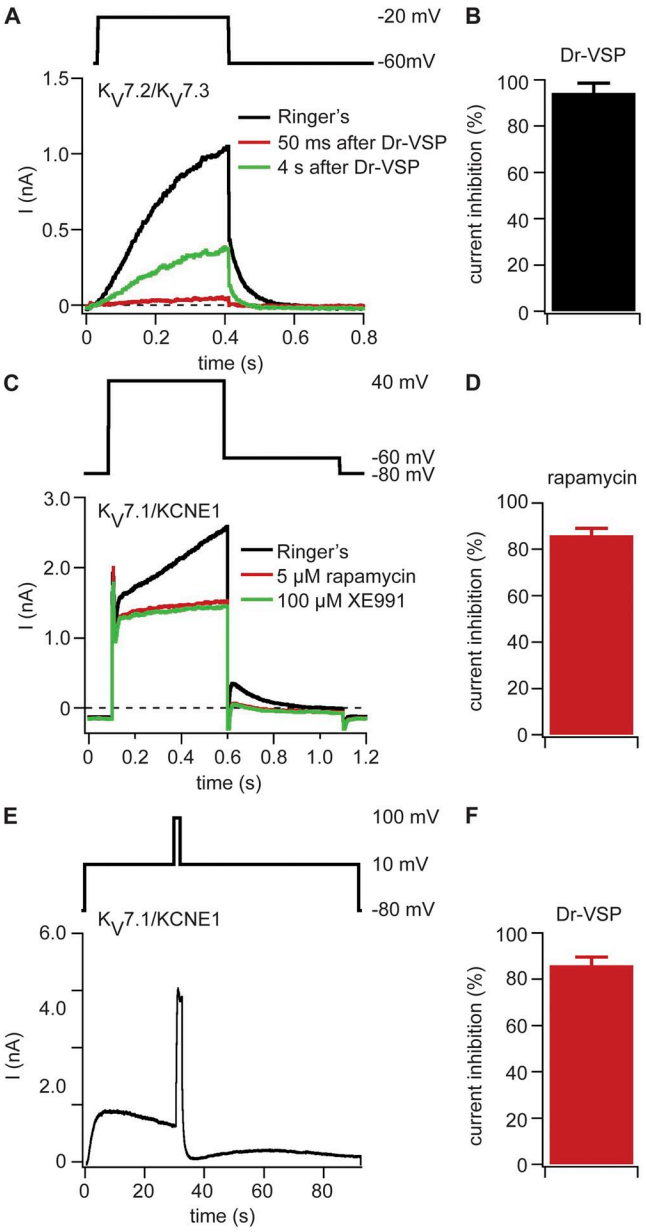

Figure 1. $PI(4,5)P_2$ depletion at the plasma membrane inhibits $K_V7.x$ channels. (A) Currents with coexpression of $K_V7.2$, $K_V7.3$, and Dr-VSP in tsA-201 cells. The black current trace was recorded before Dr-VSP activation, the red trace was recorded 50 ms after Dr-VSP activation by a 2-s pulse 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) Currents 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 µM rapamycin (black), after 60 s of rapamycin application (red), and after application of 100 µM 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 ±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)P_2 levels returned to their original state. Fig. S1 shows a comparable inhibition of K_V7.2/K_V7.3 channels upon activation of coexpressed M_1 muscarinic receptors (M_1R), as in previous work (Shapiro et al., 2000). Thus, activation of Dr-VSP or of M_1R depletes PI(4,5)P_2 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 coexpression 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)P_2 (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)P_2 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 µM 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)P_2 suppressed the K_V7.1/KCNE1-mediated currents by 86 ± 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)P_2 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 ± 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)P_2 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)P_2. We score the effects of PI(4,5)P_2 depletion on K_V7 channels as strong inhibition in summary Table 1.

TABLE 1

M_1 receptor and phosphatase effects on K_V channel

α subunit	Auxiliary subunit	M_1R	Dr-VSP	Pseudojanin or FKBP-Inp54p (in case of K_V7.1)
K_V1.1	K_Vβ1.1	Slowing of inactivation[a]	No effect	No effect
K_V1.3	None	No effect	ND	No effect
K_V1.4	None	No effect	No effect	No effect
K_V1.5	± K_Vβ1.3	No effect	No effect	No effect
K_V2.1	None	∼22% decrease	No effect	No effect
K_V3.4	None	Slowing of inactivation[a]	No effect	No effect
K_V4.2/K_V4.3	None	No effect	No effect	ND
K_V4.2/K_V4.3	KChIP1	ND	No effect	ND
K_V4.2/K_V4.3	KChIP1 + DPP6-s	ND	No effect	ND
K_V4.2/K_V4.3	KChIP2	ND	No effect	ND
K_V7.1 (KCNQ1)	KCNE1	ND	Strong inhibition	Strong inhibition
K_V7.2/7.3 (KCNQ2/Q3)	None	Strong inhibition[a]	Strong inhibition[a]	Strong inhibition
K_V11.1 (hERG)	None	ND	No effect	ND
K_V11.1 (hERG)	KCNE2	∼30% decrease	No effect	No effect
K_{ir}2.1	None	No effect	∼45% decrease	Strong inhibition

[a]These 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 µM; Zhang et al., 2003; Li et al., 2005) or containing $K_{ir}2.1$ (apparent K_d = 5 µM; 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 ± 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

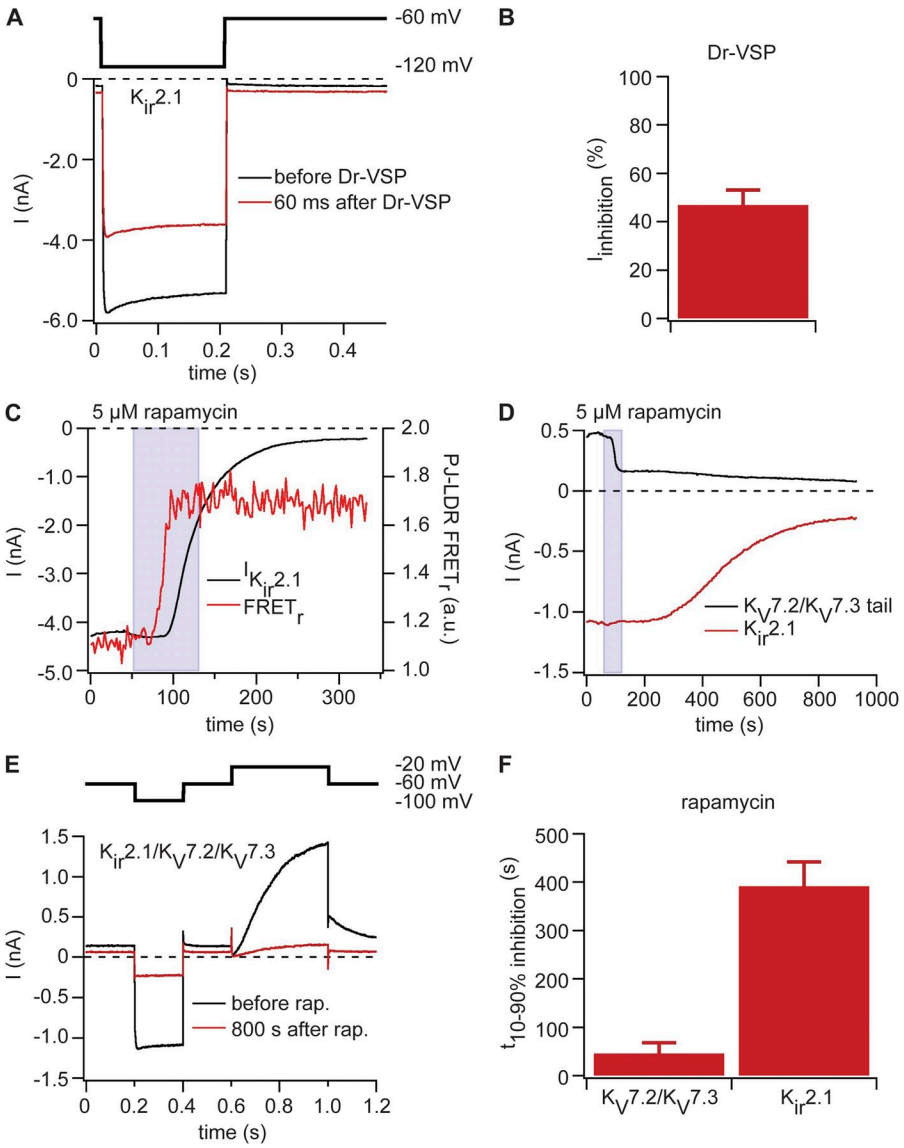

Figure 2. Dr-VSP and pseudojanin can inhibit $PI(4,5)P_2$-sensitive $K_{ir}2.1$ channels. (A) Currents in $K_{ir}2.1$ channels coexpressed with Dr-VSP. Black indicates current trace before activation of Dr-VSP, and red indicates current trace 60 ms after activation of Dr-VSP by a 2-s pulse to 100 mV. 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 ±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 $PI(4,5)P_2$.

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 $PI(4,5)P_2$, resulting in different kinetics of channel inhibition as $PI(4,5)P_2$ 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 ∼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

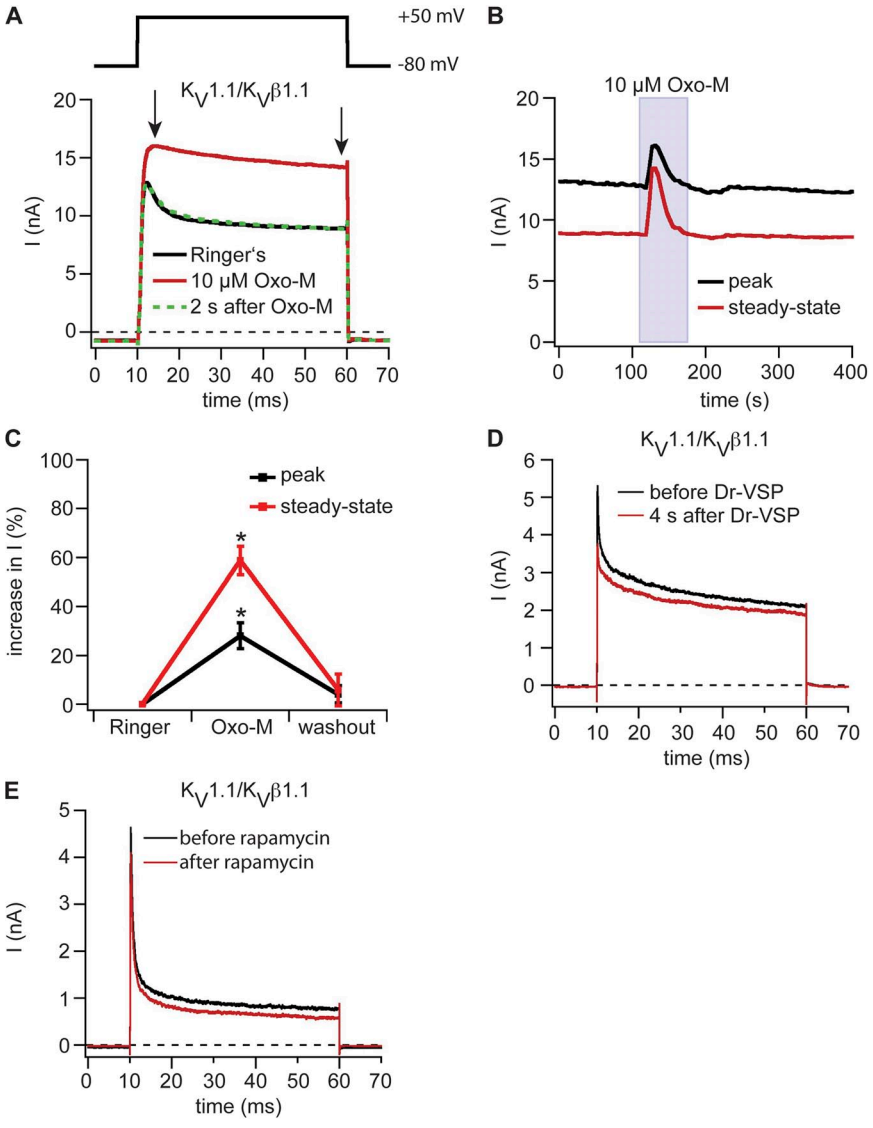

Figure 3. Modulation of K_V1 family channels by depletion of $PI(4,5)P_2$ at the plasma membrane. (A) Potassium outward currents in $K_V1.1/K_V\beta1.1$ channel complexes coexpressed with M_1R. Black indicates control current trace in Ringer's solution, red indicates during application of 10 µM Oxo-M, 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 $K_V1.1/K_V\beta1.1$ channel complexes coexpressed with M_1R upon stimulation with Oxo-M ($n = 7$). Error bars represent ±SEM. *, $P < 0.05$. (D) Current time course in a cell expressing $K_V1.1/K_V\beta1.1$ 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 ± 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 ± 5%, and steady-state currents increased 59 ± 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

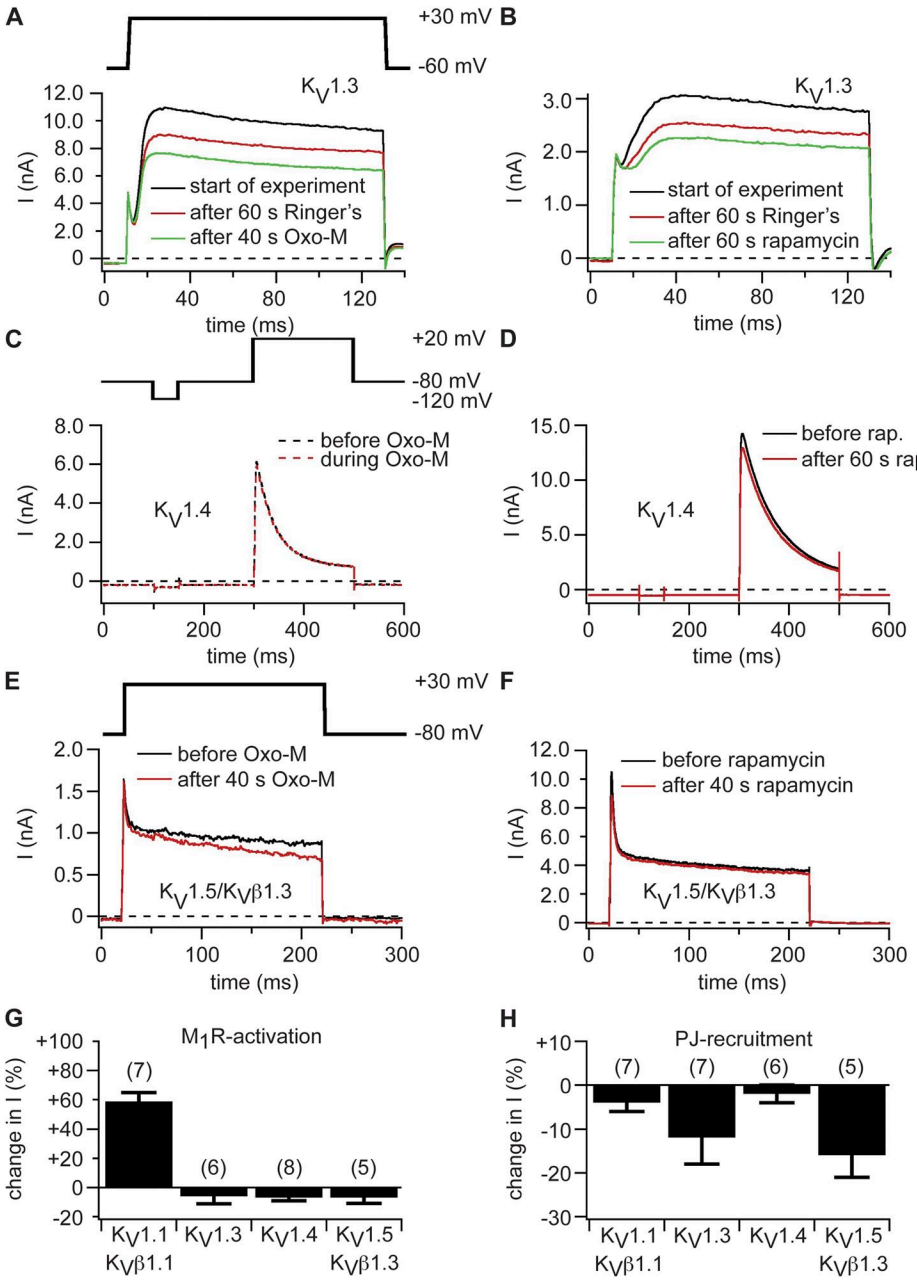

Figure 4. $K_V1.3$, $K_V1.4$, and $K_V1.5$ channels are insensitive to depletion of $PI(4,5)P_2$ at the plasma membrane. (A) Current traces of $K_V1.3$ channels coexpressed with M_1R. Black indicates traces at start of experiment, red indicates after 60 s in Ringer's, and green indicates traces after 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 ±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_V1 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(PLCδ1) and eYFP-PH(PLCδ1) 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

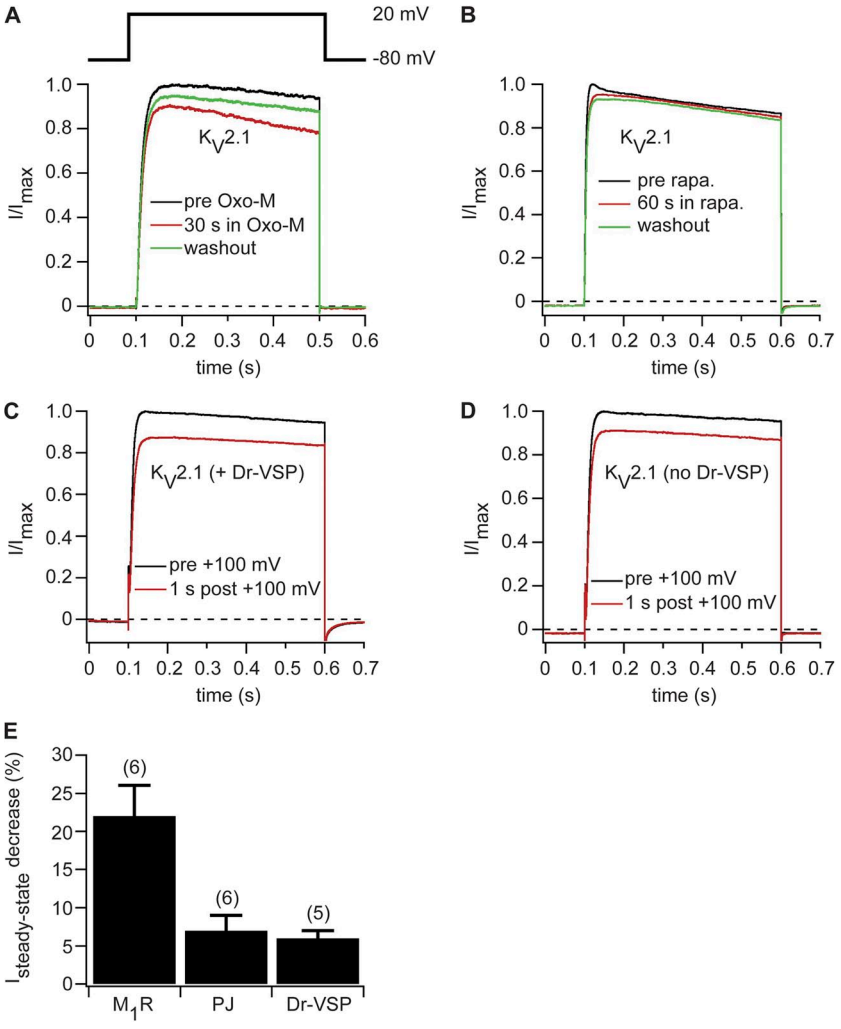

Figure 5. $K_V2.1$ channels are not sensitive to depletion of $PI(4,5)P_2$ by activation of pseudojanin or Dr-VSP. (A) Normalized current traces recorded before (black), during (red), and after (green) application of 10 µM Oxo-M in a cell transiently transfected with $K_V2.1$ and M_1R. (B) Current traces before (black), after 60 s of application (red), and after washout of 5 µM 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 ±SEM.

YFP-tagged PH probes with M_1R, Dr-VSP, or pseudojanin and performed line scan measurements with confocal laser microscopy before and after activation of these $PI(4,5)P_2$-depleting enzymes. As expected, we observed a strong localization of PH probes at the plasma membrane before $PI(4,5)P_2$ depletion and a reduction in YFP fluorescence at the plasma membrane of >70% after $PI(4,5)P_2$ 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 $PI(4,5)P_2$ depletion. The confocal images (Fig. S5, A–F, insets) show that our three tools for $PI(4,5)P_2$ depletion act uniformly along the membrane. They do not leave patches of unaltered $PI(4,5)P_2$ 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 M_1R to confirm that Ca^{2+} transients occurred during activation of M_1R (see Fig. 8 D for example). With these internal controls, we feel confident in scoring the tested K_V1 channels as not sensitive to $PI(4,5)P_2$ depletion (Table 1).

$K_V2.1$ channels are not sensitive to $PI(4,5)P_2$ depletion

Next, we asked whether $K_V2.1$ channels are sensitive to $PI(4,5)P_2$ depletion. First, we expressed $K_V2.1$ channels together with M_1R. Currents were elicited by a depolarizing test pulse to 20 mV from a holding potential of −80 mV. Upon activation of M_1R, 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 washout 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 $PI(4,5)P_2$ or a result of downstream signaling pathways after $PI(4,5)P_2$ hydrolysis by PLC, we expressed $K_V2.1$ channels together with either pseudojanin or Dr-VSP, which deplete $PI(4,5)P_2$ 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 M_1R activation is caused by signaling pathways downstream of $PI(4,5)P_2$ hydrolysis by activation of PLC or is a result of a direct loss of $PI(4,5)P_2$.

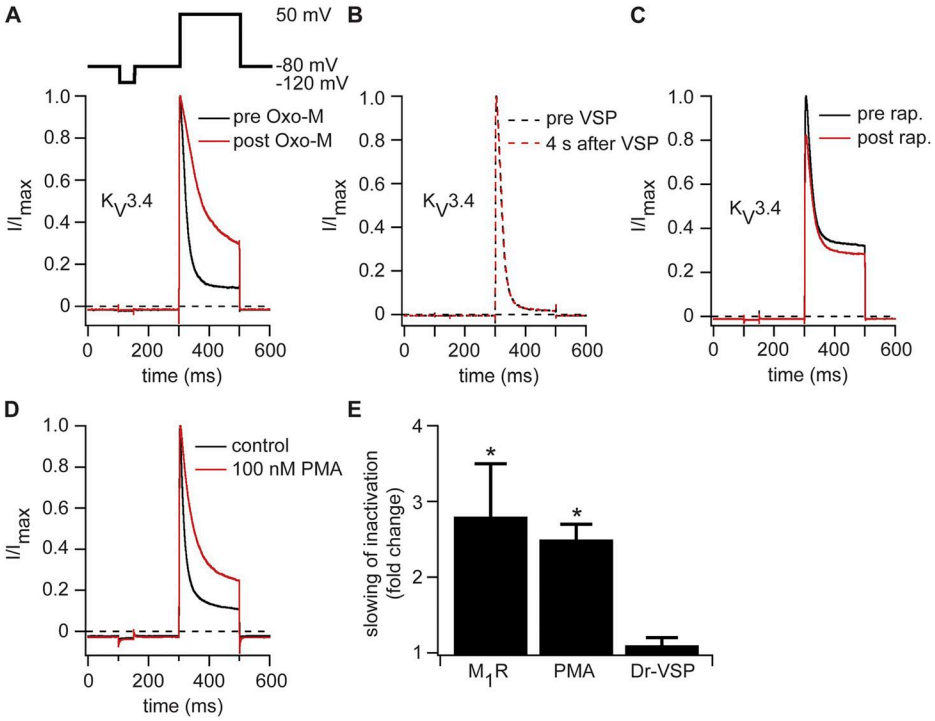

Figure 6. $K_V3.4$ channels are modulated by activation of M_1R but not by activation of Dr-VSP or pseudojanin. (A) Normalized current traces recorded before (black) and during (red) application of 10 µM Oxo-M in a cell transiently transfected with $K_V3.4$ and M_1R. (B) Same as in A but with expression of Dr-VSP and an activating pulse instead of M_1R. (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 M_1R, application of 100 nM PMA, or activation of Dr-VSP ($n = 4$ [M_1R] 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 M_1R activation

In a similar approach, we expressed $K_V3.4$ channels together with M_1R, 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 M_1R 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 M_1R, 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 $K_V1.1/K_V\beta1.1$ channels, these experiments find no direct regulation of $K_V3.4$ channels by depletion of $PI(4,5)P_2$, but they do find a large effect of M_1R, suggesting a possible role for the products of $PI(4,5)P_2$ 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 M_1R activation could be the result of an activation of PKC by the DAG produced during $PI(4,5)P_2$ 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 M_1R 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 M_1R activation.

Complexes of $K_V4.2$ and $K_V4.3$ channels with KChIPs and DPP6-s are not sensitive to $PI(4,5)P_2$ 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 $PI(4,5)P_2$. 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

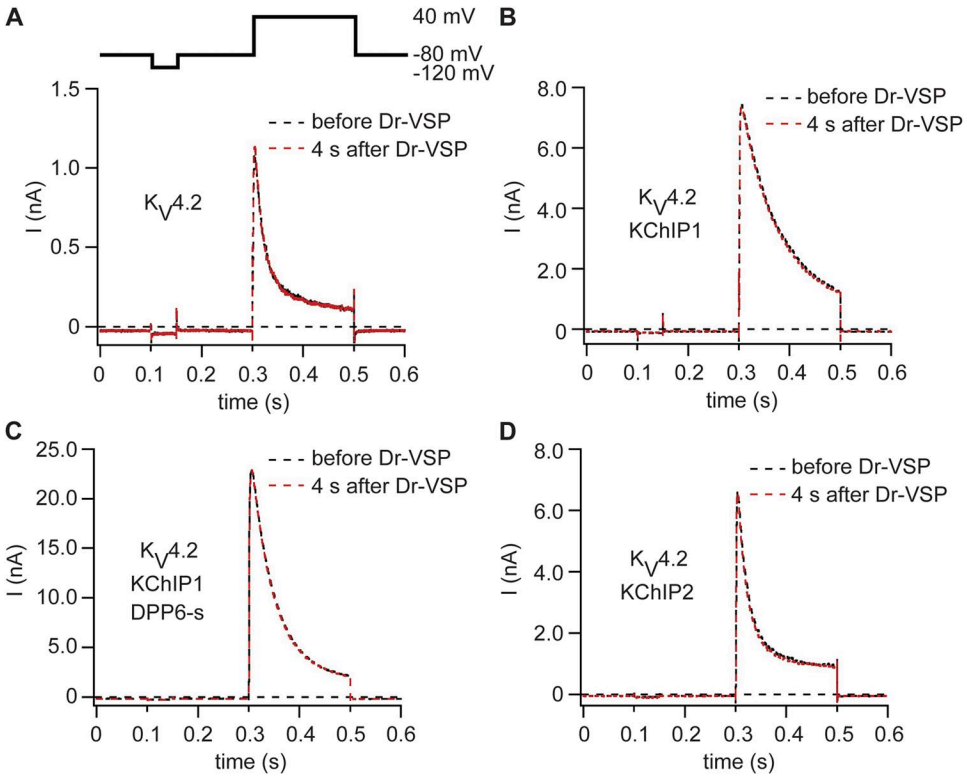

Figure 7. $K_V4.2$ channels are insensitive to depletion of $PI(4,5)P_2$ by Dr-VSP. (A) Current traces of a cell expressing $K_V4.2$ and Dr-VSP. Black indicates before activation of Dr-VSP, and red indicates 4 s after activation of Dr-VSP. (B–D) Same protocol as in A but with coexpression of KChIP1 (B), 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

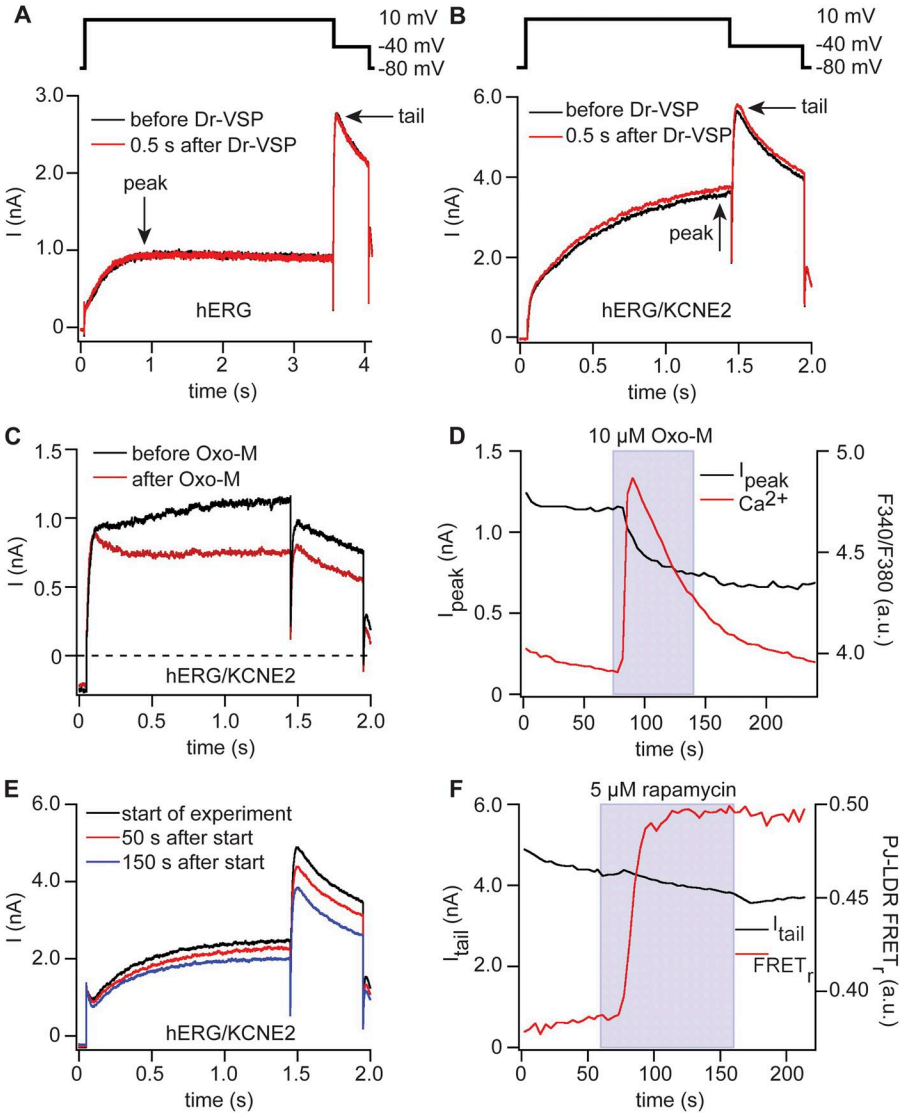

Figure 8. hERG channels are sensitive to M_1R activation but not to activation of Dr-VSP or pseudojanin. (A) Currents in hERG channels coexpressed with Dr-VSP. Black indicates before activation of Dr-VSP, and red indicates directly after activation of Dr-VSP. Arrows indicate points at which peak and 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 µM 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 M_1R modulates channel activity. With hERG and KCNE2, M_1R 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 Ca^{2+} (Fig. 8 D), but, when the Ca^{2+} transient relaxed back to its original level, current amplitudes did not recover.

The depression of hERG current during M_1R 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 M_1R activation but give no evidence for a direct modulation of hERG channels by $PI(4,5)P_2$ depletion.

DISCUSSION

We have studied the $PI(4,5)P_2$ 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 $PI(4,5)P_2$ 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 $PI(4,5)P_2$ sensitivity of several of these channels, $K_V1.1$ with $K_V\beta1.1$, $K_V1.4$, $K_V1.5$ with $K_V\beta1.3$ 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 $PI(4,5)P_2$ 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 $PI(4,5)P_2$ as that with $PI(4,5)P_2$ perfusion onto excised patches? Are different K^+ channels localized to different plasma membrane subdomains where they experience different pools of $PI(4,5)P_2$? Could superfusion of $PI(4,5)P_2$ 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 $PI(4,5)P_2$ and PI(4)P be keeping K_V channels active?

Depletion of $PI(4,5)P_2$

Our results with activation of phosphatases were unambiguously negative, so it was essential to show that our tools actually depleted plasma membrane $PI(4,5)P_2$. In many experiments, we recorded FRET from PH domains of PLCδ1 simultaneously with the current traces and found that $PI(4,5)P_2$ 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 $PI(4,5)P_2$ depletion. Right after activation of Dr-VSP, M_1R, 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 $PI(4,5)P_2$ depletion was uniform at the plasma membrane. It should be noted that because phosphatases do not generate IP_3, the translocation of PH domains during phosphatase activation really reflects loss of free membrane $PI(4,5)P_2$ rather than any gain of cytoplasmic IP_3. In some experiments when activating M_1R, we also validated activation of PLC by measuring the concomitant rises in Ca^{2+}. 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 M_1R 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 $PI(4,5)P_2$ dependence of K_V channels even if they have a high affinity to $PI(4,5)P_2$ by using Dr-VSP and pseudojanin.

We can attempt to calibrate the membrane $PI(4,5)P_2$ levels in terms of equivalent diC_8-$PI(4,5)P_2$ concentrations in solution. In these terms, the equivalent diC_8-$PI(4,5)P_2$ 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 diC_8-$PI(4,5)P_2$ suffices to support 10% $K_{ir}2.1$ channel activity (Du et al., 2004). These parameters define the window of $PI(4,5)P_2$ levels we have studied in terms of an equivalent diC_8-$PI(4,5)P_2$ scale.

Are other investigators exploring a different range of available $PI(4,5)P_2$ than we are? It was when adding 10 µM brain $PI(4,5)P_2$ to patches from *Xenopus* oocytes with $K_V1.1$ with $K_V\beta1.1$, $K_V1.4$, and $K_V1.5$ with $K_V\beta1.3$, 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 $PI(4,5)P_2$ 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 $PI(4,5)P_2$. Consistent with our phosphatase results, they did not describe any rundown that accompanied gradual loss of endogenous $PI(4,5)P_2$. When these authors then applied $PI(4,5)P_2$, they used long-chain brain $PI(4,5)P_2$ 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, $PI(4,5)P_2$, and $PI(3,4,5)P_3$ were equally effective.

Could superfusion of excised patches with long-chain $PI(4,5)P_2$ induce nonspecific effects? One possibility is that it leads to supraphysiological high concentrations of $PI(4,5)P_2$ in the patch, above the concentration range we studied with our tools. At these elevated concentrations, $PI(4,5)P_2$ might interact with domains in the channel proteins that would not normally couple to endogenous $PI(4,5)P_2$. 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 meditated 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 $PI(4,5)P_2$. It would also contain the M_1Rs, PLC, PH domains, VSP, and the membrane anchor LDR-CFP so that activation of PLC or recruiting phosphatases to this domain would deplete $PI(4,5)P_2$, 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 $PI(4,5)P_2$ because, according to our chemical measurements in M_1R-expressing CHO cells, 93% of the total cell $PI(4,5)P_2$ 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 $PI(4,5)P_2$ and not be influenced by the $PI(4,5)P_2$ depletion. However, like the other domain, it would accept applied $PI(4,5)P_2$, 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 $PI(4,5)P_2$ 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 $PI(4,5)P_2$ that have at least an acyl chain and a phosphate head group (Telezhkin et al., 2012). However, $PI(4,5)P_2$ 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 M_1R, 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 M_1R activation ($K_V1.3$, $K_V1.4$, and $K_V1.5 \pm K_V\beta1.3$), but the inactivation gating kinetics of two of the K_V channels ($K_V1.1/K_V\beta1.1$ and $K_V3.4$) were strongly slowed. From our observations, we conclude that slowing of inactivation for $K_V1.1/K_V\beta1.1$ and $K_V3.4$ during PLC activation is not caused by the resulting depletion of $PI(4,5)P_2$. 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; Winklhofer et al., 2003). For example, consider $K_V\beta1.1$, 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; Winklhofer 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 M_1R activation. This finding agrees with the published studies and reiterates the importance of DAG as a PKC-activating product downstream of $PI(4,5)P_2$ hydrolysis after activation of G_q-coupled receptors.

In contrast, in *Drosophila melanogaster*, the *shab* channel (K_V2 family) apparently is inhibited by $PI(4,5)P_2$ 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 IP_3 or a calcium rise and with the same rapid kinetics as phototransduction. Direct application of 40 µM diC_8-$PI(4,5)P_2$ 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 M_1R reduced hERG current amplitude slightly; however, activating Dr-VSP yielded no detectable change. With pseudojanin, 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 $PI(4,5)P_2$ dependence. In CHO cells, hERG current amplitude increases upon dialysis of 10 µM brain $PI(4,5)P_2$ into cells via the patch pipette, and it decreases upon activation of G_q-coupled $\alpha1_A$-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 $\alpha1_A$-adrenergic receptor stimulation resemble ours with M_1R, but our experiments did not find evidence for regulation by $PI(4,5)P_2$. We conclude that $PI(4,5)P_2$ 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 $PI(4,5)P_2$ 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 $PI(4,5)P_2$ requirement and that $PI(4,5)P_2$ 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 Ca^{2+} channels in sympathetic neurons (Shapiro et al., 1994; Suh et al., 2010) upon activation of endogenous M_1 muscarinic receptors. Some K_V channels, like $K_V1.1/K_V\beta1.1$, $K_V3.4$, and hERG, are modulated by apparently conventional second-messenger signaling pathways downstream of $PI(4,5)P_2$ hydrolysis. Unexpectedly, many K_V channels showed no change in properties when $PI(4,5)P_2$ was manipulated by lipid phosphatases. We feel we have explored the range of free $PI(4,5)P_2$ levels that might be experienced physiologically in the plasma membrane. Thus, any ability to modulate some of them with applications of brain $PI(4,5)P_2$ as micelles may be unphysiological.

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