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The phosphoinositide sensitivity of the K_V channel family

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Recently, we screened several K_V channels for possible dependence on plasma membrane phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$). The channels were expressed in tsA-201 cells and the $PI(4,5)P_2$ was depleted by several manipulations in whole-cell experiments with parallel measurements of channel activity. In contrast to reports on excised-patches using *Xenopus laevis* oocytes, we found only K_V7, but none of the other tested K_V channels, to be strongly dependent on $PI(4,5)P_2$. We now have extended our study to $K_V1.2$ channels, a K_V channel we had not previously tested, because a new published study on excised patches showed regulation of the voltage-dependence of activation by $PI(4,5)P_2$. In full agreement with those published results, we found a reduction of current amplitude by ~20% after depletion of $PI(4,5)P_2$ and a small left shift in the activation curve of $K_V1.2$ channels. We also found a small reduction of $K_V11.1$ (hERG) currents that was not accompanied by a gating shift. In conclusion, our whole-cell methods yield a $PI(4,5)P_2$-dependence of $K_V1.2$ currents in tsA-201 cells that is comparable to findings from excised patches of *Xenopus laevis* oocytes. We discuss possible physiological rationales for $PI(4,5)P_2$ sensitivity of some ion channels and insensitivity of others.

Keywords: Voltage-gated potassium channel, phosphatidylinositol 4,5-bisphosphate, $K_V1.2$

Abbreviations: FKBP, FK506-binding protein; FRB, rapamycin-binding domain of mTOR; GPCR, G-protein coupled receptor; K_V channel, voltage-gated potassium channel; LDR, Lyn_{11}-targeted FRB; M_1R, M_1 muscarinic (acetylcholine) receptor; Oxo-M, oxotremorine methiodide; $PI(4,5)P_2$, phosphatidylinositol 4, 5-bisphosphate; PJ, pseudojanin recruitable phosphatase; PLC, phospholipase C

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Introduction

Here we revisit the regulation of voltage-gated potassium (K_V) channels by plasma membrane phosphoinositide phospholipids. Although not in high abundance, the phosphoinositides of eukaryotic biological membranes regulate many membrane proteins through protein-lipid interaction domains.[1-3]

At the plasma membrane, phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) is the dominant phosphoinositide. It enhances the activity of many ion channels, and, for some channels, is necessary for activity.[3-6] Thus, it is obligatory for function of all 5 members of the K_V7 channel family and of nearly all inward rectifiers and TRP channels.[3,7-9] In recent reviews, the number of ion channels said to be regulated by $PI(4,5)P_2$ has grown so large (> 80,[3,6]) that one might anticipate that all plasma membrane channels are sensitive. However, using whole-cell recording and enzymatic methods to deplete endogenous $PI(4,5)P_2$, our laboratory failed to find $PI(4,5)P_2$ sensitivity in several channels. For example, we found that only 4 out of 8 tested voltage-gated calcium (Ca_V) channel subtypes were significantly depressed when $PI(4,5)P_2$ levels were enzymatically lowered, and some of these sensitive Ca_V channels became nearly insensitive when coexpressed with a different $Ca_V\beta$ subunit.[10,11]

Recently, we screened for $PI(4,5)P_2$ sensitivity of 8 voltage-gated potassium (K_V) channels from the K_V1, 2, 3, and 4 families, again using whole-cell methods and enzyme recruitment.[12] Three of the channels we tested, $K_V1.1$, 1.4, and 3.4, had been studied before in excised patches from *Xenopus laevis* oocytes.[13] The authors had reported interesting changes of current kinetics and amplitude when exogenous brain $PI(4,5)P_2$ was applied to the cytoplasmic face. Thus, we assumed our screen would identify many lipid-sensitive channels, yet we saw no sensitivity to $PI(4,5)P_2$ depletion for any of them ($K_V1.1$, 1.3, 1.4, 1.5, 2.1, 3.4, 4.2, and 4.3). For large test depolarizations, neither the current amplitude nor the gating

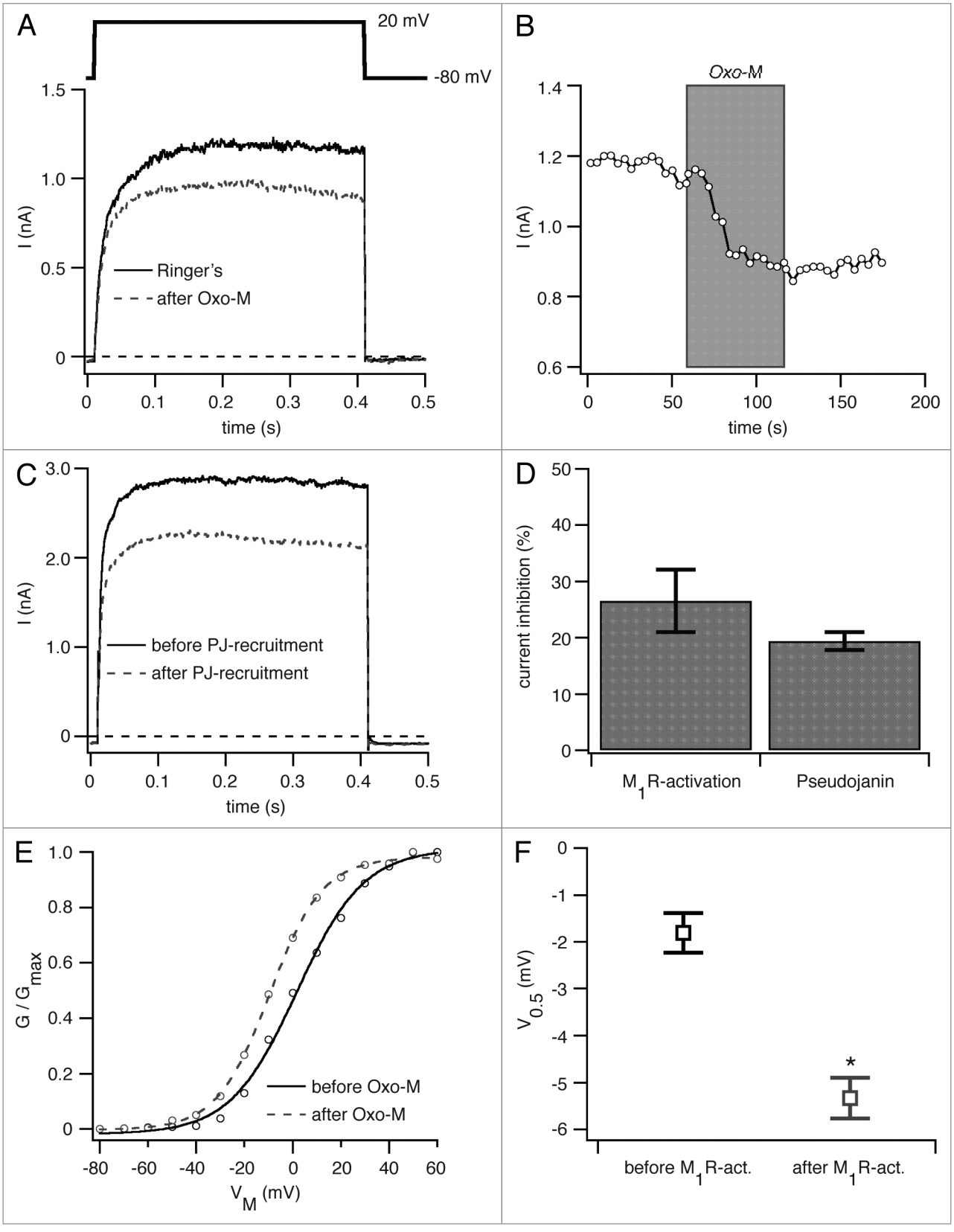

Figure 1. For figure legend, see page 532.

Figure 1 (see page 531). PI(4,5)P_2 dependence of K_V1.2 channels. (**A**) K_V1.2 channels and M_1R were transiently expressed in tsA-201 cells. Currents were recorded in the whole-cell configuration (pulse protocol shown above current traces). Figure shows current traces before (solid black) and after (dashed gray) application of 10 µM Oxo-M. (**B**) Time course of K_V1.2 mediated currents at +20 mV for the experiment shown in (**A**). (**C**) Current traces of K_V1.2 channels coexpressed with pseudojanin-YFP and LDR-CFP before (solid black) and after (dashed gray) addition of rapamycin. (**D**) Percentage inhibition of K_V1.2 channels by activation of M_1R or by recruitment of pseudojanin to the plasma membrane. (**E**) Representative G-V curve generated from test pulses to membrane potentials of – 80 to +60 mV from a holding potential of – 80 mV before (solid black) and after (dashed gray) activation of M_1R by Oxo-M application. (**F**) Voltage of half-maximal activation ($V_{0.5}$) for K_V1.2 channels before and after M_1R-activation. *$p < 0.05$.

kinetics were changed. In the same study, we did confirm that our methods easily resolved the well-known lipid sensitivity of K_V7.1, 7.2, and 7.3 and K_{ir}2.1 channels. Subsequently, using mostly different approaches, Rodriguez-Menchaca et al. reported that K_V1.2 channels are sensitive to PI(4,5)P_2 depletion.[14] They found a ~30% decrease in current amplitude from *Xenopus* oocytes by depleting excised patches of PI(4,5)P_2 and a restoration of the original current amplitude by perfusing PI(4,5)P_2 onto the inside-out patches. They recognized a dual effect of depleting PI(4,5)P_2: First, a decrease of maximum open probability and, second, a left-shift of ~14 mV in the voltage dependence of the activation curve. This result was not in contradiction to ours since we had not tested K_V1.2 channels in our screen. Nevertheless, we were stimulated by this new work to check whether our whole-cell assay system, which had given negative results with other channels, would confirm PI(4,5)P_2 sensitivity of K_V1.2.

Results

As in our previous paper,[12] the experimental design was to study ion channels transfected in mammalian tsA-201 cells by whole-cell voltage clamp. Plasma membrane phosphoinositides were depleted by 2 enzymatic maneuvers: (1) by stimulating a G protein coupled receptor (GPCR) coupled to phospholipase C (PLC), and (2) by using chemical dimerization to recruit lipid phosphatases to the plasma membrane.

PI(4,5)P_2 dependence of K_V1.2 channels

We first tested PI(4,5)P_2 depletion by PLC. K_V1.2 channels were co-expressed with PLC-coupled M_1 muscarinic receptors (M_1R) in tsA-201 cells, and depolarizing pulses to 20 mV elicited outward K^+ currents (**Fig. 1A**). Application of the muscarinic agonist oxotremorine methiodide (Oxo-M) led to a clear decrease in the current amplitude (**Fig. 1A and B**) on average by 27 ± 6% (n = 5, **Fig. 1D**). Activation of phospholipase C (PLC) is a complex stimulus. It generates several intracellular signals including depletion of PI(4,5)P_2, rise of cytoplasmic inositol trisphosphate (Ins(1,4,5)P_3) and calcium, production of diacylglycerol (DAG) and activation of protein kinase C (PKC).

To check whether the effects were really due to PI(4,5)P_2 depletion, we turned to recruitment of the lipid phosphatase pseudojanin (PJ) to the plasma membrane as another tool to deplete PI(4,5)P_2. Pseudojanin is an engineered fusion protein containing a rapamycin-binding domain (FKBP) and 2 lipid phosphatase domains in tandem (derived from Inp54p and Sac1 enzymes), which dephosphorylate PI(4,5)P_2 at the 5-position (Inp54p) and PI(4)P at the 4-position (Sac1) to yield phosphatidylinositol (PI).[12,15] Addition of the membrane-permeable drug rapamycin dimerizes the FKBP domain with the coexpressed membrane anchor Lyn-FRB-CFP, thus recruiting the pseudojanin phosphatases to the plasma membrane.[16] This dimerization strategy depletes PI(4,5)P_2 at the plasma membrane without generating downstream signaling molecules like Ins(1,4,5)P_3 or DAG. As the FKBP-rapamycin-FRB complex is very stable, the recruitment of pseudojanin to the plasma membrane is irreversible and results in a lasting depletion of PI(4,5)P_2.[12,15,16] Rapamycin addition to cells coexpressing K_V1.2, pseudojanin, and LDR-CFP resulted in a clear 19 ± 2% decrease in current amplitude (n = 5, **Fig. 1C and D**). These experiments show that K_V1.2 channel current is PI(4,5)P_2 sensitive as previously reported.[14]

We next asked whether the voltage-dependence of activation can be shifted by turning on PLC. We coexpressed K_V1.2 with M_1R and measured the conductance-voltage (G-V) relation before and after activation of M_1R (**Fig. 1E**). There was a small, but significant left shift in the normalized activation curve by 3.5 ± 0.4 mV (n = 5) (**Fig. 1F**). Thus, we confirm the observations of Rodriguez-Menchaca et al.[14]

PI(4,5)P_2 dependence of hERG channel activation

We and others have reported a change in the voltage-dependence of activation of erg channels if PI(4,5)P_2 levels are altered.[17,18] Bian et al. reported a left shift of about ~19 mV for the activation of hERG channels upon dialysis of 10 µM PI(4,5)P_2 into the cells via the patch pipette,[18] while we found a right shift of about ~5 mV in the activation curve for rat erg1 channels upon depletion of PI(4,5)P_2 by activating M_1R.[17] We decided to extend our previously published recordings on rat erg1 channels to hERG channels to test whether we would observe a similar right shift in the voltage-dependence.

We co-expressed hERG and M_1R in tsA-201 cells and measured current amplitudes before and after M_1R activation. After addition of Oxo-M we observed an inhibition of hERG mediated current of 31 ± 7% (n = 5) (**Fig. 2A and B**), in good agreement with our work on rat erg1 channels after M_1R activation.[17] We next asked whether this decrease in current amplitude is accompanied by a change in the activation curve for hERG channels. Again, we co-expressed hERG channels with M_1R and measured G-V curves before and after addition of Oxo-M to deplete PI(4,5)P_2. We detected a right shift of the activation curve of about ~7 mV (n = 5), which correlates very well with the 5 mV right shift Hirdes et al. had observed for rat erg1 channels (**Fig. 2C**).[17]

Our next step asked whether the observed right shift in the activation curve is caused by the depletion of PI(4,5)P_2 at the plasma membrane or by signaling pathways downstream of PI(4,5)P_2 hydrolysis, such as activation of protein kinases. Unlike K_V1.2 channels, it had been shown for hERG channels that

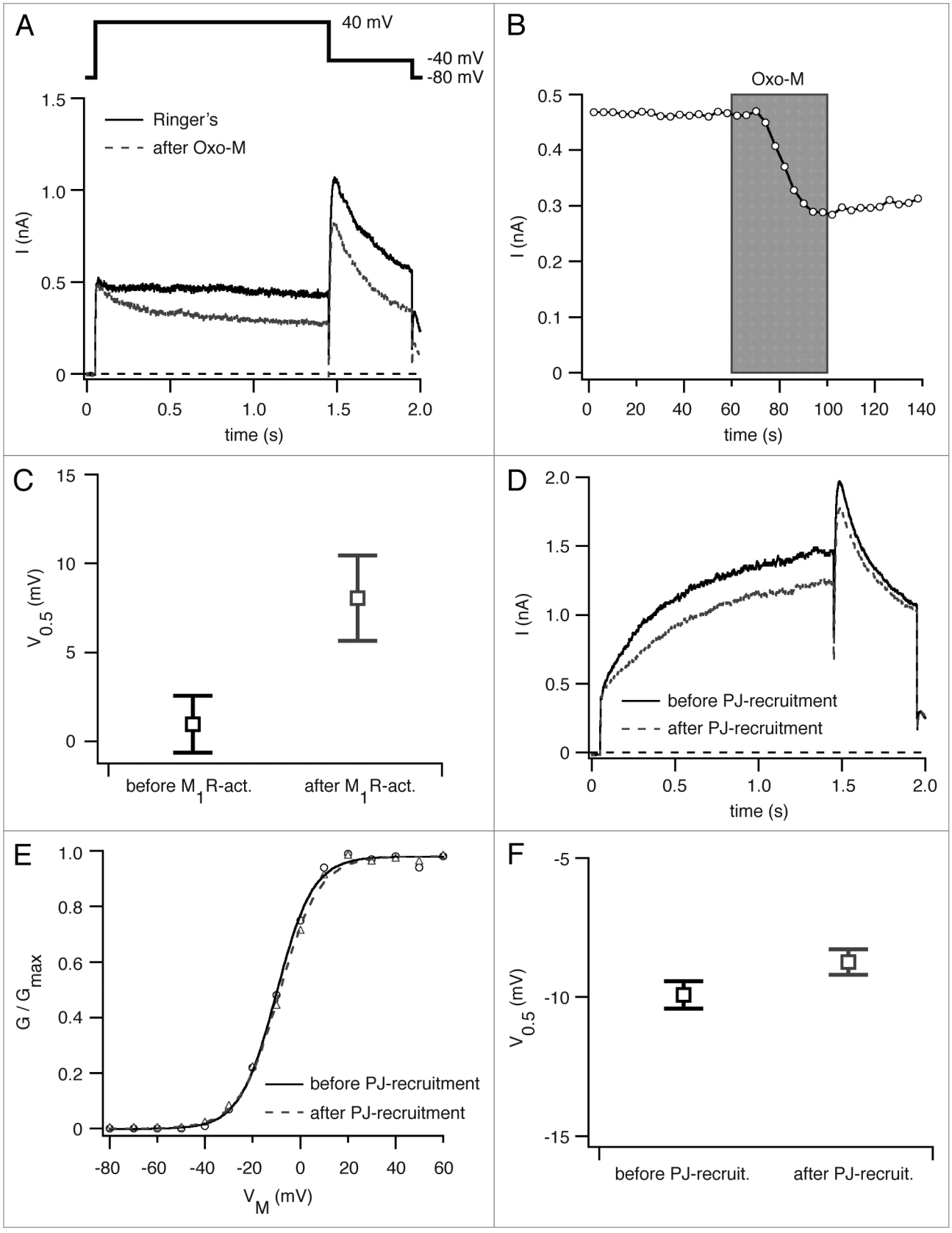

Figure 2. For figure legend, see page 534.

Figure 2 (see page 533). Voltage-dependence of activation of hERG channels before and after PI(4,5)P_2 depletion. (**A**) Figure shows current traces for hERG channels expressed in tsA-201 cells together with M_1R before (solid black) and after (dashed gray) application of 10 μM Oxo-M. Pulse protocols as shown above current traces. (**B**) Time course of hERG channel mediated current at +40 mV from the experiment shown in (**A**). (**C**) Voltages of half-maximal activation ($V_{0.5}$) of hERG channels before and after M_1R-activation. (**D**) Current traces for hERG channels expressed together with pseudojanin-YFP (PJ) and LDR-CFP before (solid black) and after (dashed gray) recruitment of PJ to the plasma membrane by rapamycin-application. (**E**) Representative G-V curve of hERG channels generated from test pulses to membrane potentials of – 80 to +60 mV from a holding potential of – 80 mV before (solid black) and after (dashed gray) recruitment of PJ to the plasma membrane. (**F**) Voltages of half-maximal activation ($V_{0.5}$) for hERG channels before and after PJ-recruitment.

activation of PKC leads to a right shift of the activation curve.[19] We expressed hERG channels together with pseudojanin-YFP and LDR-CFP in tsA-201 cells and applied rapamycin to induce translocation of pseudojanin-YFP to the plasma membrane. Recruiting PJ to deplete PI(4,5)P_2 led to a significant decrease (15 ± 1%, n = 5) of hERG mediated current amplitude (**Fig. 2D**) but, in the same cells, no significant shift in the voltage-dependence of activation (**Fig. 2E and F**). We conclude from this result that a depletion of PI(4,5)P_2 does not alter the voltage-dependence of activation of hERG channels and that our finding of a right shift in the activation curve after M_1R-activation should be attributed to other signals downstream of PI(4,5)P_2 cleavage by PLC.

Discussion

We now review 2 broad questions briefly: (1) Are K_V channels sensitive to plasma membrane PI(4,5)P_2; and (2) is there a physiological benefit from such sensitivity or insensitivity?

For excitable cells, the K_V channels whose PI(4,5)P_2 sensitivity is best studied are the K_V7 (KCNQ) family.[7,20,21] It is widely accepted that the 5 members of this family absolutely require PI(4,5)P_2 to function. They bind PI(4,5)P_2 with low enough affinity that when the lipid is depleted enzymatically by 90–95% either by PLC or by 5-phosphatases, the current falls by 80–95%. In addition, K_V1.2 channels have clear PI(4,5)P_2 sensitivity. In whole-cell experiments, currents decrease and gating is shifted in response to M_1R or voltage-sensing phosphatase activation, and in excised patches, the same effects are induced by rundown, by anti-PI(4,5)P_2 antibodies, and by blocking lipid kinases, and current is restored by application of PI(4,5)P_2.[14,22,23] With K_V1.2 however, rather than eliminating current, PI(4,5)P_2 depletion modulates channel properties more gently, reducing the amplitude by 25–30% and shifting gating. Possibly with a more severe elimination of PI(4,5)P_2, the channel could be shown to require the lipid absolutely, but the published experiments and ours never attenuate the current by as much as 50%. Similarly, with hERG (K_V11) channels we found a small depression of current (15%) but in this case no shift of gating attributable to PI(4,5)P_2 depletion.

Lipid effects on other members of the K_V1, 2, 3, and 4 families are less pronounced. In the simple whole-cell recording tests we did with K_V1.1, 1.3, 1.4, 1.5, 2.1, 3.4, 4.2, and 4.3, neither the current amplitude at large depolarizations nor the kinetics were sensitive to enzymatic depletion of PI(4,5)P_2.[12] We did not check for possible gating shifts at intermediate voltages, but we would say at least that these channels do not require physiological levels of PI(4,5)P_2 to function. This relative insensitivity of so many K_V channels was unexpected since earlier reports with membrane patches excised from Xenopus oocytes had indicated a striking loss of open-channel inactivation of K_V1.1 (with K_Vβ1.1), 1.4, and 3.4 channels when brain PI(4,5)P_2 was added to the patch.[13] Is this a discrepancy? Probably not, since our experiments involved enzymatic removal of PI(4,5)P_2 in whole-cell recording of mammalian cells, whereas the others used addition of PI(4,5)P_2 to membrane patches excised from oocytes. We would suggest that these 3 responding K_V channels may have little sensitivity to membrane PI(4,5)P_2 in the physiologically accessible range. The application of additional brain PI(4,5)P_2 to the cytoplasmic face might be able to force the membrane phosphoinositide level into a higher non-physiological range or might expose the channels to lipid micelles and solvents that had other actions. However such speculations are untested.

Hence, we suggest that besides the very strongly PI(4,5)P_2-dependent K_V7 channels, very few K_V channels, namely K_V1.2 and K_V11.1 channels so far, show significant physiological sensitivity to changes in PI(4,5)P_2 levels below the endogenous resting level. This brings us to the question, what are physiological benefits of such sensitivity or insensitivity?

We envision 2 broad physiological advantages for PI(4,5)P_2 sensitivity of ion channels. (1) Sensitivity specifically to PI(4,5)P_2 allows ion channel function and cell excitability to be modulated in response to receptor activation of PLC, and (2) as first suggested by Hilgemann,[24] a requirement for particular phosphoinositides could keep ion channels silenced or at low activity during trafficking whenever they are not in an appropriate membrane compartment.

On the first advantage, regulation by PLC, we are already well informed since this mechanism involves modulation of ion channels at the plasma membrane and classical electrophysiology. KCNQ2/KCNQ3 channels gate slowly. They are already partly open at typical resting potentials and are non-inactivating. Muscarinic cholinergic stimulation of superior cervical ganglion cells results in a profound suppression of KCNQ2/KCNQ3-mediated resting currents because PLC is activated and PI(4,5)P_2 becomes depleted. The cells depolarize a little, become more excitable, and fire repetitively to long depolarizing stimuli.[21] A similar story could be developed for K_{ATP} channels, found in many cells. These inward rectifier channels (K_{ir}6) are PI(4,5)P_2 sensitive[25] and regulate the cell resting potential and excitability as well. We can suggest that in excitable cells expressing PI(4,5)P_2-sensitive channels regulating the resting potential, it is still appropriate that each action potential repolarize quickly. Despite increased excitability, a reserve of potassium channel activity is needed. For that reason, the PI(4,5)P_2 insensitivity of e.g., K_V1.1/K_Vβ1.1, K_V1.5/K_Vβ1.3, K_V3, and K_V4, which are rapidly-gating, repolarizing channels[13,26,27] might be adaptive

to maintain action potential repolarization. Similarly, a loss of inactivation gating of $K_V1.1/K_V\beta1.1$ channels as well as of $K_V3.4$ channels with M_1R stimulation[12,28] could add repolarizing reserve. In these latter cases, the effect is not from depletion of $PI(4,5)P_2$ but probably rather by channel phosphorylation stimulated by diacylglycerol.[12,28,29] Finally, a loss of $PI(4,5)P_2$ increases the availability of $K_V1.2$ channels by shifting their voltage-dependence of activation to the left and would increase their contribution to repolarization.

How might cardiac $K_V7.1$/KCNE1 and hERG channels fit into this interpretation? Both have some $PI(4,5)P_2$ sensitivity.[12,17,18,20] In the human heart, these channels are the molecular basis for the 2 most important repolarizing currents, I_{Ks} and I_{Kr}.[30] Therefore, a decrease in $PI(4,5)P_2$ could prolong the cardiac action potential considerably. Indeed, mutations in the genes for $K_V7.1$ and hERG have been associated with a loss of $PI(4,5)P_2$ binding and critical pathological conditions like long QT syndrome and cardiac arrhythmias.[3,18,20] The heart seems to avoid this potentially serious problem in a different way. It has $PI(4,5)P_2$ at the plasma membrane to keep the channels active, but the endogenous PLC-coupled receptors do not deplete the $PI(4,5)P_2$ very much.[24] Apparently, $PI(4,5)P_2$ resynthesis can keep up with $PI(4,5)P_2$ breakdown in cardiac cells. In conclusion, $PI(4,5)P_2$ regulates the activity of voltage-gated potassium channels in different tissues by different mechanisms. They allow dynamic regulation of cellular excitability through slowly-gating channels like KCNQ2 and KCNQ3 in neurons while preserving the activity of action-potential-repolarizing fast K_V channels of neurons and the slow $K_V7.1$ and hERG channels in the heart.

The second potential advantage of $PI(4,5)P_2$ sensitivity concerns silencing during trafficking of channels in intracellular membranes, a subject we know less about. We would need further information about the physiology of intracellular compartments. What are the ion gradients across their membranes? What is their membrane potential? Is this electrical potential important for the compartment's function? Does it change during cellular activities? From such information we might be able to deduce which ion channels would be good to silence and which to promote as they traffic through that compartment. Phosphoinositides would be likely candidates to accomplish such regulation since each compartment has different lipids.[1] We already know of compartments with lumens that are acidic or have high sodium or calcium concentrations, and we know of stimuli that release stored calcium. This knowledge probably only scratches the surface of a much fuller understanding that will eventually emerge about compartmental electrophysiology.

We envision several possible outcomes of these inquiries. Internal membranes may have membrane potentials more positive than the resting potential of the plasma membrane. If so, ion channels with voltage-dependent inactivation might already be inactivated during internal trafficking, and that category of channels would not need a lipid-based mechanism to ensure lack of activity. In addition, when we understand the membrane potential of a compartment, we should recognize some transiting ion channels that would be compatible with that membrane potential and others that are not that must be kept silent there by some mechanism.

In sum, we propose hypotheses for why some channels are sensitive and some channels are not sensitive to the lipid $PI(4,5)P_2$.

Materials and Methods

Cell culture and plasmids

All experiments were performed in tsA-201 cells cultured at 37 °C and 5% CO_2 in DMEM (Invitrogen) supplemented with 10% FBS (PAA) and 0.2% penicillin/streptomycin (Invitrogen). Transient transfection of cells was performed as previously described.[12]

The following plasmids were generously given to us: M_1R (M_1 muscarinic receptor) –YFP from Neil Nathanson (University of Washington); pseudojanin-YFP from Gerald Hammond and Robin Irvine (University of Cambridge); $K_V1.2$ from Diomedes Logothetis (Virginia Commonwealth University); LDR (Lyn_{11}-targeted FRB)-CFP from Tamas Balla (National Institute of Health); and hERG from Olaf Pongs (University of Hamburg).

Electrophysiology

Whole-cell recordings were performed as previously described.[12]

Data analysis and statistics

Data analysis was performed using Igor Pro (Wavemetrics) and Excel (Microsoft). Statistical data are presented as mean ± SEM unless otherwise stated. The Student t-test was used to test for statistical significance. We considered p-values of < 0.05 as significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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