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Dual-Mode Phospholipid Regulation of Human Inward Rectifying Potassium Channels

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ABSTRACT The lipid bilayer is a critical determinant of ion channel activity; however, efforts to define the lipid dependence of channel function have generally been limited to cellular expression systems in which the membrane composition cannot be fully controlled. We reconstituted purified human Kir2.1 and Kir2.2 channels into liposomes of defined composition to study their phospholipid dependence of activity using $^{86}Rb^+$ flux and patch-clamp assays. Our results demonstrate that Kir2.1 and Kir2.2 have two distinct lipid requirements for activity: a specific requirement for phosphatidylinositol 4,5-bisphosphate (PIP_2) and a nonspecific requirement for anionic phospholipids. Whereas we previously showed that PIP_2 increases the channel open probability, in this work we find that activation by POPG increases both the open probability and unitary conductance. Oleoyl CoA potently inhibits Kir2.1 by antagonizing the specific requirement for PIP_2, and EPC appears to antagonize activation by the nonspecific anionic requirement. Phosphatidylinositol phosphates can act on both lipid requirements, yielding variable and even opposite effects on Kir2.1 activity depending on the lipid background. Mutagenesis experiments point to the role of intracellular residues in activation by both PIP_2 and anionic phospholipids. In conclusion, we utilized purified proteins in defined lipid membranes to quantitatively determine the phospholipid requirements for human Kir channel activity.

INTRODUCTION

Recent advances in lipidomics have revealed cell membranes to be composed of diverse lipid species that are heterogeneously organized and constantly remodeling (1). Ion channels are embedded in the membrane bilayer, and the intimate interaction of ion channels with the cell membrane is a major determinant of function (2–7). However, it has been difficult to characterize how the lipid bilayer affects the function of ion channels, in part because studies have generally been limited to cellular expression systems in which the membrane lipid composition cannot be fully controlled (8). Over the past decade, efforts to achieve recombinant expression of ion channels have not only led to the first high-resolution crystal structures but have also made functional studies of reconstituted channels in defined liposomes more feasible. Recently, the structures of four different potassium channels cocrystallized with lipid or detergent molecules have provided images of channel-lipid interactions (9–12). These structures affirm that ion channels are intimately surrounded by lipids, some of which make apparently specific and relatively tight interactions. One crystal structure of the prokaryotic channel KcsA revealed two partial bound lipids, one of which was modeled as a diacylglycerol, consistent with the observation that KcsA requires anionic phospholipids for channel activity in lipid bilayers (4,9). However, for the other three channels for which a crystal structure shows bound detergent or lipid molecules (Kir3.1-KirBac1.3, and KirBac3.1 and Kv1.2-Kv2.1 chimeras, respectively), no functional description of their regulation by lipids is available.

One of the most well-studied lipid modulators of ion channel activity is phosphatidylinositol 4,5-bisphosphate (PIP_2) (13), which activates inward rectifying potassium (Kir) channels (2,3,14). The results of electrophysiological (15–19), biochemical (3,20–22), and molecular simulation (23,24) studies collectively suggest that PIP_2 and other phosphatidylinositol phosphates (PIPs) (15,25) directly activate Kir channels with variable sensitivity by binding to positively charged residues in the intracellular side of these channels. However, none of these studies definitively demonstrated direct modulation by PIP_2 in a pure in vitro system, as was recently reported for the TRPM8 channel reconstituted in lipid bilayers (26). There are also reports of modulation of Kir channels by lipids other than PIPs, such as phosphatidylserine (PS) (14) and cholesterol (27). Whether other lipids might also modulate Kir channel function or whether any such lipids act through regulatory modes distinct from PIP_2 remains unknown.

To address these problems, we purified human Kir2.1 (KCNJ2) and Kir2.2 (KCNJ12) recombinantly expressed in *Saccharomyces cerevisiae* and reconstituted the channels into liposomes of defined composition for use in $^{86}Rb^+$ flux and patch-clamp assays. Using this reconstitution technique in a previous study (28), we definitively showed that PIP_2 directly activates Kir channels without the need for intermediary proteins, and quantitatively assessed the PIP_2 dependence of Kir2.1 and Kir2.2 activity. In the study presented

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here, we performed a detailed analysis of the dependence of channel activity on the bulk lipids in the membrane. We demonstrate that Kir2.1 and Kir2.2 have two distinct lipid requirements for channel activity: a specific requirement for PIP_2 and a previously unrecognized, nonspecific requirement for anionic phospholipids. Mutagenesis studies suggest that positively charged intracellular residues in Kir2.1 are involved not only in mediating PIP_2 activation but also in activation by the nonspecific anionic phospholipid requirement.

MATERIALS AND METHODS

Purification of Kir2.1 and Kir2.2 channels from *S. cerevisiae*

Human Kir2.1 (KCNJ2) and Kir2.2 (KCNJ12) were subcloned into the pND-CTFH vector, expressed in the FGY217 strain of *S. cerevisiae*, and purified as previously described (28,29). Mutations were made with the Quikchange Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) and confirmed by DNA sequencing.

Liposomal $^{86}Rb^+$ flux assay

The following lipids were purchased from Avanti Polar Lipids: synthetic POPE (1-palmitoyl 2-oleoyl phosphatidylethanolamine), POPS (1-palmitoyl 2-oleoyl phosphatidylserine), POPA (1-palmitoyl 2-oleoyl phosphatidic acid), POPC (1-palmitoyl-2-oleoyl-phosphatidylcholine), EPC (1-palmitoyl-2-oleoyl-ethylphosphatidylcholine), POPG (1-palmitoyl 2-oleoyl phosphatidyl glycerol), CL (18:1 cardiolipin), and DGS-NTA (1,2-dioleoyl-glycero-3-[(N-(5-amino-1-carboxypentyl) iminodiacetic acid) succinyl]), and natural PI (phosphatidylinositol from bovine liver), PI(4)P (phosphatidylinositol 4-phosphate from porcine brain), and PIP_2 (phosphatidylinositol 4,5-bisphosphate from porcine brain). The desired mixture of lipids in mass % were solubilized in buffer A (450 mM KCl, 10 mM HEPES, 4 mM NMG, pH 7) with 35 mM CHAPS at 10 mg/mL. For each sample, 5 μg of Kir2.1 or Kir2.2 protein were added to 100 μL of lipid (1 mg) and liposomes were formed by spinning the protein-lipid solution through a partially dehydrated column packed with Sephadex G-50 beads (Sigma-Aldrich, St. Louis, MO) preequilibrated in buffer A at 1000 $\times$ *g* (Beckman (Brea, CA) TJ6 centrifuge), and then spun through a second column containing partially dehydrated beads preequilibrated in buffer B (450 mM sorbitol, 10 mM HEPES, 4 mM NMG, pH 7). Uptake was initiated by adding 400 μL of buffer B with 1–5 μL of 1 μCi $^{86}Rb^+$ (PerkinElmer, Waltham, MA). At various time points, aliquots were flowed through 0.5 mL Dowex cation exchange columns, mixed with scintillation fluid, and counted on a liquid scintillation counter. For each experiment, a sample without protein was included as a measure of background $^{86}Rb^+$ counts, and this background was subtracted from all channel-mediated $^{86}Rb^+$ uptake counts. All $^{86}Rb^+$ uptake counts were then normalized to counts of valinomycin-mediated uptake as a measure of maximum liposome capacity (30). Data obtained at 15 min (immediately before the uptake plateau) were then plotted relative to one specific experimental condition, as indicated in the figure legends.

Giant liposome patch-clamp

The desired mixture of lipids in mass % (total 2 mg) was solubilized in K-MOPS buffer (10 mM MOPS, pH 7.4, 158 mM KCl) and 35 mM CHAPS. Then 30 μg of protein were solubilized with the lipids, and liposomes were formed as described for the flux assay except that this time Sephadex beads soaked in K-MOPS buffer were used. Giant liposomes were formed by a dehydration-rehydration cycle procedure (31). All recordings were made in symmetrical K-MOPS buffer. Patch pipettes were pulled from soda lime glass microhematocrit tubes (Kimble) to a resistance of ~2–3 MΩ. Membrane patches were voltage-clamped using a CV-4 headstage, an Axopatch 1D amplifier, and a Digidata 1322A digitizer board (Axon Instruments, Sunnyvale, CA). Single-channel data were digitized at a sampling rate of 10 kHz and a low-pass analog filter was set to 1 kHz. Analysis of single-channel data was performed with the pClamp 9.2 software suite (Axon Instruments).

Homology modeling of Kir2.1

A homology model of human Kir2.1 was generated using the ICM Homology add-on of ICM-Pro (Molsoft L.L.C.) after sequence alignment to the chicken Kir2.2 structure (PDB: 3JYC) (32).

Statistical analysis

Statistical significance was assessed by unpaired *t*-test or one-way analysis of variance as appropriate, and statistical significance (reported by an asterisk) indicates $p < 0.05$.

RESULTS

Kir2.1 and Kir2.2 are modulated by PIP_2 and POPG

Purified human Kir2.1 and Kir2.2 proteins from *S. cerevisiae* were reconstituted in liposomes and assayed for activity by means of an $^{86}Rb^+$ flux assay (28,29). Experiments were performed on liposomes composed of the neutral phospholipid POPE with replacement by varying amounts of other lipids, reported as a mass/mass ratio. A significant fraction (10–20%) of eukaryotic cell membranes is composed of anionic phospholipids (33–36) such as POPG. PIP_2 is also typically present at $\leq 1\%$ in eukaryotic membranes (37). As we previously showed (28), PIP_2 in the range of 0.01–1% is required for activity of Kir2.1 and Kir2.2 in membranes consisting of 75% POPE and 25% POPG (Fig. 1), and is highly selective for PIP_2 over other PIPs (28). Kir2.1 activity is also strongly inhibited by Ba^{2+} and spermine in the flux assay (see Fig. S1 in the Supporting Material), confirming that >96% of $^{86}Rb^+$ accumulation is mediated by Kir2.1. As shown in Fig. 1, however, the dependence of channel activity on PIP_2 is not fixed: in the absence of POPG, the PIP_2 dependence of activity for Kir2.1 and Kir2.2 is significantly right-shifted ~100$\times$, such that at least 1% PIP_2 is needed to detect $^{86}Rb^+$ flux (Fig. 1). These data suggest that Kir2.1 and Kir2.2 have both a primary PIP_2 requirement and a novel (to our knowledge) secondary POPG requirement for activity.

Kir2.1 and Kir2.2 have a nonspecific secondary requirement for anionic phospholipids

POPG sensitizes Kir2.1 and Kir2.2 to PIP_2 activation (Fig. 1). To determine whether this second lipid effect is specific to POPG, we screened channel activity in multiple

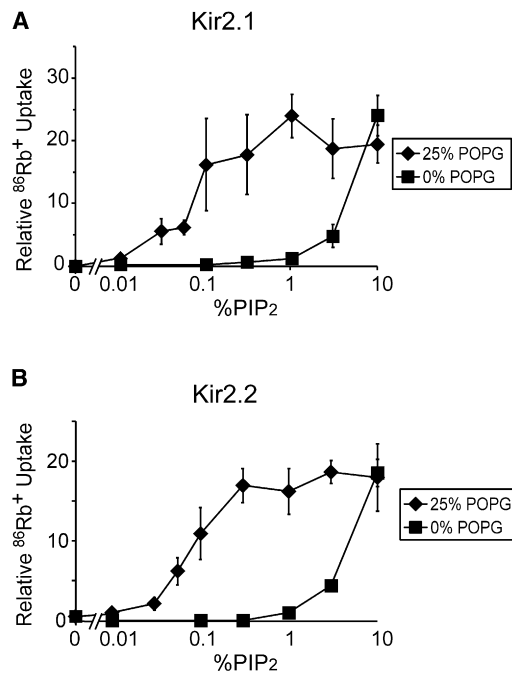

FIGURE 1 Reconstituted human Kir2.1 and Kir2.2 activity is regulated by POPG. (*A*) Kir2.1 activity-% PIP_2 relationship obtained from $^{86}Rb^+$ uptake counts at 15 min (all counts are reported from this time point), in 25% POPG or POPE-only liposomes ($n = 3$, mean $\pm$ SE). The valinomycin-normalized counts are plotted relative to counts in 0.01% PIP_2 and 25% POPG. (*B*) Same as in *A* except for Kir2.2, and $^{86}Rb^+$ uptake counts taken at 20 min (for POPE-only liposomes, $n = 3$, mean $\pm$ SE).

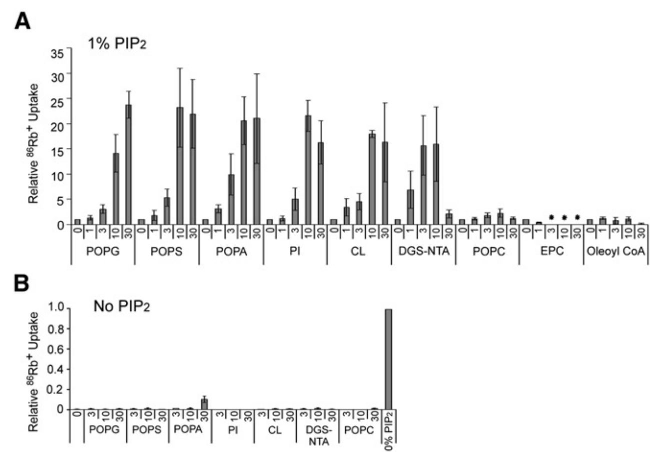

FIGURE 2 Kir2.1 has a secondary, nonspecific requirement for anionic phospholipids. (*A*) $^{86}Rb^+$ uptake counts of Kir2.1 in liposomes composed of increasing % of the indicated lipid and 1% PIP_2 ($n = 3$, mean $\pm$ SE). The valinomycin-normalized counts are plotted relative to counts in 1% PIP_2. The asterisks indicate conditions in which liposomes failed to form. $^{86}Rb^+$ uptake counts for 3%, 10%, and 30% POPG, POPS, POPA, PI, CL, and DGS-NTA are statistically different from control ($p < 0.05$). (*B*) $^{86}Rb^+$ uptake counts of Kir2.1 in liposomes composed of POPE and increasing % of the indicated lipid. The valinomycin-normalized counts are plotted relative to counts in 10% PIP_2 ($n = 3$, mean $\pm$ SE).

lipids with varying headgroup properties. As shown in Fig. 2 *A* and Fig. S2, the Kir2.1 and Kir2.2 channels demonstrate a clear requirement for lipids with negatively charged headgroups. The chemical nature of the headgroup appears to be noncritical, since multiple negatively charged lipids, including POPS, POPA, PI, CL, and the PIP_2 analog DGS-NTA can all activate these channels similarly to POPG (Fig. 2 *A* and Fig. S2). By contrast, neither the neutral POPC nor the cationic EPC activate either channel (Fig. 2 *A* and Fig. S2). Of note, ~100× more anionic lipid (~10%) than PIP_2 is required to substantially activate Kir2.1 and Kir2.2. It is conceivable that these anionic lipids are acting as weak PIP_2 surrogates and do not represent a secondary mode of regulation. However, the fact that none of these anionic phospholipids activate Kir2.1 at up to 30% in the absence of PIP_2 on a POPE-only background (Fig. 2 *B*) suggests not only that PIP_2 activation of Kir2.1 is specific (38) but that other anionic phospholipids cannot simply substitute for PIP_2 (28).

Activation by POPG increases Kir2.1 open probability and unitary conductance

Our patch-clamp analysis of single-channel currents showed that an increase in POPG activates channels at least partially by increasing the channel open probability (Fig. 3, *A* and *B*). Surprisingly, and in contrast to the singular effect of PIP_2 on P(o) in the physiological range (28), increasing POPG from 15% to 25% on a 1% PIP_2 background increases not only the open probability but also the unitary conductance (Fig. 3, *A–C*). This result is reminiscent of the effect of anionic phospholipids on the prokaryotic K^+ channel, KcsA, for which there is evidence that binding of anionic phospholipids to positively charged residues on the extracellular side of the KcsA activates the channel by increasing both the open probability and unitary conductance (39–41). An effect on the unitary conductance may indicate a direct electrostatic effect on the local permeant ion concentration, or an effect through changes in protein conformation. Of importance, this differential action on conductance is consistent with the conclusion that POPG is not acting simply as a PIP_2 surrogate.

Because we do not observe any openings in 15% POPG to the larger conductance present in 25% POPG, it seems unlikely that changing from 15% to 25% POPG stabilizes two different open conformations with different conductances. For this reason, we speculate that the effect of POPG on conductance is likely to be graded. Unfortunately, we are unable to test this because we are unable to form giant liposomes when $<$10–12% total anionic phospholipids are present. We would also note that the change in single-channel current (Fig. 3) is not likely to be linearly reflected in a change in total $^{86}Rb^+$ uptake (as in Fig. 2 *A*). Since $^{86}Rb^+$ uptake experiments are cumulative by nature, they are not as sensitive as direct current recordings and provide a nonlinear measure of activity.

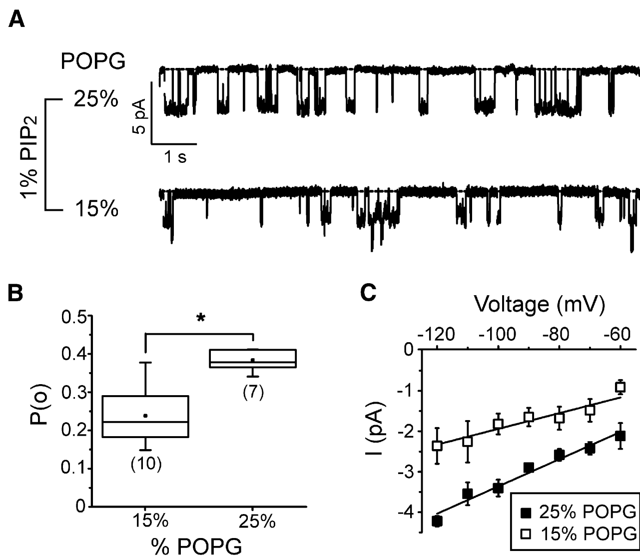

FIGURE 3 Secondary anionic phospholipid activation increases open probability and unitary conductance. (*A*) Representative continuous recordings of Kir2.1 currents at −100 mV from giant liposomes composed of the indicated % POPG and 1% PIP_2. (*B*) Box-and-whisker plot of measured Kir2.1 open probabilities from giant liposomes composed of 1% PIP_2 and either 15% or 25% POPG. The mean open probabilities, represented by the square box, are 0.24 ± 0.02 for 0.15% POPG and 0.38 ± 0.01 for 25% POPG (mean ± SE). The number of recordings for each condition is indicated in brackets, the whiskers indicate the data range, the box shows the upper and lower quartile values and median, and the asterisk indicates statistical significance ($p < 0.05$). (*C*) Current-voltage plot of Kir2.1 analyzed from the same recordings as in *B*. The derived slope conductances are 19.4 ± 0.6 pS for 15% POPG and 34.5 ± 1.5 pS for 1% PIP_2 (mean ± SE).

Inhibition of Kir2.1 by oleoyl CoA and EPC

Neither oleoyl CoA or EPC activate Kir2.1 or Kir2.2 on a 1% PIP_2 lipid background (Fig. 2 *A* and Fig. S2). However, it has been suggested that long-chain acyl CoAs such as oleoyl CoA antagonize PIP_2 activation of Kir2.1 in cellular membranes (42), and that cationic molecules such as polylysine inhibit Kir channel activity, presumably by screening the negative charge of anionic phospholipids such as PIP_2 (3,14). To determine the mode of action of oleoyl CoA and EPC, we determined the concentration dependence of inhibition on lipid backgrounds where Kir2.1 is active. On a 1% PIP_2/10% POPG background, 1–3% oleoyl CoA effectively inhibits Kir2.1 activity, whereas on a 10% PIP_2 background, ~10% oleoyl CoA is needed for inhibition (Fig. 4 *A*), consistent with oleoyl CoA competitively inhibiting PIP_2 activation. By contrast, on a 1% PIP_2/10% POPG background, 10% EPC is required to inhibit Kir2.1, and there is no inhibitory effect on a 10% PIP_2 background (Fig. 4 *B*). EPC may be inhibiting channel activity by counteracting the negative charge conferred by PIP_2 or POPG. When we compare EPC inhibition in a 1% PIP_2/5% POPG or 1% PIP_2/30% POPG background, we can see that EPC is clearly less effective at inhibiting Kir2.1 when 30% POPG is present (Fig. 4 *C*). Although these data do not exclude the possibility that EPC can also antagonize PIP_2 activation, they indicate that EPC, a positively charged phospholipid, counteracts the negative charge conferred by POPG and thus antagonizes the secondary anionic lipid requirement.

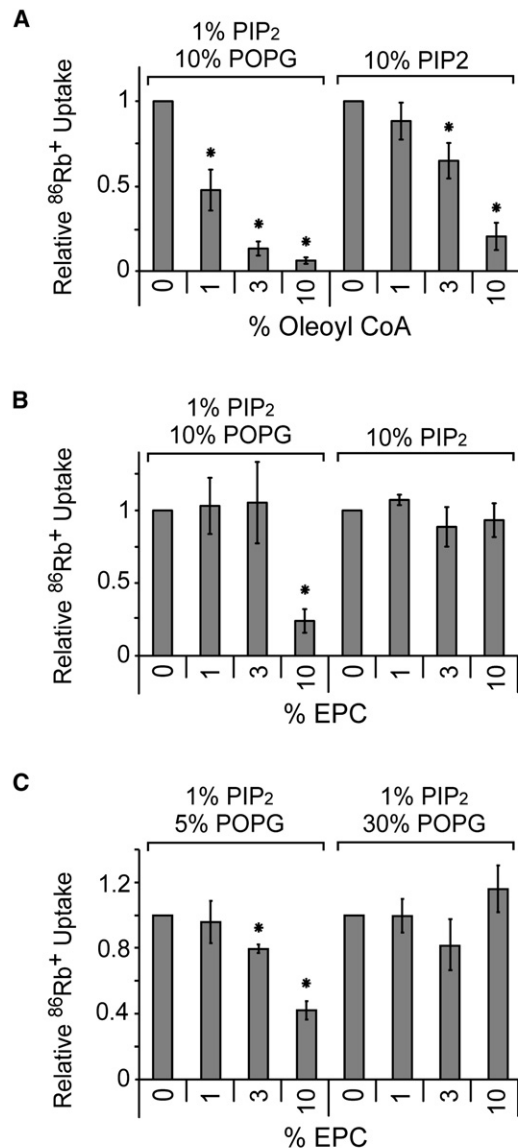

FIGURE 4 Effects of oleoyl CoA and EPC on Kir2.1 activity. (*A*) $^{86}Rb^+$ uptake counts of Kir2.1 in liposomes with increasing % oleoyl CoA and the indicated lipids shown above the graph ($n = 3$, mean ± SE). Valinomycin-normalized counts are plotted relative to counts in 0% oleoyl CoA. Asterisk indicates a statistically significant difference compared with 0% oleoyl CoA ($p < 0.05$). (*B*) Same as in *A* except with increasing % EPC ($n = 3$, mean ± SE). Asterisk indicates a statistically significant difference compared with 0% EPC ($p < 0.05$). (*C*) Same as *B* except with a background lipid composition of 1% PIP_2 and 5% POPG or 1% PIP_2 and 30% POPG ($n = 3$, mean ± SE).

PIPs can have dual regulatory roles in Kir2.1 activity

Collectively, the above data demonstrate two distinct modes of anionic phospholipid regulation in Kir2.1: a primary

specific requirement for PIP_2 and a secondary, nonspecific requirement for anionic phospholipids. Because the secondary requirement for anionic phospholipids is nonspecific, this raises the question of whether PIP_2 activates Kir2.1 on a POPE-only background (Fig. 1) by meeting both lipid requirements. In liposomes composed of 5% PIP_2, Kir2.1 is partially activated and the effect of POPG is diminished (Fig. 5 *A*), consistent with the notion that PIP_2 at high levels meets both lipid requirements and thus has dual regulatory roles in Kir2.1.

The finding that other PIPs can have competing effects on Kir2.1 activity also suggests that PIP_2 has dual regulatory roles. On a 1% PIP_2 background, all PIPs at 10% can activate Kir2.1 (Fig. 5 *B*), but with variable efficacy. PI is the most effective, followed by PI(3)P, PI(4)P, and PI(5)P, and the least effective are the multiphosphorylated PIPs (PI (3,4)P_2, PI(3,5)P_2, and PI(3,4,5)P_4). By contrast, on a 1% PIP_2/25% POPG background, all PIPs (but not PI) inhibit Kir2.1 activity, and the multiphosphorylated PIPs are the most effective (Fig. 5 *C*). PIPs most likely inhibit Kir2.1 activity (Fig. 5 *C*) by antagonizing the primary PIP_2 requirement as opposed to the secondary requirement (i.e., POPG), since the secondary requirement is nonspecific for anionic lipids (Fig. 2 *A*).

These results suggest that PIPs act on both modes of lipid regulation. PIPs activate Kir2.1 on a 1% PIP_2 background by meeting the secondary requirement (Fig. 5 *A*); however, this activation is less effective compared with that exhibited by PI because PIPs also antagonize the primary PIP_2 requirement (Fig. 5 *B*). Consistent with this proposed mechanism, the multiphosphorylated PIPs (e.g., PI(3,4)P_2), which are the most effective inhibitors of PIP_2 (Fig. 5 *C*), are the least effective in activating Kir2.1 on a 1% PIP_2 background (Fig. 5 *B*). Thus, PIPs can have dual regulatory roles in Kir2.1 activity, with the overall effect of a PIP on Kir2.1 activity depending on the background lipid composition.

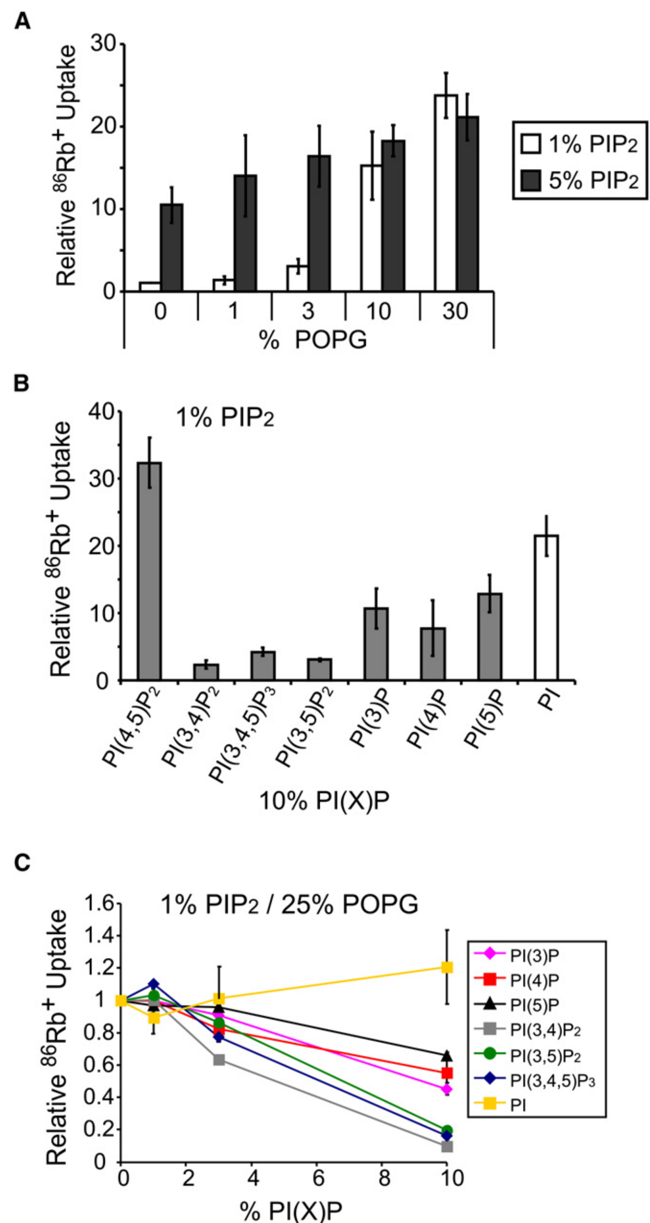

FIGURE 5 PIPs act on both modes of lipid regulation. (*A*) $^{86}Rb^+$ uptake counts of Kir2.1 in liposomes composed of increasing % POPG and 5% PIP_2 ($n = 3$, mean $\pm$ SE). POPG dependence from Fig. 3 *A* is shown in white. Valinomycin-normalized counts are plotted relative to counts in 1% PIP_2. (*B*) $^{86}Rb^+$ uptake counts of Kir2.1 in liposomes composed of 1% PIP_2 and 10% of the indicated lipids ($n = 3$, mean $\pm$ SE). Valinomycin-normalized counts are plotted relative to counts in 1% PIP_2 only, and PI data in dashed lines are from Fig. 2 *A*. $^{86}Rb^+$ uptake at 10% PI(4,5)P_2 is statistically different from all other PI(X)Ps ($p < 0.05$). (*C*) $^{86}Rb^+$ uptake counts of Kir2.1 in liposomes composed of 1% PIP_2, 25% POPG, and increasing % of the indicated lipids ($n = 3$, mean $\pm$ SE). Valinomycin-normalized counts are plotted relative to counts in 1% PIP_2 and 25% POPG. The difference in $^{86}Rb^+$ uptake between 10% PI, single-phosphorylated PIPs, and multiphosphorylated PIPs is statistically significant ($p < 0.05$).

Intracellular residues mediate anionic phospholipid regulation

There is evidence that in KcsA, anionic phospholipids act in the outer leaflet by headgroup interaction with two arginine residues (R64 and R89) on the extracellular side of the channel (39–41). Although there are multiple positively charged residues in the intracellular side of Kir2.1, there are only two positive charges (K117 and K120) in the extracellular region that conceivably could be involved in charge-dependent anionic phospholipid activation of Kir2.1 (Fig. 6 *A*). To test the effect of these residues on the lipid dependence of reconstituted Kir2.1 activity, we expressed and purified three mutants: K117A, K120A, and the double mutant K117A/K120A (Fig. S3). However, none of these mutants affected the PIP_2 or POPG dependence of activity (Fig. 6, *B* and *C*), which strongly argues that the secondary requirement for anionic phospholipids in Kir2.1 is not mediated by charge interactions on the extracellular side of the channel, and suggests instead that the secondary requirement may involve residues on the cytoplasmic domain.

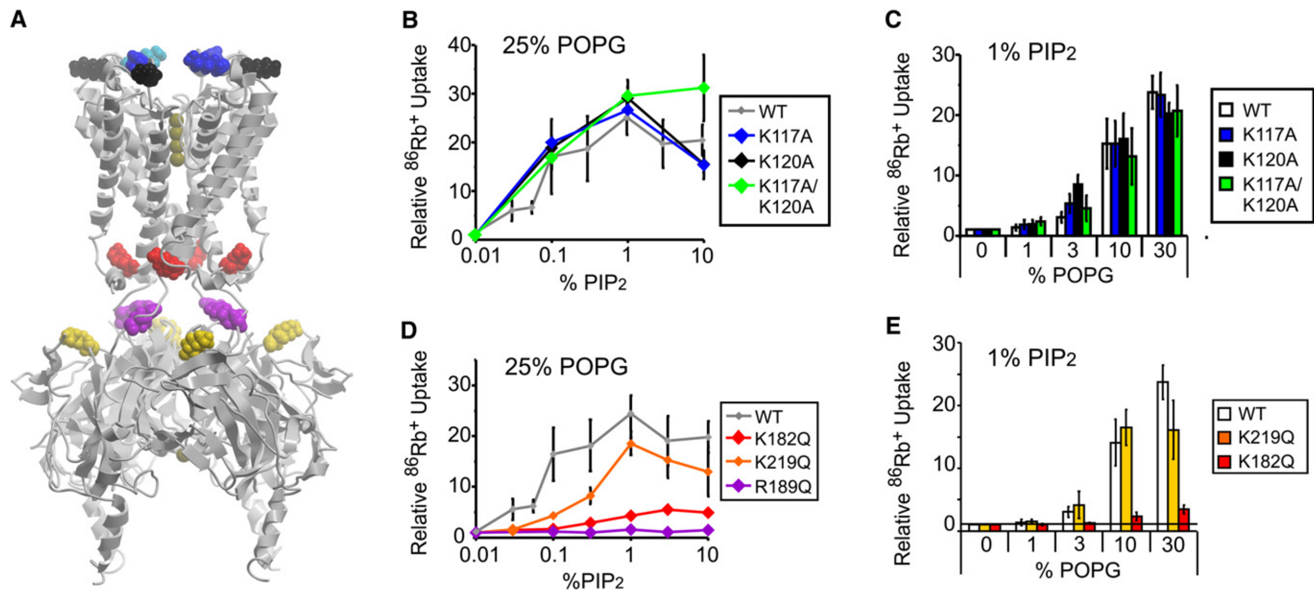

FIGURE 6 Activation by anionic phospholipids is dependent on intracellular basic residues. (*A*) Homology model structure of Kir2.1 derived from the Kir2.2 crystal structure (3JYC). Space-filling representations of amino acid side chains are shown for K117 (*blue*), K120 (*black*), K182 (*red*), R189 (*pink*), and K219 (*yellow*). (*B*) $^{86}Rb^{+}$ uptake counts of Kir2.1 mutants K117A, K120A, and K117A/K120A in liposomes composed of increasing % PIP_2 and 25% POPG ($n = 3$, mean $\pm$ SE). Valinomycin-normalized counts are plotted relative to counts in 0.01% PIP_2 and 25% POPG. WT Kir2.1 PIP_2 dependence from Fig. 1 *A* is shown in gray with dotted lines (*C*) Same as in *B* except in liposomes composed of 1% PIP_2 and increasing % POPG. Valinomycin-normalized counts are plotted relative to counts in 1% PIP_2 and 0% POPG. WT Kir2.1 POPG dependence from Fig. 2 *A*. (*D* and *E*) Same as in *B* and *C* except for the Kir2.1 mutants K182Q, R189Q, and K219Q ($n = 3$, mean $\pm$ SE).

In eukaryotic Kir channels, many residues that reduce PIP_2 sensitivity have been identified (16,17,20), and some of these may form a specific binding site in the cytoplasmic domain near the surface of the membrane (24). Of the 13 positively charged residues in Kir2.1 that have been identified as controlling PIP_2 sensitivity (16), we tested the effects of three mutants (R189Q, K182Q, and K219Q) because of their disparate effects on PIP_2 sensitivity and their proximity to the membrane interface. In Kir2.1, R189 is located on the TM2-cytoplasmic domain flexible linker, and the equivalent residue is highly conserved in all eukaryotic Kir channels. K182 is located on the bottom of TM2 just below the bundle crossing, and K219 is on the CD loop on the top of the cytoplasmic domain (Fig. 6 *A*). R189Q Kir2.1 channels show no detectable fluxes up to 10% PIP_2, whereas K182Q channels are minimally active and K219Q shifts the PIP_2 dependence of activation by ~10-fold (Fig. 6 *D* and Fig. S3), in agreement with the relative effect of these mutations on the sensitivity of Kir2.1 to PIP_2 activation in cellular membranes (16). The POPG dependence of these mutants (normalized to activity measured in the absence of POPG) shows a similar trend: K182Q is only weakly activated by POPG, in contrast to K219Q, which is activated by POPG with a dependence similar to that of the wild-type (WT; Fig. 6 *E*). It is not possible to distinguish from these data whether these mutants disrupt PIP_2 or POPG interactions, or whether they alter channel open-state stability. Collectively, however, the data do suggest that positively charged residues on the cytoplasmic side, as opposed to the extracellular side of the channel, are important for mediating both modes of anionic phospholipid regulation in Kir2.1.

DISCUSSION

Physiological regulation of Kir channels by lipids

The cell membrane, in which ion channels are embedded, is a complex and dynamic environment that is predicted to play a critical role in regulating membrane excitability (3–7,14). Kir channels are widely expressed in the human body, including the nervous system (43,44), and Kir2.1 has been implicated in epileptogenesis and regulation of cerebral blood flow (45). In this study, we have shown that human Kir2.1 and Kir2.2 channels are directly activated by anionic phospholipids via two distinct modes of action: a specific requirement for PIP_2 and a secondary, nonspecific requirement for anionic phospholipids. By reconstituting channel proteins in model liposomes, we were able to generate a quantitative description of the lipid dependence of channel activity. Kir2.1 activity is modulated over a large range of PIP_2 concentrations (from 0.01% to at least 1%; Fig. 1) (28), covering the estimated levels of PIP_2 in a cell membrane (~0.25% of total phospholipids) (13,37,46). Total anionic phospholipids in cell membranes are on the order of 10–20% of total lipid, which is also within the range we find for modulation by the secondary lipid requirement (Fig. 3 *A*) (33–36).

In cell membranes, the anionic phospholipid content (in mammalian cells, primarily PI and its derivatives, and PS) is constantly being remodeled to regulate numerous cellular functions. PIP_2 and other PIPs play important roles in signaling, membrane trafficking, and regulating various protein effectors, including ion channels and components of the actin cytoskeleton (47–49). PS is also a bioactive lipid that regulates enzymes (e.g., protein kinase C) and tags apoptotic cells for recognition by phagocytes (50–52). Many diseases are associated with altered metabolism of phospholipids. For example, mice with diabetic cardiomyopathy show dramatic changes in anionic phospholipid composition that are correlated with contractile dysfunction in the heart (53,54), where Kir2.1 and other inward rectifiers are critical repolarizing forces in the action potential. Specifically, a major depletion of cardiolipin occurs at the earliest stages of disease, followed by a large (40%) increase in PI species and a smaller though significant increase in PG and PS species (54,55). Given the relationship we have identified, such changes in PI species (including the phosphorylated derivatives) would clearly have complex effects on Kir2.1 channel function. Significant changes in anionic phospholipid content have also been observed in the myocardium of spontaneously hypertensive rats (56), hypoxemic myocardium of children with congenital heart disease (57), and brains of patients with Alzheimer's disease (58–60). Our results demonstrate that such alterations to lipid membrane content will significantly affect Kir channel activity, and it is important to clarify the physiological and pathophysiological consequences of these effects in future studies.

Complex dual-mode regulation of Kir2.1

Previous studies of lipid regulation of Kir channels have been limited to cellular expression systems in which the membrane composition cannot be controlled. To manipulate lipids in the membrane, lipid micelles are typically perfused onto membrane patches, which may restrict investigations to lipid modulators that act at low concentrations, such as PIP_2. By reconstituting purified human Kir2.1 and Kir2.2 proteins in model liposomes of defined lipid composition, we were able to identify a novel (to our knowledge) mode of regulation by anionic phospholipids and add another dimension of complexity to Kir2.1 regulation by lipids, especially given that PIPs can act on both the primary PIP_2 requirement and the secondary requirement. Certain PIPs have been reported to only activate (i.e., $PI(3,5)P_2$, $PI(3,4,5)P_3$, and PI(4)P) (38) or inhibit (i.e., $PI(3,4)P_2$) (42) Kir2.1. To the contrary, however, we find that all PIPs except $PI(4,5)P_2$ can either activate or inhibit Kir2.1 depending on the lipid background (Fig. 5). The net effect of a given PIP on Kir2.1 activity results from a combination of 1), its effect as an inhibitor of the PIP_2-specific requirement; and 2), its contribution to the overall anionic lipid content in the membrane.

In addition to anionic phospholipids, cholesterol has been implicated as an inhibitor of Kir channel activity (27). We anticipate that other lipid variants, such as lysophospholipids, sphingolipids, or polyunsaturated fatty acids, may also regulate Kir2.1 activity, and the overall effect of a single lipid species will depend on all the regulatory modes that it acts upon and the lipids that make up the rest of the membrane.

Molecular basis of activation by anionic phospholipids

Activation of the Kir2.1 secondary requirement by POPG increases both open probability and unitary conductance (Fig. 3), with the major driver of enhanced channel activity being the effect on open probability. Further studies will be required to confirm that the single-channel conductance effect is also observed for the other secondary anionic lipids, but in considering the possible molecular basis, it is notable that this dual effect is reminiscent of anionic phospholipid activation of KcsA. In this channel, anionic phospholipids also increase both single-channel conductance and open probability, and crystallographic and fluorescence studies have identified an intersubunit phospholipid-binding site on the extracellular side of the channel (40,41). However, we found that although mutation of the only two basic residues (K117 and K120) on the extracellular side of the Kir2.1 channel had no effect on lipid modulation (Fig. 5, *B* and *C*), mutations of intracellular residues, especially R189Q and K182Q, significantly affected activation of Kir2.1 by PIP_2 and POPG (Fig. 5, *D* and *E*). Thus, the molecular mechanism of anionic phospholipid modulation of Kir channels differs from that of KcsA, despite their functional similarities. Perhaps there has been a similar evolutionary pressure for Kir2.1 and KcsA to adopt a requirement for anionic phospholipids (61), but different structural components are apparently utilized. This is consistent with the fact that anionic phospholipids are almost exclusively localized to the inner leaflet of eukaryotic plasma membranes (52,62), in contrast to prokaryotic membranes (63). We hypothesize that some of the 13 basic residues previously identified in the intracellular side of Kir2.1 to affect PIP_2 sensitivity (16) may also be involved in mediating the secondary requirement for anionic phospholipids. This is consistent with molecular-dynamics simulations that suggested that only a subset of these residues are likely required to form a specific binding site for PIP_2 (24).

CONCLUSIONS

In conclusion, we have demonstrated that Kir2.1 and Kir2.2 have two distinct lipid requirements for channel activity: a specific requirement for PIP_2 and a nonspecific requirement for anionic phospholipids. Considering the high sequence and structural similarities, it seems highly likely

that this mechanistic scenario will hold for other Kir channels. However, examination of the general applicability to other K^+ channels or to other cation channel superfamily members will require further advances in expression and purification. This study represents a technical breakthrough in the use of model liposomes to study lipid regulation of purified ion channels by radiotracer flux assays and patch-clamping, and demonstrates how the effect of a given lipid species can vary significantly depending on the lipid composition of the membrane. As we expand our knowledge of cellular lipidomes and our ability to purify novel integral membrane proteins, further studies utilizing such approaches will help elucidate how the membrane bilayer influences excitability in physiology and disease.

SUPPORTING MATERIAL

Three figures are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(10)05297-5.

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