Correlation between structure and function in phosphatidylinositol lipid–dependent Kir2.2 gating

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Inward rectifier K⁺ (Kir) channels regulate cell membrane potential. Different Kir channels respond to unique ligands, but all are regulated by phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂). Using planar lipid bilayers, we show that Kir2.2 exhibits bursts of openings separated by long quiescent interburst periods. Increasing PI(4,5)P₂ concentration shortens the Kir2.2 interburst duration and lengthens the burst duration without affecting dwell times within a burst. From this, we propose that burst and interburst durations correspond to the cytoplasmic domain (CTD)–docked and CTD-undocked conformations observed in the presence and absence of PI(4,5)P₂ in atomic structures. We also studied the effect of different phosphatidylinositol lipids on Kir2.2 activation and conclude that the 5’ phosphate is essential to Kir2.2 pore opening. Other phosphatidylinositol lipids can compete with PI(4,5)P₂ but cannot activate Kir2.2 without the 5’ phosphate. PI(4)P, which is directly interconvertible to and from PI(4,5)P₂, might thus be a regulator of Kir channels in the plasma membrane.

Significance

Phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂) levels regulate cell membrane voltage by gluing two halves of a K⁺ channel together and opening the pore. PI(4)P competes with this process. Because both of these lipids are relatively abundant in the plasma membrane and are directly interconvertible through the action of specific enzymes, they may function together to regulate channel activity.


We first looked at the dependence of channel activity on PI(4,5)P₂ concentration. In the absence of C8-PI(4,5)P₂, no channel openings were observed. Upon addition of C8-PI(4,5)P₂, current was increased (Fig. 1B and C), indicating that PI(4,5)P₂ is both necessary and sufficient for channel opening, as was found previously in a different reconstitution system (20). Kir2.2 activity increased over the C8-PI(4,5)P₂ concentration range 0 to 15 μM and approached a maximum by 15 μM, as shown in three separate experiments (Fig. 1B and C). The analysis of channel activity dependence on C8-PI(4,5)P₂ was limited by the following property: C8-PI(4,5)P₂-induced current begins to decrease spontaneous-
ously after about 30 min, as shown (Fig. 1C). The disappearance of channel activity over time introduced uncertainty to the concentration-dependence of Kir2.2 activity. The disappearance also imposed a limitation on the kinetic analysis; however, as we will demonstrate, this limitation did not prevent us from extracting rate constants using the multichannel analysis described below.

Influence of C8-PI(4,5)P₂ on the Gating Kinetics of Kir2.2. We next looked at how C8-PI(4,5)P₂ influences the kinetics of Kir2.2 gating. Fig. 2A shows a single-channel trace recorded in the presence of 3, 6, and 15 μM C8-PI(4,5)P₂. The channel opened in bursts of activity separated by quiescent intervals. We saw from this trace two processes that operated on very different timescales. The relatively fast process, occurring on the subsecond timescale, accounted for rapid channel opening and closing within a burst of activity (Fig. 2A, Insets). The slow process, occurring on the minute timescale, accounted for the appearance and disappearance of bursts. Lifetime histograms for events within bursts showed single open-time and single closed-time distributions, which corresponded to the relatively rapid gating transitions that occurred within a burst (Fig. 2A–D). Note that the histograms were essentially unchanged when the concentration of C8-PI(4,5)P₂ was increased from 6 μM to 15 μM (compare Fig. 2B with Fig. 2D and Fig. 2C with Fig. 2E). This observation indicates that gating transitions within a burst were insensitive to the C8-PI(4,5)P₂ concentration over a range that influenced open probability (Fig. 1B). A second, longer closed dwell time exists because we saw it in the raw trace as long-duration time intervals free of channel activity (Fig. 1B); however, the occurrence of this slow process was too low to accumulate enough events during the recording, which was limited in duration owing to the phenomenon of channel disappearance over time. Fig. 2F shows the connectivity diagram for two closed and one open state (Fig. 2F, Inset). One linear kinetic subscheme, 2, was incompatible with the channel record because in this case openings would not be interrupted by brief closures. The remaining two linear kinetic subschemes, 3 and 4, were compatible with the channel record, which cannot distinguish among them.

In the context of the compatible kinetic schemes, we next asked which transitions are affected by the concentration of C8-PI(4,5)P₂. Given the low frequency of interburst intervals, confounded by channel disappearance over time, we studied bilayer membranes with several channels present at once. While this approach does not possess the intuitive simplicity of single-channel analysis, it is perfectly valid and, in this case, was enabling because it permitted a sufficient number of events to estimate the rate constants before the channels disappeared. Fig. 3A shows a multichannel membrane in the presence of 3, 6, and 15 μM C8-PI(4,5)P₂. The 15-μM record was used to estimate the total number of channels in the membrane (see Materials and Methods), while 3- and 6-μM records were subject to multichannel kinetic

Results

Dependence of Kir2.2 Opening on C8-PI(4,5)P₂ Concentration. Fig. 1A shows a schematic of the planar lipid bilayer system used in this study (21–23). A lipid bilayer with a defined composition separates the top and bottom chambers, each filled with electrolyte solution. Liposomes containing Kir2.2 channels are fused with the bilayer, resulting in some channels with the CTD facing the top chamber and others with the CTD facing the bottom chamber (Fig. 1A). Soluble reagents such as C8-PI(4,5)P₂ (PI(4,5)P₂ with 8-carbon acyl chains) are added to the top chamber. Because C8-PI(4,5)P₂ is membrane impermeant, only channels with their CTD facing the top chamber are activated (Fig. 1A) (23).

gating properties with the known structural changes. Furthermore, given our detailed chemical knowledge of the PI(4,5)P₂ binding site on Kir2.2, we characterized and interpreted the influence of different phosphatidylinositol lipid derivatives on channel gating. We found that competition for the PI(4,5)P₂-binding site by PI(4)P inhibited Kir2.2 activity. Because both PI(4)P and PI(4,5)P₂ contribute substantially to the pool of plasma membrane phosphatidylinositol lipids, their competition and interconversion might be relevant to Kir2.2 in vivo (24–26).
The functional analysis leads to the simple conclusion that only the slow kinetic process of transition between the burst and quiescent states is sensitive to C8-PI(4,5)P2. The structural studies showed that the binding of C8-PI(4,5)P2 was associated with a large conformational change between the CTD-undocked and CTD-docked structures (8, 16, 17). We thus proposed that the slow gating transitions in the channel recordings correspond to CTD engagement and disengagement and that when the CTD is engaged, the pore can open (Fig. 3B). The rapid gating transitions within a burst would then represent C8-PI(4,5)P2-independent conformational changes that occur elsewhere along the ion conduction pathway. According to this model, the channel record provides a dynamic readout of the CTD engagement and disengagement process, whose equilibrium is shifted by the C8-PI(4,5)P2 concentration.

**Effect of Phosphatidylinositol Lipid Derivatives on Gating.**

Earlier studies showed that phosphatidylinositol lipids with different phosphate substituents can also interact with Kir channels, including Kir2.2 (26). Fig. 4A shows the main, direct chemical interactions between C8-PI(4,5)P2 and Kir2.2 derived from the crystal structure (16). Individual membrane recordings using the planar bilayer system show the effects of several different phosphatidylinositol lipids (Fig. 4B–G). During each recording, application of the lipid under examination was followed by application of C8-PI(4,5)P2 to ensure the presence of Kir2.2 channels in the membrane. Soluble (C8) phosphatidylinositol analysis (Table 1) (27). The analysis assumes that all channels are identical in their behavior. For most channel types we have studied, including Kir2.2, this assumption is only approximately true. There appeared to be a fraction of outlier channels with lower or higher than average open probability, which undoubtedly contributed to the variation in rate constant values between different experiments reported in Tables 1 and 2. This limitation notwithstanding, Table 1 shows that C8-PI(4,5)P2 affected only the rate constants for transitions into and out of long closed state (C_{long}). In detail, when C8-PI(4,5)P2 was increased, the burst periods lengthened and the quiescent periods shortened. Consistent with the single-channel trace and histograms in Fig. 2, rate constants for opening and closing within a burst were insensitive to C8-PI(4,5)P2 concentration: Within a burst, only the mean number of transitions was affected by C8-PI(4,5)P2. Table 1 reports rate constant values for subscheme 3, but subscheme 4 would yield a similar conclusion, that only rate constants into and out of C_{long} are sensitive to C8-PI(4,5)P2 concentration (Fig. 2F).
Kinetics of Kir2.2 gating based on subscheme 3 ($C_{\text{Long}} = O$) in the presence of 3 and 6 μM C8-PI(4,5)P$_2$. Rate constant (k), open probability ($P(o)$), mean burst duration ($T_{\text{burst}}$), mean interburst interval ($T_{\text{interburst}}$), mean duration of $C_{\text{Short}}$ state, and mean number of short closures per burst (No. $C_{\text{Short}}$ per burst) were calculated from three different recordings.

Table 1. Kinetics of Kir2.2 gating in the presence of 3 and 6 μM C8-PI(4,5)P$_2$

| Recording no. | Channel no. | C8-PI(4,5)P$_2$ (μM) | C8-PI(4)P (μM) | $k_{12}$ (s$^{-1}$) | $k_{21}$ (s$^{-1}$) | $k_{23}$ (s$^{-1}$) | $k_{32}$ (s$^{-1}$) | P(o) | $T_{\text{burst}}$ (s) | $T_{\text{interburst}}$ (s) | Mean duration of $C_{\text{Short}}$ (ms) | No. $C_{\text{Short}}$ per burst |
|---------------|-------------|----------------------|----------------|----------------------|----------------------|----------------------|----------------------|------|------------------|---------------------|-----------------------------|-----------------------------|
| 1             | 8           | 3                    |                | 0.0097 ± 0.0007      | 1.7 ± 0.2            | 81 ± 2               | 5.52 ± 0.11          | 0.097 | 9.722           | 105.195             | 12.05                       | 49                         |
|               | 6           | 3                    |                | 0.021 ± 0.003       | 1.2 ± 0.2            | 77 ± 2               | 5.22 ± 0.08          | 0.160 | 13.570          | 49.146              | 12.87                       | 65                         |
| 2             | 9           | 3                    |                | 0.0056 ± 0.0006     | 2.7 ± 0.4            | 92 ± 3               | 6.1 ± 0.2            | 0.049 | 6.110           | 184.571             | 10.51                       | 34                         |
|               | 6           | 3                    |                | 0.017 ± 0.004       | 0.8 ± 0.2            | 84 ± 2               | 4.85 ± 0.07          | 0.266 | 22.669          | 59.364              | 11.83                       | 103                        |
| 3             | 4           | 3                    |                | 0.0054 ± 0.0014     | 7 ± 2                | 77 ± 2               | 6.3 ± 0.5            | 0.015 | 2.140           | 201.661             | 11.95                       | 12                         |
|               | 6           | 3                    |                | 0.020 ± 0.023       | 1.2 ± 0.2            | 85 ± 4               | 5.3 ± 0.2            | 0.112 | 15.136          | 50.156              | 11.58                       | 74                         |

Do phosphatidylinositol lipids that do not activate Kir2.2 fail to bind altogether, or do they bind but fail to open the gate? An earlier work based on a flux assay with Kir2.1 (29) and the data in Fig. 5 for Kir2.2 suggest the latter, that certain phosphatidylinositol lipids inhibit Kir2.2 activation by competing with PI(4,5)P$_2$ for its binding site. Following Kir2.2 activation by 6 μM C8-PI(4,5)P$_2$, addition of 20 μM C8-PI(3,4,5)P$_3$ to the lipid bilayer system. It also presents a mechanistic model derived by correlating C8-PI(4,5)P$_2$-dependent changes in the atomic structure of Kir2.2 with C8-PI(4,5)P$_2$—dependent changes in the activation of Kir channels in general (26). The present study advances our understanding by analyzing the phosphatidylinositol lipid-dependent gating of Kir2.2 in the compositionally defined lipid bilayer system. It also presents a mechanistic model derived by correlating C8-PI(4,5)P$_2$—dependent changes in the atomic structure of Kir2.2 with C8-PI(4,5)P$_2$—dependent changes in the kinetics of gating. The accuracy of the kinetic data is limited for reasons described above. Nevertheless, the conclusion that C8-PI(4,5)P$_2$ influences only the relatively slow transitions that govern exchange between the burst and quiescent periods is robust. Furthermore, C8-PI(4,5)P$_2$ influenced both the rates of entry and exit from the long quiescent periods for Kir2 channels. Given that Kir2.2 is the most thoroughly understood Kir channel from a structural point of view, this kinetic analysis has value. In the model, we connected the large conformational change observed in structural studies—C8-PI(4,5)P$_2$—mediated engagement between the CTD and TMD—with the very slow burst-quiescent period interconversions. The magnitude of conformational change documented in the structure was compatible with slow kinetic transitions. This idea is depicted in cartoon form in Fig. 3B. Of course, this is a hypothesis and not a claim that the model is necessarily true. In the CTD-docked conformation, the pore opens and closes rapidly in a C8-PI(4,5)P$_2$—independent manner. Other Kir channels also exhibit burst kinetics (18, 19), and in KATP, ATP-

Table 2. Kinetics of the C8-PI(4)P competition

| Recording no. | Channel no. | C8-PI(4,5)P$_2$ (μM) | C8-PI(4)P (μM) | $k_{12}$ (s$^{-1}$) | $k_{21}$ (s$^{-1}$) | $k_{23}$ (s$^{-1}$) | $k_{32}$ (s$^{-1}$) | P(o) | $T_{\text{burst}}$ (s) | $T_{\text{interburst}}$ (s) | Mean duration of $C_{\text{Short}}$ (ms) | No. $C_{\text{Short}}$ per burst |
|---------------|-------------|----------------------|----------------|----------------------|----------------------|----------------------|----------------------|------|------------------|---------------------|-----------------------------|-----------------------------|
| 1             | 5           | 6                    |                | 0.013 ± 0.002       | 0.90 ± 0.14          | 52 ± 2               | 4.20 ± 0.11          | 0.134 | 14.907          | 77.772              | 19.04                       | 57                         |
|               | 6           | 6                    |                | 0.005 ± 0.002      | 13 ± 4              | 57 ± 8               | 3.0 ± 0.5            | 0.006 | 1.865           | 266.014             | 14.36                       | 4                          |
| 2             | 4           | 6                    |                | 0.007 ± 0.002      | 0.62 ± 0.13          | 53 ± 2               | 3.35 ± 0.09          | 0.170 | 27.597          | 153.769             | 18.59                       | 86                         |
|               | 6           | 6                    |                | 0.0013 ± 0.0003    | 1.8 ± 0.4           | 43 ± 2               | 3.7 ± 0.2            | 0.046 | 7.475           | 784.335             | 22.26                       | 25                         |
| 3             | 5           | 6                    |                | 0.0027 ± 0.0007    | 0.14 ± 0.05         | 43.9 ± 1.2           | 3.72 ± 0.08           | 0.235 | 94.889          | 370.114             | 22.72                       | 324                        |
|               | 6           | 6                    |                | 0.0005 ± 0.0005    | 0.39 ± 0.09         | 42.1 ± 1.2           | 3.03 ± 0.08           | 0.117 | 38.427          | 2,271.968           | 23.54                       | 108                        |

Kinetics of Kir2.2 gating based on subscheme 3 ($C_{\text{Long}} = C_{\text{Short}} = O$) in the presence of 6 μM C8-PI(4,5)P$_2$ and after application of 6 μM C8-PI(4)P. Rate constant (k), open probability ($P(o)$), mean burst duration ($T_{\text{burst}}$), mean interburst interval ($T_{\text{interburst}}$), mean duration of $C_{\text{Short}}$ state, and mean number of short closures per burst (No. $C_{\text{Short}}$ per burst) were calculated from three different recordings.
cated short-chain phosphatidylinositol lipid (or 1.5% long-chain PI(5)P) to was calculated as the ratio of average current induced by 20 μM of various phosphatidylinositol lipids on Kir2.2 gating. Here, the activation of multiple open states were observed from membranes with 1.5% (mole fraction) PI(5)P. Further application of 20 μM C8-PI(4,5)P2 resulted in a significant increase of current. The membrane was held at −100 mV. (C) C8-PI(3,5)P2 can activate Kir2.2. Further application of 20 μM C8-PI(4,5)P2 resulted in a significant increase of current. The membrane was held at −100 mV. (D) C8-PI(3,4,5)P3 activates Kir2.2 to a similar extent as C8-PI(4,5)P2. The membrane was held at −100 mV. (E–G) 20 μM C8-PI(4,5)P2 (F), or C8-PI(3,4)P2 (G) failed to activate Kir2.2 despite the presence of Kir2.2 channels in the membrane, demonstrated by further addition of 20 μM C8-PI(4,5)P2. The membranes were held at −100 mV. Large current deflections were caused by mixing. Zero-current level is marked with a dashed line. (H) Summary of the effect of variable phosphatidylinositol lipids on Kir2.2 gating. Here, the activation was calculated as the ratio of average current induced by 20 μM of the indicated short-chain phosphatidylinositol lipid (or 1.5% long-chain PI(5)P) to the average current following subsequent addition of 20 μM C8-PI(4,5)P2. (I–L, Indicated phosphatidylinositol lipid and indicated C8-PI(4,5)P2, mean ± SEM, n = 3). PIP, phosphatidylinositol lipid.

Fig. 4. Effect of different phosphatidylinositol lipids on Kir2.2. (A) C8-PI(4,5)P2-binding site on Kir2.2 (PDB 3SPH). The channel is shown as a gray ribbon. C8-PI(4,5)P2 is shown as sticks and is colored according to atom type: oxygen, red; phosphorus, orange; and carbon, yellow. Sidechains of residues that form hydrogen bonds with 4 phosphate are shown as sticks and are colored teal, IF helix, interfacial helix. Data for Fig. 4A adapted from Hansen et al. (16). (B) PI(5)P can activate Kir2.2. Short openings (inset) were observed from membranes with 1.5% (mole fraction) PI(5)P. Further application of 20 μM C8-PI(4,5)P2 can activate Kir2.2. Further application of 20 μM C8-PI(3,5)P2 resulted in a significant increase of current. The membrane was held at −100 mV. (C) C8-PI(3,5)P2 can activate Kir2.2. Further application of 20 μM C8-PI(4,5)P2 resulted in a significant increase of current. The membrane was held at −100 mV. (D) C8-PI(3,4,5)P3 activates Kir2.2 to a similar extent as C8-PI(4,5)P2. The membrane was held at −100 mV. (E–G) 20 μM C8-PI(4,5)P2 (F), or C8-PI(3,4)P2 (G) failed to activate Kir2.2 despite the presence of Kir2.2 channels in the membrane, demonstrated by further addition of 20 μM C8-PI(4,5)P2. The membranes were held at −100 mV. Large current deflections were caused by mixing. Zero-current level is marked with a dashed line. (H) Summary of the effect of variable phosphatidylinositol lipids on Kir2.2 gating. Here, the activation was calculated as the ratio of average current induced by 20 μM of the indicated short-chain phosphatidylinositol lipid (or 1.5% long-chain PI(5)P) to the average current following subsequent addition of 20 μM C8-PI(4,5)P2. (I–L, Indicated phosphatidylinositol lipid and indicated C8-PI(4,5)P2, mean ± SEM, n = 3). PIP, phosphatidylinositol lipid.

Fig. 5. Competitive inhibition of C8-PI(4,5)P2 activation by various phosphatidylinositol lipids. (A–D) C8-PI(3)P (A), C8-PI(4)P (B), C8-PI(3,4)P2 (C), or C8-PI(3,5)P2 (D) inhibited Kir2.2 currents that were activated by C8-PI(4,5)P2. Following Kir2.2 activation by 6 μM C8-PI(4,5)P2, application of 20 μM C8-PI(3)P (A), C8-PI(4)P (B), C8-PI(3,4)P2 (C), or C8-PI(3,5)P2 (D) caused a reduction in current. The membranes were held at −100 mV. Zero-current level is marked with a dashed line. (E) Summary of the inhibition by different phosphatidylinositol lipids. Percentages of current decrease after application of 20 μM of the indicated phosphatidylinositol lipids are plotted (i.e., 100% − % inhibited current/100 μM C8-PI(4,5)P2). (F) Current recorded from a bilayer containing multiple Kir2.2 channels after application of 6 μM C8-PI(4,5)P2 followed by additional 6 μM C8-PI(4)P. An expanded trace is shown in the inset. The membrane was held at −100 mV. C, closed; PIP, phosphatidylinositol lipid.

independent gating within burst periods has been attributed to processes inside the selectivity filter (30). The basis for rapid gating in Kir2.2 is still unknown.

A G protein–independent GIRK channel was found to exhibit a more complex dependence on PI(4,5)P2 than we describe here for Kir2.2 (19). In the mutant GIRK channel, in addition to influencing the burst (but not the interburst) duration, multiple open states were deduced and attributed to different degrees of PI(4,5)P2 occupation. In Kir2.2, we observed only a single open state and can say only the following regarding the functional stoichiometry of PI(4,5)P2 activation: 1) that the quiescent periods shortened with increasing PI(4,5)P2 concentration, together with no current observed in the absence of PI(4,5)P2, suggests that at least one bound PI(4,5)P2 molecule is required to enter a burst and 2) that the burst periods lengthened with increasing PI(4,5)P2 concentration suggests that after a sufficient number of PI(4,5)P2 molecules have bound to enter a burst, at least one more PI(4,5)P2 can still bind (Fig. 3B). If this were not the case, then the burst duration would be independent of the PI(4,5)P2 concentration. In conclusion, somewhere between one and three PI(4,5)P2 molecules would seem to be required to stabilize the structure underlying the burst state, which we propose is the CTD-docked state. We know from the structures that four PI(4,5)P2 molecules can bind to the CTD-docked conformation, but if the model is correct, fewer than four can support the CTD-docked conformation.
with PI(4,5)P₂, it is abundant in the plasma membrane (25). Moreover, PI(4)P is a precursor in the synthesis of PI(4,5)P₂, and dephosphorylation of PI(4,5)P₂ generates PI(4)P (24–26). Because PI(4,5)P₂ activates and PI(4)P competitively inhibits, changes in lipid metabolism could give rise to a very steep change in the level of Kir2.2 activity.

**Materials and Methods**

**Cloning, Expression, and Purification.** A synthetic gene fragment (Bio Basic, Inc) encoding residues 38 to 369 of chicken Kir2.2 (Kir2.2) channel (GI:118097849) was subcloned into a modified pEG BacMan vector with a C-terminal green fluorescent protein (GFP)-TID4 tag linked by a preScission protease tease site (31). This construct was used in all experiments in this study.

Bacmid containing Kir2.2 gene was generated according to the manufacturer's instructions (Invitrogen) by transforming the cKir2.2 pEG BacMan construct into Escherichia coli DH1OBCell. The Bacmid was then transfected into Spodoptera frugiperda Sf9 cells to produce baculoviruses using Cellfectin II (Invitrogen). After two rounds of amplification, P3 viruses (1:10 v:v ratio) were added to suspension cultures of HEK293S GnTI cells (American Type Culture Collection) at a density around 1.5 to 3 x 10⁶ cells/mL in Freestyle 293 media (GIBCO) supplemented with 2% fetal bovine serum (GIBCO) at 37 °C with 5% CO₂ and 80% humidity.

**Electrophysiology.** The bilayer experiments were performed as previously described with minor modifications (23, 36). A piece of polyethylene terephthalate transparency film separated the two chambers of a polyoxymethylene block, which were filled with buffer containing 10 mM potassium phosphate, pH 7.4, 150 mM KCl, and 2 mM MgCl₂.

Decane-lipid mixture of 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine:1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine:1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (Avanti) (w:v: w) was used to form 100-μm hole on the transparency film. Voltage was controlled with an Axopatch 2008 amplifier in whole-cell mode. The analog current signal was low-pass filtered at 1 kHz (Bessel) and digitized at 10 kHz with a Digidata 1322A digitizer. Digitized data were stored on a computer using the software PClamp (Molecular Devices, Sunnyvale, CA). Experiments were performed at room temperature.

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**Kinetic Analysis.** Recordings containing one to nine channels were idealized through half-amplitude threshold crossing and analyzed using Clampfit software (Molecular Devices).

For single-channel recordings, open or closed dwell-time distributions for events within the burst were fitted to an exponential probability density function. Models with different term numbers were compared, and the best model was selected (37).

Multichannel recordings were analyzed as described (27). Event lists were fitted using a three-state linear scheme (Scheme 1). Dead time was 0.27 ms. Channel numbers used in the multichannel kinetic analysis were estimated using the maximum number of observed open channel levels (from 15 μM C₈-P(4,5)P₂ recordings). Rate constants between all states (k₁₂, k₂₁, k₂₃, and k₃₂) were obtained by simultaneous fit to the dwell-time histograms of all conductance levels (27). The mean number of short closures per burst, mean burst, and interburst duration were then calculated based on the rate constants — mean number of short closures per burst = \( \frac{k_{32}}{k_{23}} \), mean interburst duration = \( \frac{k_{23}}{k_{21}} \), and mean burst duration = \( \frac{k_{21}}{k_{21}} \). Under our condition, that is k₃₂ ≫ (k₂₃ + k₃₂), mean interburst duration can be approximated as \( \frac{1}{k_{21}} \), and mean burst duration can be approximated as \( \frac{1}{k_{21}} \).

**Estimation of the Channel Number Used in the Multichannel Kinetic Analysis.** To evaluate the validity of using the maximum number of observed open channel levels (from 15 μM C₈-P(4,5)P₂ recordings) as the channel number (N) in the multichannel kinetic analysis, we asked what is the probability of observing N channels at least once during the duration of the record? This probability is a function of N, the rate constants, and the initial condition. This probability is given by the integral of the first passage time probability density function for the appearance of the rightmost state for the scheme, for n = 8, shown in Scheme 2.

**Scheme 1.** Three-state linear scheme. The closed states are denoted as C₁ and C₂, and the open state is denoted as O₃. Rate constants between these states are denoted as k₁₂, k₂₁, k₂₃, and k₃₂.

**Scheme 2.** This scheme, with the rate constants weighted as shown, models a membrane with n = 8 identical channels. The quiescent state of each channel is denoted as C_{close}, and the burst is denoted as C_{open}. The leftmost state represents the membrane with eight closed channels. The rightmost state represents the membrane with eight open (i.e., burst) channels.
Table 3. Reanalysis of recording 3 from Table 1, using various channel numbers (N)

| Recording no. | Channel number | C8-PI(4,5)P2 (µM) | k_{12} (s^{-1}) | k_{21} (s^{-1}) | k_{23} (s^{-1}) | k_{32} (s^{-1}) | P(O) | T_{burst} (s) | T_{interburst} (s) | C_{short} per burst (ms) | No. C_{Short per burst} |
|---------------|----------------|-------------------|-----------------|-----------------|-----------------|-----------------|------|---------------|-------------------------|--------------------------|------------------------|
| 3             | 4              | 0.0054 ± 0.0014    | 7 ± 2           | 77 ± 7          | 6.3 ± 0.5       | 0.015           | 2.140 | 201.661       | 11.95                    | 11.52                    |                        |
| 6             | 5              | 0.020 ± 0.003      | 1.2 ± 0.2       | 85 ± 4          | 5.3 ± 0.2       | 0.112           | 15.136 | 50.156       | 11.58                    | 73.92                    |                        |
| 3             | 6              | 0.0044 ± 0.0016    | 7 ± 2           | 75 ± 8          | 7.1 ± 0.6       | 0.0117          | 2.110 | 246.782       | 12.12                    | 11.30                    |                        |
| 6             | 6              | 0.016 ± 0.003      | 1.3 ± 0.3       | 86 ± 4          | 5.33 ± 0.19     | 0.0892          | 13.353 | 63.729       | 11.43                    | 66.13                    |                        |
| 3             | 7              | 0.0036 ± 0.0014    | 7 ± 2           | 76 ± 7          | 6.2 ± 0.6       | 0.0098          | 2.096 | 300.129       | 12.12                    | 11.22                    |                        |
| 6             | 7              | 0.013 ± 0.003      | 1.4 ± 0.3       | 87 ± 4          | 5.4 ± 0.2       | 0.0743          | 12.551 | 78.305       | 11.37                    | 62.42                    |                        |
| 3             | 8              | 0.0031 ± 0.0009    | 7 ± 2           | 76 ± 7          | 6.2 ± 0.5       | 0.0084          | 2.085 | 353.833       | 12.12                    | 11.15                    |                        |
| 6             | 8              | 0.011 ± 0.002      | 1.4 ± 0.3       | 87 ± 4          | 5.37 ± 0.17     | 0.0637          | 12.036 | 92.779       | 11.33                    | 60.01                    |                        |
| 3             | 9              | 0.0027 ± 0.0013    | 7 ± 2           | 76 ± 7          | 6.2 ± 0.5       | 0.0073          | 2.077 | 407.228       | 12.12                    | 11.11                    |                        |
| 6             | 9              | 0.0095 ± 0.0015    | 1.5 ± 0.3       | 87 ± 4          | 5.39 ± 0.18     | 0.0557          | 11.678 | 107.161      | 11.31                    | 58.34                    |                        |
| 3             | 10             | 0.0024 ± 0.0006    | 7 ± 2           | 76 ± 7          | 6.2 ± 0.6       | 0.0065          | 2.072 | 460.095       | 12.12                    | 11.08                    |                        |
| 6             | 10             | 0.0084 ± 0.0016    | 1.5 ± 0.3       | 87 ± 4          | 5.4 ± 0.2       | 0.0496          | 11.438 | 121.653      | 11.27                    | 57.25                    |                        |
| 3             | 10             | 0.0021 ± 0.0009    | 7 ± 2           | 76 ± 7          | 6.2 ± 0.6       | 0.0059          | 2.067 | 513.606       | 12.12                    | 11.05                    |                        |
| 6             | 10             | 0.0075 ± 0.0015    | 1.5 ± 0.3       | 87 ± 4          | 5.4 ± 0.2       | 0.0446          | 11.247 | 136.103      | 11.26                    | 56.36                    |                        |

Kinetics of Kir2.2 gating based on subscheme 3 (\( CLong = C_{Short} = O \)) in the presence of 3 and 6 µM C8-PI(4,5)P2. Rate constant (k), open probability (P(O)), mean burst duration (\( T_{burst} \)), mean interburst interval (\( T_{interburst} \)), mean duration of \( C_{Short} \) state, and mean number of short closures per burst (No. \( C_{Short per burst} \)) were calculated from recording 3 from Table 1 using the channel numbers listed in column 2.

Using Mathematica (Wolfram), we estimated that the mean first passage time was 268 s and that the probability of observing eight channels at least once during the duration of the record (10 min) was 0.9. Given the 10% chance that we could not see all eight channels open within 10 min and the problem of channel disappearance over time, it is possible that we underestimated the channel number. Therefore we asked, if we assign the incorrect value for N, will our conclusion that C8-PI(4,5)P2 affects only the burst and interburst periods be wrong? Using recording 3 (Table 1), in which we assigned \( n = 4 \) on the basis of direct observation, we reanalyzed the record for \( n = 4 \) to 10 (Table 3). The value of \( N \) had little influence on the determination of kinetic values within the burst and had a small influence on \( k_{12} \) and mean burst duration. The main effect was on \( k_{12} \) and mean interburst duration. However, importantly, when C8-PI(4,5)P2 was increased, the burst periods strengthened and the quiescent periods shortened. Thus, our general conclusion that C8-PI(4,5)P2 concentrations influenced the transitions between the burst and interburst states holds even if we underestimated \( N \).

Data Availability. All study data are included in the main text.

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