Research Paper

Stoichiometry of Kir channels with phosphatidylinositol bisphosphate

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Phosphatidylinositol bisphosphate (PIP2) is the most abundant phosphoinositide in the plasma membrane of cells and its interaction with many ion channel proteins has proven to be a critical factor enabling ion channel gating. All members of the inwardly rectifying potassium (Kir) channel family depend on PIP2 for their activity, displaying distinct affinities and stereospecificities of interaction with the phosphoinositide. Here, we explored the stoichiometry of Kir channels with PIP2. We first showed that PIP2 regulated the activity of Kir3.4 channels mainly by altering their bursting behavior. Detailed burst analysis indicates that the channels assumed up to four open states and a connectivity of four between open and closed states depending on the available PIP2 levels. Moreover, by controlling the number of PIP2-sensitive subunits in the stoichiometry of a tetrameric Kir2.1 channel, we showed that characteristic channel activity was obtained when at least two wild-type subunits were present. Our studies support a kinetic model for gating of Kir channels by PIP2, where each of the four open states corresponds to the channel activated by one to four PIP2 molecules.

Introduction

Numerous reports since the pioneering work of Hilgemann and Ball1 and Fan and Makielski2 have implicated phosphoinositides in the direct control of the activity of just about every type of ion channel (reviewed in refs. 3–6). The first channel whose activity was shown to depend on phosphoinositides was the inwardly rectifying ATP-inhibited potassium (KATP) channel.1,2 Subsequently, several additional inwardly rectifying K (Kir) channels were also shown to depend directly on phosphoinositides for maintenance of their activity,7,8 and by now, all Kir members have exhibited similar dependencies.9,10 Kir channels are the ion channel family, whose dependence on phosphoinositides, and in particular on phosphatidylinositol bisphosphate (PIP2) that is the most abundant plasma membrane phosphoinositide, has been studied most comprehensively (reviewed in refs. 11–19). Despite intense studies of Kir channel interactions with phosphoinositides, the number of PIP2 molecules controlling channel activity is not known. We pursued this question using two different Kir channels and two distinct approaches: first we used Kir3 channels and characterized their unitary kinetics of interaction with PIP2; next we measured both the resulting microscopic and macroscopic currents of Kir2.1 tetramers containing a varying number of mutant subunits that when in a homotetramer showed impaired sensitivity to activation by PIP2.

Kir3 channels were the first effector proteins shown to be activated directly by the Gβ subunits of G proteins (Gβγ).20 It was later shown that intracellular Na+ could also serve as a gating molecule for Kir3 channels21 and that the activity of these channels required PIP2 in the presence of either Gβγ or Na+.22 A pore helix mutant of Kir3.4, Kir3.4-S143T (Kir3.4*) displays high homomeric activity with unaltered Gβγ sensitivity.23 Previous studies have shown that proline mutations in the mid-region of the pore-lining TM2 helix rendered Kir3 channel activity insensitive to overexpression of Gβγ or depletion of Gβγ molecules due to overexpression of scavenger molecules.24,25 These studies suggested that, the Kir3.4*–S176P mutation functionally mimicked the effects of saturating Gβγ interactions with the channel.24 In contrast, this mutation was found to display unaltered PIP2 sensitivity.25

In this manuscript, we first describe studies of single-channel kinetics of S176P under various levels of channel-PIP2 interactions. We chose the S176P mutant to study the effects of PIP2 on unitary kinetics because of its simpler single-channel kinetics, due to insensitivity to Gβγ and unaltered channel-PIP2 interactions. This mutant did not display closed-time components in the range of tens of milliseconds, typically found in Kir3 channel activity26 (Jin, Logothetis et al., unpublished results). Thus, the S176P mutant
the sensitivity of the channels to PIP2.27 One of the residues critical for Kir2.1 channels is the R218 residue.27,28 Heterologous expression of excess Kir2.1(R218Q) relative to Kir2.1 wild type has been shown to yield unitary activity with distinct characteristics, suggesting that tetrameric channels contained one, two or three of the mutant subunit.27 In order to study the effects of PIP2 on the unitary kinetics of Kir2.1 channels at progressively attenuated channel-PIP2 interactions, we studied the single-channel kinetics of Kir2.1 channels and tandem tetrameric channels containing one Kir2.1-R218Q (referred to as Q) (QRRR), two (QRQR), and three (QQQR) mutant subunits. Our results showed that QRQR and QRRR displayed similar open times and conductance characteristics as Kir2.1, while we were not able to detect neither microscopic nor macroscopic QQQR activity.

Our results indicate that PIP2 had saturating effects on the mean open time of the Kir channels at moderate endogenous PIP2 levels. The endogenous PIP2 levels also had saturating effects on the activity of Kir2.1 channels, but further increases of PIP2 levels dramatically increased the channel activity of Kir3.4*-S176P channels by altering their bursting behaviors. Detailed single-channel burst analysis indicated that the channel could assume up to four open states. The number of recognized open states varied from two to four depending on the available PIP2 levels. Moreover, our analysis of unitary kinetic components and bursting parameters yielded a consistent scheme for the number of open states and the connectivity between open and closed states.34 These results have led us to propose a gating model with four open states each corresponding to the channel activated by one to four PIP2 molecules.

RESULTS

Open- and closed-time kinetics from cell-attached recordings of S176P. Figure 1 shows single-channel traces and dwell-time fitting results of a representative cell-attached recording of S176P. As can be visually appreciated in Figure 1A, S176P typically displayed relatively homogeneous openings, and the distribution of the open times contained one single component as shown in Figure 1B. The recording displayed four closed-time components (Fig. 1C). Table 1 shows fitting results of open and closed times of a total of five cell-attached recordings. Four out of the five recordings (#1–4) exhibited a single open-time component and one recording (#5) exhibited two open-time components. The open-time constant of recording #1 is similar to the shorter open-time constant of recording #5, while the open-time constant of recordings #2,3,4 were very similar to the longer open-time constants of recording #5. The representative traces shown in Figure 1A correspond to recording #4.

As can be seen from Table 1, the open probabilities of different recordings differed by as much as twenty fold, although most recordings showed very similar mean open times. The large variation in the open probability was mainly due to the variation in the mean closed-time. One can also see that despite more than 10-fold differences in the mean closed times, the four closed-time constants were very similar in different recordings. We present our fitting results of the dwell-time distributions using the time constant and the frequency of each component. The frequency of each kinetic component is deduced from the weight of the component (which is most commonly used to present the dwell-time fitting results) and the number of total events normalized by the recording length (see Materials and Methods). The frequency of an open- (closed-) time component is the calculated value of the frequency of the opening (closed) events of the particular component taking place in the recordings. The frequencies of the two longer closed times (Fc3 and Fc4) also exhibited smaller variations compared to the frequencies of the two short close-time components (Fc1 and Fc2). The main contributor toward the variation in the mean closed-time was the variation in the frequencies of the shortest closed-time components.

Bursting behaviors of S176P under cell-attached conditions. Figure 2 compares the details of single-channel traces and the bursting behaviors of relatively “low” and “high” activity recordings, which are visualized in Figure 2A. Figure 2B shows the distribution of the bursts with solid bars and the open times are distributed with red bars above the segments.

Figure 1. Single-channel traces (A) and the distributions of open (B) and closed (C) times of a representative cell-attached recording of Kir3.4*(S176P). Single-channel traces are displayed in various time scales as indicated in the figure. The segments shown in further expanded time scales are indicated with red bars above the segments.
displayed open probabilities 0.6% and 9.4%, respectively. The two recordings shown displayed the lowest and highest open probabilities among the cell-attached recordings that exhibited a single open-time component (Table 2, recordings #1 and #4). As can be visually appreciated from Figure 2A, the higher activity recording displayed much longer bursting behaviors. The mean burst durations of the low and high activity recordings were 20.5 and 249.9 ms, respectively. The difference in the burst durations accounted primarily for the difference in the open probabilities. Since the closed events of the two short closed-time components (τ_{c1} and τ_{c2}) appeared within the channel bursts, the longer burst durations of the high activity recordings displayed a greater number of short closed events, thus an increased frequency of the two short closed-time components, as seen in Table 1. The number of openings per burst (NOPB) of the low activity recording exhibited two components, while the high activity recording exhibited one extra long component (Fig. 2B and C, right panels). The left panels of Figure 2B and C show the distributions of the total opening time per burst (TOTPB). Consistent with the distribution of NOPB, the TOTPB of the high activity recordings also exhibited three components, one more component than the low activity recording.

Table 2 lists some bursting parameters such as the bursting probability (BPo), mean burst duration (MBTo), mean interburst interval (MBTc), and open probability within bursts (Powb). As can be seen in Table 2, the interburst intervals in different recordings are very similar despite the dramatic variations in the open probability and the mean burst durations. One can readily see that differences...
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in the burst durations are responsible for differences in the open probability. Application of PIP2/Na however did not significantly change the single-channel kinetic properties within bursts such as MTo and Powb (the ratio of MTo and Powb under PIP2/Na application and CA was 1.06 ± 0.09 and 1.05 ± 0.03, respectively). As can be seen in Figure 4C and 4D, the distributions of both TOTPB (4C) and NOPB (4D) exhibited four components.

Wortmannin treatment shortens channel openings and bursting. Figure 5 shows a recording after the endogenous PIP2 levels were downregulated by Wortmannin (WTMN) treatment. As can be...
Open and closed-time kinetics of Kir3.4*–S176P under PIP2/Na applications

| Po  | MTc | MTo | τc1  | τc2  | τc3  | τc4  | σc1  | σc2  | σc3  | σc4  | σc5  | Length |
|-----|-----|-----|------|------|------|------|-------|-------|-------|-------|-------|--------|
| 0.641 | 3.48 | 0.44 | 1.13 | 23.52 | 3248.91 | 595.98 | 21.39 | 0.36 | 0.04 |
| 0.446 | 2.41 | 0.80 | 1.59 | 102.39 | 466.47 | 9.89 | 0.44 | 2.01 | 33.29 | 1118.05 | 563.86 | 4.38 | 0.28 | 0.33 |
| 0.484 | 3.18 | 1.43 | 2.65 | 155.27 | 252.60 | 11.91 | 0.39 | 1.20 | 15.70 | 521.46 | 6713.69 | 398.33 | 8.94 | 0.39 | 0.18 |
| 0.551 | 2.82 | 1.23 | 2.14 | 172.98 | 368.51 | 5.20 | 0.46 | 1.23 | 12.23 | 100.25 | 1854.72 | 524.39 | 16.39 | 0.56 | 0.13 |
| 0.404 | 2.99 | 1.34 | 2.34 | 117.71 | 247.33 | 13.91 | 0.43 | 1.38 | 19.00 | 471.35 | 13162.95 | 360.20 | 4.28 | 0.46 | 0.09 |
| 0.489 | 2.40 | 1.11 | 1.71 | 270.10 | 359.42 | 8.21 | 0.41 | 1.24 | 15.56 | 311.30 | 435046.6 |

Open- and closed-time kinetics of Kir3.4*–(S176P) under the application of PIP2/Na. The open probability (Po), mean open time (MTo), mean closed time (MTc) are listed together with the two open time constants (τc1 and τc2) and their frequencies (Fo1 and Fo2), the five close time constants (τc1, τc2, τc3, τc4 and τc5) and their frequencies (Fc1, Fc2, Fc3, Fc4 and Fc5). The unit of time constants, mean dwell times, and recording length (indicated by “Length”) is in ms, while the unit of frequency of each open- or closed-time component is in Hz.

### Four Components

Visualized in Figure 5A, the channel displayed extremely low activity (Po = 0.13%) and short bursting behavior. The channel more often displayed isolated single openings and the bursts tended to be short when the channel was in a bursting mode (as can be seen in the middle and bottom traces at an expanded time scale). As shown in Figure 5B and Table 4, the open-time component of the WTMN-treated channels displayed a single component with time constants shorter than channels not treated with WTMN (Fig. 1A and Table 1). The closed-time kinetics displayed up to four components (Fig. 5B, right panel and Table 4). Except the shortest closed-time constants (τc1), all other closed-time constants displayed large variations and not all recordings displayed all four components. One can also see the low frequencies of the open-time component and the shortest closed-time component of channels after WTMN treatment (Table 4) compared to channels under control conditions (Table 1). As can be seen from Figure 5C, both TOTPB (left panel) and NOPB (right panel) exhibited two components. Therefore, the channel displayed shorter open times, and shorter bursts after WTMN treatment.

**Whole-cell currents of tandem tetrameric channels containing a different number of channel-PIP2 interaction defective mutant subunits.** Our detailed burst analysis of Kir3.4*–S176P indicated that the number of the recognizable open states varied between two and four depending on the PIP2 levels. We reasoned (see Discussion) that the different number of open states reflects activation of the...
channel by different numbers of PIP$_2$. Surprisingly, the channel exhibited two open states after Wortmannin treatment, despite the low open probabilities. This observation may indicate that the Wortmannin treatment did not lower the PIP2 levels to the extent that binding of only one PIP2 molecule was possible. In order to test whether Kir channels display measurable activities when only one PIP2 molecule bound the channel and in order to study the effects of the PIP2 on the unitary kinetics of Kir channels at progressively attenuated channel-PIP2 interactions, we turned to the highly expressing Kir2.1 channels. We utilized the Kir2.1(R218Q) mutant that is defective in channel-PIP2 interactions$^{27,28}$ to control the number of PIP2-sensitive subunits (wild-type) in a tetrameric Kir2.1 channel. We have previously shown that the R218Q mutation does not alter expression of the mutant channel on the surface of Xenopus oocytes.$^{27}$

Since the R218Q homomers yield very small currents$^{27,28}$ compared to the wild-type controls, we set out to test the magnitude of macroscopic currents resulting from mixtures of 1:1 ratios of wild-type to R218Q Kir2.1 channels. With 1 ng total injected RNA in 24 hours, we obtained currents that were -37% of the control (Fig. 7A). Assuming a binomial distribution of subunit assembly into tetramers, we would expect that if all four subunits needed to be wild-type in order to form functional channels, only 1/16 or -6% of the tetramers would yield currents in the 1:1 mixture. These crude mixing experiments suggested that no more than three PIP2-binding subunits may be required to yield functional channels.

In order to test whether two PIP2-sensitive subunits could form active channels, we constructed a R218Q/wild-type (QR) dimer and expressed it in Xenopus oocytes. The resulting QRQR tetramers expected from assembly of the QR dimers gave comparable currents to those obtained from the mixing experiments (Fig. 7B), suggesting that even two PIP2-sensitive subunits could form active channels.

In order to compare the relative currents produced in tetramers bearing 0–4 wild-type subunits, we constructed tandem tetramers of Kir2.1 channels that contained different numbers of subunits containing the Kir2.1(R218Q) mutation. Figure 7C and D shows respective summary and representative whole-cell currents of tandem tetramers containing one, two and three mutated subunits, compared with homomeric tetramers of the mutant R218Q and the wild-type Kir2.1. Our results indicate that increasing the number of mutant subunits progressively decreased whole-cell currents. The currents obtained from tandem tetramers containing a single wild-type subunit did not yield significant whole-cell currents compared to Kir2.1(R218Q) channels or uninjected controls.

Comparison of the whole-cell currents suggests that introducing progressively more wild-type subunits into the tetramer has a synergistic effect on channel activity. When normalizing the whole-cell currents of all constructs to the whole-cell currents of QRQR (the tandem channel containing two wild-type subunits), the normalized whole-cell currents of QRQR, QRRR and Kir2.1 were 1, 4.6 and 9.1, respectively. These results indicate that starting from the first two wild-type subunits, introduction of one additional wild-type subunit into the tandem QRQR channels increases the channel whole-cell currents by approximately 4.6-fold.

Effect of the different number of mutant subunits on the single-channel kinetics of Kir2.1 channels. Figure 8 shows single-channel traces and the summary of the single-channel kinetic parameters, open probability (Po), mean open time (MTo) and mean closed-time (MTC) for the tandem tetramers bearing two or three R218Q subunits compared to wild-type Kir2.1 channels. As can
Table 4  The open- and closed-time kinetics of WTMN treated Kir3.4*-S176P

| Po      | MTo  | τo  | Po (Hz) | MTo  | τc1 | τc2 | τc3 | τc4 | Fc1 (Hz) | Fc2 (Hz) | Fc3 (Hz) | Fc4 (Hz) | Length   |
|---------|------|-----|---------|------|-----|-----|-----|-----|---------|---------|---------|---------|----------|
| 0.0041  | 1.0  | 1.2 | 1.696   | 249.1| 0.7 | 445.2| 0.643| 2.233| 0.7      | 445.2   | 0.643   | 2.233   | 812879.1 |
| 0.0007  | 1.7  | 1.7 | 0.535   | 2375.3| 0.7 | 6867.1| 0.293| 0.147| 0.7      | 445.2   | 0.643   | 2.233   | 382690.5 |
| 0.0003  | 1.3  | 1.3 | 0.127   | 4559.2| 2.0 | 10568.1| 0.041| 0.086| 0.7      | 445.2   | 0.643   | 2.233   | 684083.0 |
| 0.0021  | 0.8  | 1.0 | 0.879   | 388.9 | 0.3 | 265.0 | 0.155| 0.235| 0.8      | 445.2   | 0.643   | 2.233   | 211205.7 |
| 0.0013  | 1.3  | 1.3 | 0.574   | 1004.4| 0.6 | 2850.6 | 0.294| 0.017| 0.243   | 0.029   | 0.029   | 1546763.1 |

Open- and closed-time kinetics of Wortmannin (WTMN) treated Kir3.4*(S176P). The open probability (Po), mean open time (MTo), mean closed-time (MTc) are listed together with the open-time constants (τo) and their frequencies (Fo), the four closed-time constants (τc1, τc2, τc3 and τc4) and their frequencies (Fc1, Fc2, Fc3 and Fc4, see text for definition). All recordings displayed one open-time component. The unit of time constants, mean dwell times, and recording length (indicated by “Length”) is in ms, while the unit of frequency of each open- or closed-time component is in Hz.

Figure 6. The effects of the application of PIP2/Na on WTMN-treated Kir3.4*-S176P. (A) Single-channel traces of WTMN treated S176P in the CA mode (left) and after the channel was reactivated by PIP2/Na application (right). (B) A segment of the recording in the CA mode is displayed at different expanded time scales. The channel typically displays short bursts (I) and isolated short openings (II) at the CA mode following WTMN treatment. (C) A segment of the recording under PIP2/Na activation is displayed at different expanded time scales. The channel typically displayed long bursting behavior with openings longer than the CA mode after WTMN treatment. (D) Comparison of the fitting results of the open-time kinetics at the CA mode (A, left panel) and a segment of PIP2/Na-activated recordings (A, right panel) in which the channel did not display overlapping openings. The channel displayed a single open-time component at both conditions but with distinct time constants.
be visually appreciated from the single-channel traces displayed at an expanded time scale (Fig. 8B), the tandem tetramers containing two or more wild-type subunits (QRQR and QRRR) displayed very similar single-channel kinetics to those of the wild-type Kir2.1 when the channels were bursting. They all displayed characteristic long openings and high open probabilities (example in ref. 36). The bursting behavior and thus the open probability of QRQR displayed large variations among different recordings. Some recordings of QRQR (such as the top from the set of two traces shown in Fig. 8A and B) exhibited high open probabilities (Po) and long bursts which were almost indistinguishable from Kir2.1 wild-type channels. Other recordings exhibited much lower Po and shorter bursts (bottom traces of Fig. 8A and B). As shown in Figure 8D, the recordings can be grouped into two distinct groups according to their Po. The Po of the high activity group was similar to that of QRRR (0.749 ± 0.062, n = 2, for QRQR vs. 0.797 ± 0.026, n = 3 for QRRR), while the Po of the low activity group was similar to that of QQQR (0.19 ± 0.1, n = 3, for QQQR vs. 0.26 ± 0.03, n = 2 for Kir2.1).
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Figure 8. (A) Single-channel traces of the tandem tetramers and the wild-type Kir2.1. The tetramers containing two (QRQR) and three (QRRR) wild-type subunits displayed an apparent similar behavior within their bursting period of activity. Single-channel activity of QRQR displayed greater variability in its bursting behaviors, as with the representative two traces shown for this channel. (B) Representative segments (indicated by red bars in A) of the recordings from different channels shown in (A) are displayed in expanded time-scales. (C) Comparison of the single-channel parameters open probability (Po), mean open time (MTo) and mean closed time (Mtc) of the tandem tetramers and the wild-type Kir2.1. The tandem tetramers displayed progressively lower open probabilities as the number of subunits bearing mutation R218Q increased (asterisk indicate p < 0.05). The Po for Kir2.1 wt, QRRR and QRQR was 0.89 ± 0.012, 0.797 ± 0.026, and 0.41 ± 0.130, respectively. All functional channels displayed very similar open-time kinetics (394.7 ± 39.0, 375.4 ± 27.7 and 359.8 ± 14.6 for Kir2.1 wt, QRRR and QRQR, respectively). Differences in the Po were mainly due to differences in Mtc (46.17 ± 1.7, 102.17 ± 20.6 and 716.27 ± 241.1 for Kir2.1 wt, QRRR, and QRQR, respectively). (D) The distribution of open probabilities (Po) of the cell-attached recordings of QRQR. The 6 recordings can be grouped into two groups according to their Po. The Po of the first group is in the range between 0.2 to 0.4 (0.292 ± 0.027, n = 4), and the Po of the second group is within the range between 0.7 to 0.8 (0.749 ± 0.062, n = 2).

of the low activity group was much lower (0.292 ± 0.027, n = 4). The mean open times of the two tandem tetramers were similar to those of the wild-type channel (Fig. 8C, middle panel). The differences in the open probabilities (Fig. 8C, left panel) were mainly due to the difference in the mean closed-times (Fig. 8C, right panel). Table 5 lists the summaries of the fitting results of open-time kinetics of QRQR, QRRR and Kir2.1 wild-type channels. All channels displayed two open-time components with similar time constants. Table 6 lists the summaries of the fitting results of closed-time kinetics of QRQR, QRRR and Kir2.1 wild-type channels. All channels displayed four closed-time components. The time constants of the first three closed-time components (τc1, τc2, and τc3) and their frequencies were similar for all channels compared to the considerable differences in the mean closed time. The main contributor of the differences in the mean closed time of different channels was the longest closed-time component (τc4). Due to the small numbers of long closed events, the confidence interval of the longest component for all channels was extremely large (data not shown), so it is not meaningful to make quantitative comparisons of the longest closed-time constants and their frequencies for different channels. We were unable to detect characteristic unitary activity from either the QQQR in tandem constructs or the Kir2.1(R218Q) monomers. For these two constructs, we injected up to 16 ng per oocyte (we normally injected 0.025 ng for other channels). We tried more than twenty recordings from QQQR injected cells and 14 recordings from Kir2.1-R218Q injected cells, we did not observe any Kir2.1 characteristic single-channel activity. Macroscopic measurements using two-electrode voltage clamp yielded very small currents for QQQR or Kir2.1-R218Q that were often not significantly greater than uninjected controls. Functional expression of these channels

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appeared to depend on the specific batch of oocytes used, which made a rigorous characterization difficult.

Tandem constructs of multimeric ion channels are commonly used in studies aiming to determine subunit stoichiometry or to regulate subunit composition (examples in refs. 37–46). The subunit composition of each individual multimeric channel, however, could be different from that of the tandem constructs (examples in refs. 41, 42, 44 and 46). The single-channel recordings we obtained from QRRR (containing one channel in the patch) were quite consistent, therefore it is likely they represent channels with the same subunit composition. It has been shown that the tandem tetramers of Kir2.1 formed channels predominantly by intra-molecular assembly,42 thus again it is likely that all our recordings from QRRR were indeed from channels containing three wild-type subunits. The distribution of the open probabilities (Po) of QRQR single-channel recordings, however, contained two distinct populations (Fig. 8D). The Po of the higher activity group was quite similar to that of QRRR. Therefore it is quite possible that this group may indeed reflect activity from channels containing three wild-type subunits, resulting from an inter-molecular assembly. We were not able to obtain characteristic recordings from QQQR, suggesting that a channel containing a single wild-type subunit may be non-functional or may display an extremely low activity. Therefore we assume the recordings in the low activity group of QRQR represent activities from channels containing two-wild type subunits. Wild-type channels displayed a moderately increased Po compared to the channels containing three wild-type subunits. This was likely to be due to the fact that the later are already highly active (Po: 0.797 ± 0.026). Interestingly, oocytes expressing wild-type channels displayed whole-cell currents close to two fold greater than those expressing QRRR. The possible discrepancy may be due to differences in the surface expression efficiencies between tandem tetramers and monomers, as suggested by Schonherr and colleagues.40

**DISCUSSION**

Variations in the range of endogenous PIP2 levels are reflected in the activities of Kir3 channels. Our results showed that the open probabilities of Kir3.4*(S176P) channels were much lower in the cell-attached condition than the high open probability that could be reached by application of PIP2/Na. The open probabilities of the channels also exhibited up to a 20-fold variation under cell-attached conditions. Application of PIP2/Na activated channels to comparable conditions. These results indicate that PIP2 levels were the main limiting factor of channel activity under cell-attached conditions, and that variations in the endogenous PIP2 levels underlie the variability in channel activities. The PIP2 level-dependent variations in channel activity indicate that endogenous PIP2 levels fall into a range of sub-saturating concentration levels at which the activity of Kir3 channels is very sensitive. Since Kir3 channel activity at endogenous PIP2 levels lies at the sensitive range of the PIP2 dose-response curve, cells expressing Kir3 channels are able to regulate their channel activity by regulating PIP2 levels. Hydrolysis of PIP2 has been indeed suggested as a contributing mechanism to the slow time-scale desensitization of K_ACh currents in response to ACh in cardiac myocytes and heterologous expression systems.47,48

The durations of the openings and bursts of Kir channels displayed different sensitivities to PIP2 levels. Our single-channel studies of Kir3.4*(S176P) suggested that the open-time kinetics of

| Table 5 | Open-time kinetics of Kir2.1 and tandem tetramers |
|---------|---------------------------------------------------|
| Kir2.1  | QRRR                                             | QRQR                                             |
| τo1 (ms)| 1.5 ± 0.3 [n = 2]                                 | 3.0 ± 1.6 [n = 3]                                | 2.5 ± 1.3 [n = 2] |
| τo2 (ms)| 402.7 ± 33.5 [n = 3]                              | 384.4 ± 27.2 [n = 3]                             | 372.9 ± 16.8 [n = 4] |
| Fo1 (Hz)| 0.074 ± 0.061 [n = 2]                             | 0.047 ± 0.010 [n = 3]                            | 0.051 ± 0.003 [n = 2] |
| Fc1 (Hz)| 2.244 ± 0.168 [n = 3]                             | 2.078 ± 0.199 [n = 3]                            | 1.320 ± 0.324 [n = 4] |

Comparison of the fitting results of the open-time kinetics of the tandem tetramers and the wild-type Kir2.1 channels. All channels displayed up to two open-time components. Not all recordings displayed all two open-time components. The number of recordings that displayed each component is as indicated (n).

| Table 6 | Closed-time kinetics of Kir2.1 and tandem tetramers |
|---------|-----------------------------------------------------|
| Kir2.1  | QRRR                                             | QRQR                                             |
| τc1 (ms)| 1.6 [n = 1]                                       | 1.8 ± 0.5 [n = 3]                                | 1.4 ± 1.0 [n = 2] |
| τc2 (ms)| 13.8 ± 1.8 [n = 3]                                | 16.9 ± 1.4 [n = 3]                               | 14.0 ± 2.0 [n = 4] |
| τc3 (ms)| 88.7 ± 5.8 [n = 3]                                | 91.4 ± 4.1 [n = 3]                               | 87.1 ± 5.6 [n = 4] |
| τc4 (ms)| 4382.9 ± 1990.8 [n = 2]                           | 1.9 x 10^8 ± 1.9 x 10^8 [n = 2]                   | 1.6 x 10^8 ± 1.6 x 10^4 [n = 2] |
| Fc1 (Hz)| 0.488 [n = 1]                                     | 0.136 ± 0.064 [n = 3]                            | 0.207 ± 0.004 [n = 2] |
| Fc2 (Hz)| 1.190 ± 0.095 [n = 3]                             | 1.011 ± 0.125 [n = 3]                            | 0.814 ± 0.235 [n = 4] |
| Fc3 (Hz)| 0.896 ± 0.086 [n = 3]                             | 0.985 ± 0.091 [n = 3]                            | 0.569 ± 0.119 [n = 4] |
| Fc4 (Hz)| 0.003 ± 0.001 [n = 2]                             | 0.002 ± 0.0001 [n = 2]                           | 0.016 ± 0.005 [n = 2] |

Comparison of the fitting results of the closed-time kinetics of the tandem tetramers and the wild-type Kir2.1 channels. All channels displayed up to four closed-time components. Not all recordings displayed all four closed-time components. The number of recordings that displayed each component is as indicated (n). The three shortest closed-time components of WT and QRQR are very similar. The difference in the closed-time components was mainly caused by the longest closed-time component.
the channels are PIP₂ dependent, but the endogenous PIP₂ levels had saturating effects on the open-time kinetics of the channels in most cell-attached recordings. The mean open time of channels following Wortmannin treatment (1.3 ± 0.11 ms, n = 5, Table 4) was shorter than untreated controls obtained in the cell-attached mode, (2.40 ± 0.20 ms, n = 5, Table 1), suggesting that the channel displayed shorter mean open times at low PIP₂ levels. The effects of the PIP₂ levels on the open-time kinetics was further confirmed by application of PIP₂/Na onto patches originally displaying short openings (and extremely low open probabilities) due to Wortmannin treatment. As shown in Figure 6, applications of PIP₂/Na increased the mean open time of the channels to an extent similar to the control (i.e. without Wortmannin treatment) cell-attached recordings. The saturating effects of the endogenous levels of PIP₂ on the open-time kinetics of Kir3.4*-S176P was suggested by the observation that most cell-at- tached recordings displayed very similar open-time kinetics, even though the large differences in the open probabilities and the mean burst durations suggest considerable differences in endogenous PIP₂ levels. Furthermore, application of exogenous PIP₂ did not increase the mean open time compared to control (i.e. without Wortmannin treatment) in the cell-attached mode, but dramatically increased the channel burst durations. Therefore, unlike the open-time kinetics, the bursting behavior of the channel is far from being saturated by endogenous PIP₂ levels.

The saturating effect of PIP₂ on the open-time kinetics was also shown in the single-channel kinetics of the tandem tetramers of Kir2.1 channels. Since the mutant R218Q subunit has an impaired sensitivity to PIP₂, the progressive deviation of the single-channel kinetics of the tandem tetramers from the wild-type Kir2.1 with a progressively increasing number of mutant subunits, reflects the single-channel kinetics of Kir2.1 at different available PIP₂ levels. The tandem tetramer containing two mutant subunits (QRQR) displayed unaltered open-time kinetics, despite the lower open probability. Therefore this is consistent with the observation from Kir3.4*-S176P that the requirements for the Kir channels displaying unaltered open-time kinetics are much lower than those for channel bursting and channel open probabilities.

Plausible models for the different sensitivities of the durations of openings and bursts on the PIP₂ levels. Our results have shown that PIP₂ levels affect both open-time kinetics and bursting behaviors of the Kir channels, and that PIP₂ had saturating effects on the open-time kinetics at much lower levels than are required for having saturating effects on the bursting kinetics and the open probability. It is interesting to interpret these findings in the context of previous mutagenesis and structural studies. A number of previous studies have suggested that the open-time kinetics of Kir channels are sensitive to mutations of residues in the proximity of the selectivity filter, while mutations of residues within the region of the helix bundle crossing mainly affect the bursting behavior of the channels (reviewed in ref. 49). In one model, we can assume that a Kir channel possesses distinct high and low affinity binding sites for PIP₂, and that binding of PIP₂ to the two types of binding sites would exert specific effects. In other words, binding of PIP₂ to the high affinity site(s) would affect the open-time kinetics of the channel similar to the effects of the mutations in the region of the selectivity filter, while binding of PIP₂ to the low affinity binding site would specifically affect the bursting behavior of the channel similar to the effects of the mutations in the helix bundle crossing. Therefore, as the levels of PIP₂ increase, the high affinity binding site(s) would be occupied first and increase the mean open time of the channel, while further occupation of the low affinity binding site(s) due to increased PIP₂ levels would affect the bursting behavior of the channel.

Alternatively, our observations could also be accounted for by another model that assumes that Kir 2 and 3 channels are composed of multiple gates located in series along the permeation pathway.50,51 For a channel composed of multiple gates, the mean open time should be dictated by the gate that displays the shortest openings. Assuming one or more gates other than the PIP₂-operated gate (the “P” gate) undergoes spontaneous, PIP₂ independent transitions (which we will call the “S” gate(s) for the sake of discussion) between the open and closed states, then openings of the P gate may be the limiting factor of the open time of the channel when the P gate would not be sufficiently stabilized at low PIP₂ levels, thus causing the channel to exhibit short mean open time at extremely low levels of PIP₂. When the open state of the P gate would be sufficiently stabilized at higher PIP₂ levels, it would no longer be the limiting factor of the open-time kinetics of the channel. One opening of the P gate now could allow multiple transitions of the S gate(s) between the open and closed states, thus manifesting itself as one burst. Further increase of PIP₂ levels could further stabilize the P gate at the open state to prolong channel bursting, but would have little effect on the open-time kinetics, which would now be limited by the S gate(s).

Implication of the small variations in the interburst intervals of Kir3.4*-S176P in the cell-attached mode. As can be seen in Table 2, the interburst intervals of cell-attached recordings were comparable. One can also see that the closed-time constants in different recordings were very similar, which indicates that the life time of long closed states are insensitive to PIP₂ levels. This argues that once a channel is closed because of the unbinding of PIP₂, the rate of PIP₂ binding is not the rate limiting step in channel opening by PIP₂. If the binding of PIP₂ were the rate limiting step, then the recordings displaying longer bursting should display shorter interburst intervals, as the rate of the PIP₂ binding should be proportional to the available PIP₂ levels. Therefore our results argue that for the multiple gate model PIP₂ would stabilize the open state of the P gate, but it may have very limited effects on catalyzing its transition from the closed to the open state. The rate limiting step for opening the P gate may involve overcoming certain intrinsic stabilization factors of the closed state. This notion is consistent with recent findings by Rapedius and colleagues that an intrasubunit hydrogen bond between TM1 and TM2 of the Kir1.1 channel is responsible for the slow activation kinetics of macroscopic currents elicited by application of PIP₂ to inside-out patches.52

The increase of burst durations at higher PIP₂ levels suggests that a channel binds multiple PIP₂ molecules simultaneously. If a channel were to bind only one PIP₂ molecule, then the lifetime of the PIP₂ bound state would solely depend on the energetic nature of channel-PIP₂ interactions, i.e., the off rate of PIP₂ molecules from the channel. Changes in PIP₂ concentration would only affect the intervals it takes the channel to bind back a PIP₂ molecule after the unbinding of the last bound PIP₂ molecule. If this were true, we would predict that, at higher PIP₂ levels, a channel should display shorter interburst intervals but unaltered burst durations, which is the opposite of what we observed.
The number of open states of Kir3.4*-S176P is dependent on PIP2 levels. The number of open-time components reflects the number of open states. Our results indicate that the S176P channel exhibited up to two open-time components. This may suggest that S176P has two open states. The number of open states can be also reflected by the distributions of parameters describing the bursting behaviors of the channels, such as the number of openings per burst (NOPB) and the total open time per burst (TOTPB). The probability density function of the distribution of NOPB is a geometric function, whose number of components reflects the connectivity between open and closed states. The probability density function of the distribution of TOTPB is an exponential function, whose number of components reflects the number of open states. Our results show that both NOPB and TOTPB exhibited two and three components in low and high activity cell-attached recordings, respectively. Both NOPB and TOTPB following Wortmannin treatment and PIP2 application displayed two and four components, respectively. This indicates that the number of accessible open states depends on the available PIP2 levels, varying in the range between two (low activity cell-attached recordings or following Wortmannin treatment), to three (high activity recordings), and four (upon application of PIP2/Na) under our experimental conditions. Thus, we propose that these open states accessible at different PIP2 levels reflect the open states of the channel associated with a different number of PIP2 molecules. In many cases, the channel S176P displayed one single open-time component, although the number of components of bursting parameters, such as the NOPB and TOTPB, displayed multiple components.

A kinetic model for channel-PIP2 interactions. A PIP2-dependent number of open states reflects the stoichiometry of channel-PIP2 interactions. In Figure 9, we postulate simple kinetic diagrams that satisfy our findings of the number of open states and the connectivity between open and closed states at high (under PIP2/Na applications), moderate (cell attached mode, no Wortmannin treatment) and low (after Wortmannin treatment) PIP2 levels. We introduced C4 to represent our observation of the presence of long closed states even under application of PIP2/Na. We propose that the four PIP2-dependent open states (O1 through O4 in Figure 9) reflect channel openings upon association of 1–4 PIP2 molecules. Under this assumption, C1 through C4 represent channel closures in the presence of PIP2, and C0 represents channel closure due to the complete dissociation of PIP2. This kinetic model assumes that association of PIP2 molecules activates a Kir channel by allowing it transition to corresponding open states. It doesn’t assume a one-to-one correspondence between channel openings and PIP2 association. The basic assumption of this kinetic model, which assumes that the four open states reflect the stoichiometry of channel-PIP2 interactions, is consistent with the tetrameric subunit composition of Kir channels. This model, however, requires that a Kir channel can transition to the open state upon association of one PIP2 molecule. This requirement may seem inconsistent by two results: a) our finding that Kir3.4*-S176P displayed two open states even when the PIP2 levels were down regulated by Wortmannin treatment so that the channel displayed extremely low open probabilities, and b) our finding that it required a minimum of two wild-type subunits in the Kir2.1 tandem construct in order to record measurable channel activity. This apparent contradiction can be reconciled if the first two PIP2 molecules bind the channel cooperatively (i.e., binding of the first PIP2 eases the binding of the second PIP2), and the first PIP2 would quickly dissociate without the binding of the second PIP2) and the open probability is low when the channel binds only one PIP2 molecule. In this scenario, the channel activity could be too low to be detected when the PIP2 level is so low that simultaneous binding of two PIP2 molecules is not possible. Therefore a channel will display detectable activity only when PIP2 levels allow simultaneous binding of at least two PIP2 molecules. This scenario may account for the observation that at least two open states were observed for Kir3.4*-S176P. The channel activity could also be too low to detect if a channel is incapable of binding a second PIP2 molecule, such as in the case of QQQR, the construct containing only one wild-type subunit. This would be due to the combination of a short life time of the complex between the channel and only one PIP2 bound, together with the low open probability of the channel when the channel is bound to only one PIP2 molecule.

The proposed kinetic model naturally accounts for our observations of the dependence of number of open states on PIP2 levels that were obtained in the recordings of Kir3.4*-S176P under various recording conditions. Although a channel has the capacity of binding up to four PIP2 molecules, the actual number of PIP2 molecules being associated with the channel may constantly fluctuate due to the stochastic
process of PIP$_2$ molecules binding and unbinding the channel. We are reasoning that the maximum number of PIP$_2$ molecules associated with a channel during an actual recording depends on the PIP$_2$ levels under a given recording condition. Therefore, although a complete kinetic model contains four open states, the actual number of open states in a given recording depends on the maximum number of PIP$_2$ molecules associated with the channel during the recording. Thus, different recordings may display different number of open states depending on the PIP$_2$ levels achieved under the specific experimental condition. We propose that a Kir3 channel can associate with a maximum number of four PIP$_2$ molecules only under extreme conditions such as during application of exogenous PIP$_2$, (Fig. 4), since only in these recordings we obtained four open states. Under cell-attached conditions, the maximum number of PIP$_2$ molecules that simultaneously associate with a channel varies between two and three depending on the endogenous PIP$_2$ levels (Fig. 2). Even when PIP$_2$ levels are down regulated, such as following Wortmannin treatment, it is still possible that a channel would simultaneously associate with two PIP$_2$ molecules (Fig. 5), since the recordings displayed two open states.

The proposed model also naturally accounts for the dependence of the Kir bursting behavior on PIP$_2$ levels. A burst of a Kir channel would start from the first opening of the channel upon the association of PIP$_2$ molecule(s) (the transition from C$_0$ to C$_i$, i = 1, 2, …, Fig. 9) and it would end by the last opening before the dissociation of the last PIP$_2$ molecule(s) (C$_i$ to C$_0$ transition, i = 1, 2, …). During a burst event, the channel may undergo many transitions between PIP$_2$ bound kinetic states as the number of the associated PIP$_2$ molecules fluctuate. Therefore, the higher the PIP$_2$ levels, the less often the number of the associated PIP$_2$ molecules fluctuates to zero, thus the longer the burst durations.

Our studies indicate that even under application of exogenous PIP$_2$, the channel can still display long closures. These closures are likely to reflect other PIP$_2$-independent closed state(s). We have therefore introduced an extra closed state C$_i$ in our kinetic model to represent the possible PIP$_2$-independent closed state(s). The connection between this state and other kinetic states would require more detailed studies to substantiate.

**MATERIALS AND METHODS**

**Expression of recombinant channels in Xenopus oocytes.** The active point mutants of the human Kir3.4 (GenBank accession number U39196), Kir3.4-S143T or Kir3.4$^*$ and Kir2.1 were subcloned in the pGEMHE plasmid vector$^{29,31}$ and used as previously described.$^{23,30}$ Point mutations were generated using the Quickchange site-directed mutagenesis kit (Stratagene). The sequence of all constructs was confirmed by automated DNA sequencing (Sequencing facility, Cornell, Ithaca). The cRNA concentration of Kir3.4$^*$ was estimated from two successive dilutions, which were electrophoresed in parallel on formaldehyde gels, compared to known concentrations of RNA marker (Gibco, NY) diluted to a stock concentration of 320 ng per microliter and used as an internal control. cRNA concentrations of all other constructs were equalized to the internal control by a series of dilutions to ensure that an equal amount of control and mutant cRNA was injected. Expression of channel proteins in oocytes was accomplished by injection of the desired amount of cRNA into *Xenopus* oocytes. In all single-channel experiments, oocytes were injected with 0.02 to 0.2 ng of channel RNA. Oocytes were isolated and microinjected as previously described.$^{31}$ All oocytes were maintained at 18°C and recordings were performed 1–3 days following injection.

**Single-channel recordings and analysis in Xenopus oocytes.** The vitelline membrane of oocytes was manually removed with forceps in the recording chamber without treatment with a hypotonic solution. Single-channel activity was recorded in the cell-attached or inside-out patch configurations,$^{32,33}$ using an Axopatch 200A amplifier (Axon Instruments, CA). The bath solution was always the same as the control solution including (in mM): KCl 96, EGTA 5, and HEPES 10, pH 7.40. The pipette solution contained 10 mM EDTA in addition to the control solution. 100 μM gadolinium was routinely included in the pipette solution to suppress native oocyte stretch-activated channels. Chemicals were purchased from Sigma (St. Louis, MO). PIP$_2$ (PI-4,5-P$_2$) was purchased from Biomol (BIOMOL International LP). Five μM working solution (dissolved in the control solution) was sonicated for 10 minutes prior to use in the experiments. All microelectrodes used in the experiments were pulled from WPI-K borosilicate glass and gave resistances between 1-20 MΩ. All experiments were performed at room temperature (20–22°C). All of the recordings were performed at a membrane potential of ~80 mV, unless otherwise indicated. Single-channel currents were filtered at 1-2 KHz with a 6-pole low-pass Bessel filter, sampled at 5–10 KHz and stored directly into the computer's hard disk through the DIGIDATA 1200 interface (Axon Instruments, Foster City, CA.). The single-channel data of Kir2.1 and the tandem tetrameric constructs were filtered at 200 Hz prior to idealization of the data. pCLAMP (version 8, Axon Instruments) software were used for data acquisition. Single-channel records were analyzed using pCLAMP8 software and some of our own programs to complement this software package. The probability of the existence of the second channel without displaying overlapped openings was calculated$^{34}$ for recordings used to fit the closed-time histograms. Baseline drifts were carefully removed before idealization. Distributions of open and closed-time (exponential functions), the burst durations (exponential functions), number of openings per burst (geometric functions) and total length of open time per burst (exponential functions) were fit using computer programs developed in our lab, which maximized the logarithm of the likelihoods based on the formulas of Colquhoun and Sigworth.$^{34}$ The number of components required for each fitting was the minimal number of components such that an improvement of the fitting results using an additional component was not statistically significant (at 0.1% significance level in our fittings).$^{34}$ The mean burst duration for each recording was calculated as an arithmetic mean of the burst durations of the same record. The burst delimiters $t_r$ were determined according to the distributions of the closed-time of each recording by numerically solving the equation:

$$\exp(-t_r/\tau_{c2}) + \exp(-t_r/\tau_{c3}) = 1$$

where $\tau_{c2}$ and $\tau_{c3}$ are the second and third shortest closed-time components, respectively (example in Table 1). The fitting results of the open and closed-time distributions were presented as the time constants and the frequency of each component. The i-th open (closed) time component, $F_{oi}$ ($F_{ci}$), is the predicted value of the frequency of the observation of the open (closed) events of the
components whose lifetime is within the dwell time fitting range. \( F_i \) (\( F_{oi} \) and \( F_i \)) are calculated by the following equations:

\[
F_i = \frac{N * W_i}{L} \sum_{j=1}^{n} \left( e^{-\frac{T_j}{\tau_j}} - e^{-\frac{T_i}{\tau_i}} \right)
\]

where, \( T_j \) and \( T_i \) stand for the short and long limit of the dwell-time fitting range, respectively. \( N \) stands for the total number of events within the fitting range; \( L \) stands for the total recording time; \( \tau_i \) and \( W_i \) stand for the time constant and the weight of the i-th open-(closed-) time components, respectively.

**Two-electrode voltage-clamp recording and analysis.** Whole-cell currents were measured by conventional two-microelectrode voltage clamp with a GeneClamp 500 amplifier (Axon Instruments, Union City, CA), as previously reported. A high-potassium (HK) solution was used to superfuse oocytes (in mM: 96 KCl, 1 NaCl, 1 MgCl₂, 3 KOH/HEPES [pH 7.4]). Basal currents represent the difference was used to superfuse oocytes (in mM: 96 KCl, 1 NaCl, 1 MgCl₂, 3 KOH/HEPES [pH 7.4]). Basal currents represent the difference from those in the absence of Ba²⁺. Recordings from different batches of oocytes were normalized by the mean of whole-cell basal currents from oocytes expressing the control channel Kir2.1. Statistics (i.e., mean and standard error of the mean) of each construct were calculated from all of the normalized data recorded from different batches of oocytes.

**Tandem constructs of Kir2.1 and Kir2.1(R218Q).** We first made the dimeric constructs each with two Kir2.1 cassettes covalently joined together by a -Q10GS-linker. An XhoI site was conveniently located after the stop codon of each dimer. The dimeric constructs were carried in pGEMHE and were then modified to create two ND and CD precursors, ND and CD, for assembling the tetramers. To make the ND precursor construct, the start codon of the dimeric construct was replaced by a Q8 linker followed by EcoR5 and XhoI sites. To make the CD precursor construct, the stop codon of the dimeric construct was replaced by a Q8 linker followed by EcoR5 and XhoI sites. The appropriate ND and CD harboring R218 and R218Q in the ND and CD constructs were joined by a -Q8DI-linker. All mutations and insertions were confirmed by sequencing.

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**References**

1. Hilgemann DW, Ball R. Regulation of cardiac Na⁺,Ca²⁺ exchange and K_ATP potassium channels by PIP₂, Science 1996; 273:956-9.
2. Fan Z, Makielki JC. Anionic phospholipids activate ATP-sensitive potassium channels. J Biol Chem 1997; 272:5388-95.
3. Hilgemann DW, Feng S, Nausbogh C. The complex and intriguing lives of PIP₂ with ion channels and transporters. Sci.StKE 2001:R19.
4. Suh BC, Hille B. Regulation of ion channels by phosphatidylinositol 4,5-bisphosphate. Current Opinion in Neurobiology 2005; 15:370-8.
5. Gamper N, Shapiro MS. Regulation of ion transport proteins by membrane phosphoinositides. Nat Rev Neurosci 2007; 8:921-34.
6. Logothetis DE, Nilus B. Dynamic changes in phosphoinositide levels control ion channel activity. Pflugers Arch 2007; 455:1-3.
7. Huang CL, Feng S, Hilgemann DW. Direct activation of inward rectifier potassium channels by PIP₂, and its stabilization by Gβγ, Nature 1998; 391:803-6.
8. Sui JL, Pettit-Jacques J, Logothetis DE. Activation of the atrial KACH channel by the γ subunits of G proteins or intracellular Na⁺ ions depends on the presence of phosphatidylinositol bisphosphates. Proc Natl Acad Sci USA 1998; 95:11787-92.
9. De X, Zhang H, Lopes C, Mirshahi T, Rohacs T, Logothetis DE. Characteristic interactions with phosphatidylinositol 4,5-bisphosphate determine regulation of Kir channels by diverse modulators. J Biol Chem 2004; 279:37271-81.
10. Rohacs T, Lopes CM, Jin T, Ramdya PP, Molnar Z, Logothetis DE. Specificity of activation by phosphoinositides determines lipid regulation of Kir channels. Proc Natl Acad Sci USA 2003; 100:745-750.
11. Logothetis DE, Jin T, Lupyyn D, Rosenhouse-Dantsker A. Phosphoinositide-mediated gating of inwardly rectifying K⁺ channels. Pflugers Arch 2007; 455:83-96.
12. Logothetis DE, Lupyn D, Rosenhouse-Dantsker A. Diverse Kir modulators act in close proximity to residues implicated in phosphoinositide binding. J Physiol 2007; 582:953-65.
13. McK D, Helbitz S. G-protein mediated gating of inward-rectifier K⁺ channels. Eur.J Biochem 2000; 267:5830-6.
14. Rohacs T, Lopes C, Mirshahi T, Jin T, Zhang H, Logothetis DE. Assaying phosphatidylinositol bisphosphate regulation of potassium channels. Methods Enzymol 2002; 345:71-92.
15. Ruppersberg JP. Intracellular regulation of inward rectifier K⁺ channels. Pflugers Arch 2000; 441:1-11.
16. Stanfield PR, Nakajima S, Nakajima Y. Constitutively active and G-protein coupled inward rectifier K⁺ channels: Kir2.0 and Kir3.0. Rev Physiol Biochem Pharmacol 2002; 145:47-179.
17. Sui JL, Chan K, Langan MN, Vivaudou M, Logothetis DE. G protein gated potassium channels. Adv Second Messenger Phosphoprotein Res 1999; 33:179-201.
18. Takanou M, Karotum S. Regulation of cardiac inwardly rectifying potassium channels by membrane lipid metabolism. Prog Biophys Mol Biol 2007; 94:320-35.
19. Xie LH, John SA, Ribaher B, Weiss JN. Activation of inwardly rectifying potassium (Kir) channels by phosphatidylinositol-4,5-bisphosphate (PIP2): Interaction with other regulatory ligands. Prog Biophys Mol Biol 2007; 94:320-35.
20. Logothetis DE, Kurachi Y, Galper J, Neer EJ, Clapham DE. The βγ subunits of GTP-binding proteins activate the muscarinic K⁺ channel in heart. Nature 1987; 325:321-6.
21. Sui JL, Chan KW, Logothetis DE. Na⁺ activation of the muscarinic K⁺ channel by a G-protein-independent mechanism. J Gen Physiol 1996; 108:381-91.
22. Sui JL, Petit-Jacques J, Logothetis DE. Activation of the atrial K⁺,Ca⁺ channel by the βγ subunits of G proteins or intracellular Na⁺ ions depends on the presence of phosphatidylinositol bisphosphates. Proc Natl Acad Sci USA 1998; 95:3367-72.
23. Vivaudou M, Chan KW, Sui JL, Jan L, Reuvény E, Logothetis DE. Probing the G-protein regulation of GIRK1 and GIRK4, the two subunits of the KACH channel, using functional homomeric mutants. J Biol Chem 1997; 272:1553-60.
24. Jin T, Feng L, Mirshahi T, Rohacs T, Chan KW, Sanchez R, Logothetis DE. The βγ subunits of G proteins gate a K⁺ channel by pivoted bending of a transmembrane segment. Mol Cell 2002; 10:469-81.
25. Sadja R, Smadja K, Alagori N, Reuvény E. Coupling Gβγ-dependent activation to channel opening via pore elements in inwardly rectifying potassium channels. Neuron 2001; 29:660-80.
26. Yakubovic D, Pustuhoven B, Vitler A, Dessauer CW, Dascal N. Slow modul gating of single G proteinactivated K⁺ channels expressed in Xenopus oocytes. J Physiol 2000; 524:735-55.
27. Lopes CM, Zhang H, Rohacs T, Jin T, Yang J, Logothetis DE. Alterations in conserved Kir channel-PIP₂ interactions underlie cholangiopathies. Neuros 2002; 34:933-44.
28. Zhang H, He C, Yan X, Mirshahi T, Logothetis DE. Activation of inwardly rectifying K⁺ channels by distinct PtdIns(4,5)P₂, interactions. Nature Cell Biology 1999; 1:118-3.
29. Liman ER, Tyrer J, Hess B. Subunit stoichiometry of a mammalian K⁺ channel determined by construction of multimeric cDNAs. Neuron 1992; 9:861-71.
30. Chan KW, Langan MN, Sui JL, Kezak PA, Bahan H, Jadus JA, Logothetis DE. A recombinant inwardly rectifying potassium channel coupled to GTP-binding proteins. J Physiol 1996; 507:381-97.
31. Logothetis DE, Movahedi S, Sarler C, Lindpaintner K, Nadal-Ginard B. Incremental reductions of positive charge within the S4 region of a voltage-gated K⁺ channel result in corresponding decreases in gating charge. Neuro 1992; 8:531-40.
32. Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflugers Arch 1981:391-850.
33. Methfessel C, Witzemann V, Takahashi T, Mishina M, Numa S, Sakmann B. Patch clamp measurements on *Xenopus laevis* oocytes: currents through endogenous channels and implanted acetylcholine receptor and sodium channels. Pfliigers Arch 1986; 407:577-88.
34. Coleqhoun D, Sigworth F. Fitting and Statistical Analysis of Single-Channel Records. In Single-Channel Recording, B.A.N.E. Sakmann, ed. New York and London: Plenum Press, 1995; 483-585.
35. Coleqhoun D, Sakmann B. Fast events in single-channel currents activated by acetylcholine and its analogues at the frog muscle end-plate. J Physiol 1985; 369:501-57.
36. Cho H, Palmer LG, Sackin H. Structural determinants of gating in inward-rectifier K+ channels. Biophys J 1999; 76:1988-2003.
37. Isacoff EY, Jan YN, Jan LY. Evidence for the formation of heteromultimeric potassium channels in Xenopus oocytes. Nature 1990; 345:530-4.
38. Ogilvies EM, Zagotta WN, Hoshi T, Heinemann SH, Haab J, Aldrich RW. Cooperative subunit interactions in C-type inactivation of K channels. Biophys J 1995; 69:2449-57.
39. Sajda R, Alagem N, Reuveny E. Graded contribution of the Gβγ binding domains to GIRK channel activation. Proc Natl Acad Sci USA 1992; 99: 10783-8.
40. Schuehler R, Hohl S, Terlau H, Baumann A, Heinemann SH. Individual subunits contribute independently to slow gating of bovine EAG potassium channels. J Biol Chem 1999; 274:5362-9.
41. Shapiro MS, Zagotta WN. Stoichiometry and arrangement of heteromeric olfactory cyclic nucleotide-gated ion channels. Proc Natl Acad Sci USA 1988; 85:14546-51.
42. Yang J, Jan YN, Jan LY. Determination of the subunit stoichiometry of an inwardly rectifying potassium channel. Neuron 1995; 15:1441-7.
43. Zheng J, Sigworth FJ. Intermediate conductances during deactivation of heteromultimeric Shaker potassium channels. J Gen. Physiol 1998; 112:457-74.
44. McCormack K, Lin L, Iverson LE, Tanouye MA, Sigworth FJ. Tandem linkage of Shaker K+ channel subunits does not ensure the stoichiometry of expressed channels. Biophys J 1992; 63:1406-11.
45. Groot-Kormelink PJ, Broadbent SD, Boorman JP, Sivilotti LG. Incomplete incorporation of tandem subunits in recombinant neuronal nicotinic receptors. J Gen Physiol 2004; 123:697-708.
46. Liman ER, Tytgat J, Hess P. Subunit stoichiometry of a mammalian K+ channel determined by construction of multimeric cDNAs. Neuron 1992; 9:861-71.
47. Kobrinsky E, Mirshahi T, Zhang H, Jin T, Logothetis DE. Receptor-mediated hydrolysis of plasma membrane messenger PIP2 leads to K+ current desensitization. Nature Cell Biology 2000; 2:507-14.
48. Keselman I, Friebourg D, Felsenfeld DP, Logothetis DE. Mechanism of PLC-mediated Kir3 current inhibition. Channels 2007; 1:113-23.
49. Bichet D, Haas FA, Jan LY. Merging functional studies with structures of inward-rectifier K+ channels. Nat Rev Neurosci 2003; 4:957-67.
50. Nishida M, Cadene M, Chait BT, MacKinnon R. Crystal structure of a Kir3.1-prokaryotic Kir channel chimera. EMBO J 2007; 26:4005-15.
51. Pegan S, Arrabé C, Zhou W, Kosiakowski W, Collins A, Slesinger PA, Choe S. Cytoplasmic domain structures of Kir2.1 and Kir3.1 show sites for modulating gating and rectification. Nat Neurosci 2005; 8:279-87.
52. Rapedius M, Fowler PW, Shang L, Sansom MS, Tacker SJ, Baukrowitz TH. Bonding at the helix-bundle crossing controls gating in Kir potassium channels. Neuron 2007; 55:602-14.