Distinct Specificities of Inwardly Rectifying K⁺ Channels for Phosphoinositides*

(Received for publication, August 16, 1999, and in revised form, October 5, 1999)

Tibor Rohács, Jian Chen‡, Glenn D. Prestwich‡, and Diomedes E. Logothetis§

From the Department of Physiology and Biophysics, Mount Sinai School of Medicine of the New York University, New York, New York 10029 and the §Department of Medicinal Chemistry, University of Utah, Salt Lake City, Utah 84112

Activation of several inwardly rectifying K⁺ channels (Kir) requires the presence of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂). The constitutively active Kir2.1 (IRK1) channels interact with PtdIns(4,5)P₂ strongly, whereas the G-protein activated Kir3.1/3.4 channels (GIRK1/GIRK4), show only weak interactions with PtdIns(4,5)P₂. We investigated whether these inwardly rectifying K⁺ channels displayed distinct specificities for different phosphoinositides. IRK1, but not GIRK1/GIRK4 channels, showed a remarkable dependence on the phospholipid acyl residues. GIRK1/GIRK4 channels were activated with a similar efficacy by PtdIns(3,4)P₂, PtdIns(3,5)P₂, PtdIns(4,5)P₂, and PtdIns(3,4,5)P₃. In contrast, IRK1 channels were not activated by PtdIns(3,4)P₂ and only marginally by high concentrations of PtdIns(3,5)P₂. Similarly, high concentrations of PtdIns(3,4,5)P₃ were required to activate IRK1 channels. For either channel, PtdIns(4)P was much less effective than PtdIns(4,5)P₂, whereas PtdIns was inactive. In contrast to the dependence on the position of phosphates of the phospholipid head group, GIRK1/GIRK4, but not IRK1 channel activation, showed a remarkable dependence on the phospholipid acyl chains. GIRK1/GIRK4 channels were activated most effectively by the natural arachidonyl stearyl PtdIns(4,5)P₂, and much less by the synthetic dipalmitoyl analog, whereas IRK1 channels were activated equally by dipalmitoyl and arachidonyl stearyl PtdIns(4,5)P₂. Incorporation of PtdInsP₄ into the membrane is necessary for activation, as the short chain water soluble diC₈ PtdIns(4,5)P₂ did not activate either channel, whereas activation by diC₈ PtdIns(4,5)P₂ required high concentrations.

Inwardly rectifying potassium channels play an important role in regulating membrane excitability. Most of the inwardly rectifying K⁺ (Kir) channels have been cloned and classified into subfamilies Kir 1–6 (1, 2). The Kir 2.0 subfamily consists of constitutively active, strongly inwardly rectifying K⁺ channels. The first member to be cloned (3) was Kir 2.1 (IRK1), which is expressed in brain, heart muscle, and skeletal muscle (1). The Kir 3.0 subfamily consists of the G protein-activated inwardly rectifying K⁺ channels (GIRKs) (4, 5). The prototype of this channel subfamily is the atrial acetylcholine-activated K⁺ channel, K₁₅₅ᵥ, which mediates the effect of the vagus nerve on cardiac pacemaker cells. K₁₅₅ᵥ is a heterotetramer comprising two subunits, GIRK1 and GIRK4 (6). It was demonstrated that the mechanism of activation is via direct interaction of the channel with G protein βγ subunits (7–9). These channels are also activated by intracellular Na⁺ (10) and Mg²⁺ ions (11).

Phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂), although a quantitatively minor membrane component, is a key signaling phospholipid. Its hydrolysis by phospholipase C and further phosphorylation by phosphatidylinositol 3-kinases generates important second messengers. In addition to serving as a precursor for second messengers, PtdIns(4,5)P₂ itself plays an important role in such processes as the organization of the cytoskeleton and vesicular transport (12, 13).

Recently, PtdIns(4,5)P₂ has been shown to directly regulate various ion channels and transporters, such as the Na⁺/Ca²⁺ exchanger (14), the InsP₃ receptor Ca²⁺ channel (15), an Na⁺-activated nonsensitive cation channel (16), and several inwardly rectifying K⁺ channels including Kir1.1 (ROMK1) (17, 18), Kir1 (17, 19), GIRK1/GIRK4 (17, 20), and ATP-sensitive K⁺ channels (14, 17, 21–23). IRK1 channels are constitutively active, with their activity depending only on the presence of PtdIns(4,5)P₂ in the membrane (19). ROMK1 channels are regulated by protein kinase A through phosphorylation. The role of phosphorylation may be to enhance the interaction of these channels with PtdIns(4,5)P₂ (18). GIRK1/GIRK4 channels are regulated by Gβγ and intracellular Na⁺ ions. It has been proposed that both regulatory molecules exert their effects by strengthening the interaction of the channel with PtdIns(4,5)P₂ (17, 19, 24, 25). It seems, from these data, that PtdIns(4,5)P₂ may serve as a common final regulator of several inwardly rectifying K⁺ channels. Furthermore, dynamic changes in PtdIns(4,5)P₂ in the membrane can also contribute to the regulation of ion channels. Upon activation of phospholipase C by receptor stimulation in vivo, PtdIns(4,5)P₂ concentration in the membrane decreases (26–28), and this decrease leads to inhibition of GIRK1/GIRK4 channels (29). PtdIns(4,5)P₂ may therefore also serve as a control point for cross-talk between different signaling pathways.

In the present study, we have investigated the structural elements in PtdIns(4,5)P₂ responsible for the interaction with inwardly rectifying K⁺ channels. For this purpose, we examined the effects of different phosphoinositides on recombinant PtdIns(3,4)P₂, phosphatidylinositol 3,4-bisphosphate; PtdIns(3,5)P₂, phosphatidylinositol 3,5-bisphosphate; PtdIns(3,4,5)P₃, phosphatidylinositol 3,4,5-trisphosphate; InsP₃, inositol 1,4,5-trisphosphate; diP, dipalmitoyl; AAS, arachidonyl stearoyl; PH, pleckstrin homology; NPₒ, open probability of N channels; MTₒ, mean open time; NFMₒ, mean frequency of opening of N channels.
channels expressed in Xenopus oocytes. We have chosen GIRK1/GIRK4 and IRK1 because they show different affinities toward PtdIns(4,5)P2; GIRK1/GIRK4 interacts with PtdIns(4,5)P2 weakly, whereas the interaction of IRK1 with PtdIns(4,5)P2 is strong (17, 19). We found that, in addition to the different affinities for PtdIns(4,5)P2, the two channels show remarkable differences in specificity toward various phosphoinositides. Some of these results have appeared in a preliminary form as an abstract (30).

EXPERIMENTAL PROCEDURES

Materials—Dipalmitoyl (diP) PtdIns(4,5)P2, PtdIns(3,4)P2, PtdIns(3,5)P2, and PtdIns(3,4,5)P3 were synthesized as described previously (31, 32), some batches of diP PtdIns(4,5)P2 were purchased from Echelon Research Laboratories Inc. (Salt Lake City, UT), Matreya Inc. (Pleasant Gap, PA), and Calbiochem. Short chain PtdIns(4,5)P2-s (diC4 and diC8) were purchased from Echelon. PtdIns(4,5)P2 and PtdIns(4)P, purified from bovine brain, containing mostly the arachidonyl and stearyl (AASt) acyl chains, were purchased from Roche Molecular Biochemicals and from Calbiochem. PtdIns (purified from bovine liver) was purchased from Sigma. All synthetic analogs (from Echelon or synthesized in the laboratory of G. D. P.) were analyzed by 1H and 31P NMR and found to be homogenous in isomer composition to a detection limit of 2.5% and free from particulate material. The phosphate composition of these samples was established to be 75–95% of the theoretical amount by colorimetric analysis of ashed samples. Commercial PtdIns(4)P and PtdIns(4,5)P2, AAst lipids show phosphate content in the 80–95% range using the same methodology. All of the long acyl chain lipids (except PtdIns) were dispersed in water (0.5 mM) by sonication for 30 min on ice and then aliquoted and kept at 2 °C. Before experiments, a new aliquot was thawed, which was used only on that day. The lipid was diluted in the bath solution (see below under “Electrophysiology”) and sonicated for 10–30 min. This procedure results in the formation of mostly small micelles of PtdInsP2 (33). Application of these lipids to the patch activates the channels, probably by fusion of these micelles to the patch membrane. The short acyl chain, watersoluble lipids (diC4 and diC8) were dissolved in water, aliquoted, and kept at 2 °C. We also sonicated these analogs in a similar manner to the longer acyl chain forms. PtdIns was dissolved in chloroform and kept at 2 °C. Before experiments, an aliquot was taken, chloroform was evaporated under N2, and PtdIns was then dispersed in bath solution by sonicating for 30 min.

Expression of Recombinant Channels in Xenopus Oocytes—GIRK1, GIRK4 and IRK1 were subcloned in the pGMHE plasmid vector. GIRK1/GIRK4 interacts with PtdIns(4,5)P2 weakly, whereas the interaction of IRK1 with PtdIns(4,5)P2 is strong (17, 19). We found that, in addition to the different affinities for PtdIns(4,5)P2, the two channels show remarkable differences in specificity toward various phosphoinositides. Some of these results have appeared in a preliminary form as an abstract (30).

FIG. 1. Effect of the number of phosphates in the head group on the efficiency of phosphoinositides on GIRK1/GIRK4 channel activity. GIRK1/GIRK4 channels were expressed in Xenopus oocytes, measurements were performed on inside-out patches. A, representative experiments showing the effects of 2 mM free Mg2+ and 20 mM Na+, 5 mM ATP, and PtdIns(4,5)P2 in the indicated concentrations on the activity parameters of GIRK1/GIRK4 channels. The top panel shows the NPo; the middle panel, the MT50; and the bottom panel, the NPo. B, traces a–f are unitary currents from the points indicated by the arrows on the top panel of A. C, statistical summary of the data obtained with PtdIns(4,5)P2, PtdIns(4)P, PtdIns(3,4)P2, and PtdIns. The average NPo induced by Mg2+/Na+ after 3 min of lipid treatment was divided by the Mg2+/Na+-induced NPo prior to the addition of the lipid. Mean ± S.E. is shown; n = 3–13 for the different phosphoinositides, and n = 33 for ATP. Asterisks indicate a significant difference between the Mg2+/Na+-induced current in the absence (Mg/Na) and the presence (as indicated) of the lipid (*, p < 0.05; ***, p < 0.001).

All of the phosphoinositides were of the arachidonyl stearyl acyl chain composition.

Effects of Phosphoinositides on K+ Channels
Electrophysiology—Single-channel recordings on GIRK1/GIRK4 channels were performed in the inside-out patch configuration using an EPC-7 patch clamp amplifier. All microelectrodes were pulled from WPI-K borosilicate glass using a Sutter P-97 microelectrode puller and gave 2–6 megohms of resistance. The vitelline membrane of the X. laevis oocytes was removed before seal formation using fine forceps. The pipette solution contained 96 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 1 mM NaCl, and 10 mM HEPES (pH 7.35). The bath solution contained 96 mM KCl, 5 mM EGTA, and 10 mM HEPES (pH 7.35). Gadolinium at 6 mM concentration was added to the pipette solution to inhibit stretch-activated channels in the oocyte membrane. Free Mg2+ concentration was calculated as described previously (35). Single-channel recordings were performed at ~80 mV holding potential. Single-channel currents were filtered at 1 kHz and collected at 5–10 kHz for GIRK1/GIRK4 and 0.5–1 kHz for IRK1 channels. Data were stored directly on the hard drive of the computer through the Digidata 1200 interface (Axon Instruments, Foster City, CA). pCLAMP 6.01 (Axon Instruments) software was used for data acquisition and analysis. For calculation of \( N_p \) and \( N_f \), and \( M_T \) in patches with a few unitary events, we complemented pCLAMP with our analysis program as described previously (10) (see also Axon Instruments’ Web page). Results are displayed as average \( N_p \) over 5-s bins.

For macropatch recordings (36), low resistance pipettes (15–25-μm tip diameter) were used. Sampling rate was usually 30 Hz. Pipette and bath solutions were the same as in the single-channel measurements, including gadolinium in the pipette solution. The omission of gadolinium in macropatch measurements did not significantly affect the data. Recordings were performed at ~80 mV holding potential. For quantitative analysis, the currents elicited by the phosphoinositides were fit by a sigmoidal (Boltzmann) function using the curve-fitting routine of Microcal Origin. Some of these fits are shown on Figs. 5, B, and C, and 6B. All experiments were performed at room temperature (20–22 °C).

**RESULTS**

The Effects of Different Phosphoinositides on GIRK1/GIRK4 Channels—We examined first the effects of different PtdInsP2 analogs on GIRK1/GIRK4 channels expressed in Xenopus oocytes. These channels are not significantly activated by 2.5 μM PtdIns(4,5)P2 alone (20); however, the presence of PtdIns(4,5)P2 is required for other gating molecules such as Gβγ, Na+, and Mg2+ to activate the channel (11, 19). To test the interaction of the channel with PtdInsP2, we chose Na+ and Mg2+ rather than Gβγ to gate the channel because the effect of the ions is faster than that of Gβγ and is easily reversible.
Effects of Phosphoinositides on K⁺ Channels

Fig. 3. Effects of different phosphate substitutions in the inositol head group on GIRK1/GIRK4 channel activity. Experiments were performed as described for Figs. 1 and 2. All phosphoinositides were synthetic, with dipalmitoyl side chains. A, representative curves for 2.5 μM PtdIns(3,5)P₂, PtdIns(3,4)P₂, and PtdIns(3,4,5)P₃. B, summary of the data; mean ± S.E. is shown, n = 3–7 for each group. Asterisks indicate a significant difference between the Mg²⁺/Na⁺-induced current in the presence and the absence of the lipid (*, p < 0.05; ***, p < 0.01). The difference among the effects of the different phospholipids was not statistically significant with one-way analysis of variance on the p < 0.05 level.

The top panel of Fig. 1A shows the open probability (NPₒ) of channels in a typical experiment. The middle and bottom panels of Fig. 1A show the mean open time (MTO) and mean frequency of opening (MNPₒ) analysis, respectively, of the same experiment. Fig. 1B shows representative current traces from the same patch that is shown in Fig. 1A. After excision of the patch, channel activity ran down quickly, yielding very low levels of activity (trace a). This current rundown is thought to be attributable to the basal breakdown of PtdIns(4,5)P₂ in the membrane by lipid phosphatases and phospholipases, as well as the removal of cytosolic factors (Na⁺, Mg²⁺, GTP) needed for activity of the channel. The average NPₒ, after channel rundown in the absence of Mg²⁺ and Na⁺ was 0.0106 ± 0.0002 (n = 40). Application of 2 mM free Mg²⁺, 5 mM ATP, and 20 mM Na⁺ induced high channel activity (trace b), because in the presence of ATP, lipid kinases can resynthesize PtdIns(4,5)P₂ in the membrane, which together with Na⁺ and Mg²⁺ activate the channel. After removal of ATP, channel activity ran down despite the continued presence of the two gating ions. The average NPₒ, after channel rundown, in the presence of Mg²⁺ and Na⁺ was 0.0915 ± 0.0170 (n = 40). Application of PtdIns(4,5)P₂ alone thereafter did not significantly activate the channels. Reintroduction of Na⁺ and Mg²⁺ ions following patch exposure to 2.5 μM PtdIns(4,5)P₂ induced channel activity comparable with that induced by ATP, Mg²⁺, and Na⁺. PtdIns(4,5)P₂ and Mg²⁺ ATP also increased the mean open time of the channels (Fig. 1A, middle panel), consistent with our previous report (20). This increase, however, was small and contributed only slightly to the increase in NPₒ. ATP and 2.5 μM AASt PtdIns(4,5)P₂ increased MTo from 2.11 ± 0.08 to 4.49 ± 0.22 ms (n = 27, p < 0.005) and from 1.87 ± 0.11 to 4.39 ± 0.31 ms (n = 13, p < 0.005), respectively.

In experiments similar to the one shown in Fig. 1A, we examined the effect of reducing the number of phosphate groups in the head group of PtdIns(4,5)P₂ on channel activity. Fig. 1C summarizes the data comparing PtdIns(4,5)P₂, PtdIns(4)P, and PtdIns. PtdIns at 2.5 μM was inactive (Fig. 1C) and remained ineffective even at 25 μM (n = 2, data not shown).

We next studied how the acyl chain composition influences the efficiency of these lipids to activate GIRK1/GIRK4 channel current. Phosphoinositides in mammalian cells usually contain the arachidonoyl side chain in position 2 and the stearoyl side chain in position 3 (AASt) of the glycerol backbone (Fig. 2D). In studying phospholipid signaling, synthetic analogs of these lipids are frequently used, which usually contain palmitoyl chains in both positions (diP), as the arachidonoyl forms are difficult to handle during synthesis. Much to our surprise, we
found a marked difference in efficacy between the synthetic versus the natural forms. The natural AASt PtdIns(4,5)P₂ at 2.5 μM was about four times more active than the synthetic diP PtdIns(4,5)P₂, even when the latter was applied at 25 μM (Fig. 2A). DiP PtdIns(4,5)P₂ also increased MTₐ less effectively than the AASt form. MTₐ was increased from 1.98 ± 0.18 to 3.16 ± 0.47 ms (n = 7, p < 0.02) by 2.5 diP PtdIns(4,5)P₂ and from 1.74 ± 0.13 to 3.02 ± 0.44 ms (n = 4, p < 0.05) by 25 μM diP PtdIns(4,5)P₂.

The difference between diP and AASt PtdIns(4,5)P₂ was even more marked when the experiments were performed on macropatches. Macropatch recordings of GIRK1/GIRK4 channels were relatively difficult to obtain because of the moderate expression of these channels. Thus, most experiments involving GIRK1/GIRK4 channels were performed on smaller patches. Fig. 2C shows a representative macropatch measurement. After establishing the inside-out configuration, channels were allowed to run down, judging by application of Na⁺ and Mg²⁺ inducing only small currents. When the patch was treated with 2.5 μM diP PtdIns(4,5)P₂, the Mg²⁺/Na⁺-induced current became gradually higher; it was 4.90 ± 2.07 times higher 10 min after the application of the lipid than before (n = 4, p < 0.05). Subsequent application of 2.5 μM AASt PtdIns(4,5)P₂ substantially increased the Mg²⁺/Na⁺-induced current. Treatment with AASt PtdIns(4,5)P₂ for 10 min rendered the Mg²⁺/Na⁺-induced current 100.9 ± 31.8 times higher than before the application of the lipids (n = 9, p < 0.01). Similar to our results with PtdIns(4,5)P₂, the higher efficacy of AASt compared with diP PtdIns(3, 4, 5)P₃ on PDK1 protein kinase has been described (37).

We also tested the effects of two different short chain analogs, diC₈ and diC₄ PtdIns(4,5)P₂, on small patches in the presence of Mg²⁺ and Na⁺. DiC₈ PtdIns(4,5)P₂ activated GIRK1/GIRK4 channels when applied at 25 μM concentration (Fig. 2A, lower panel and 2B, right panel). This activation was immediate and quickly reversible, in contrast to the effects of the longer chain forms. DiC₄ PtdIns(4,5)P₂ at 2.5 μM was marginally active (Fig. 2B). The shortest side chain, diC₄ PtdIns(4,5)P₂, was inefficient at both 2.5 and 25 μM (data not shown).

We next tested the effect of the position of the phosphate group in the inositol ring on channel activation (Fig. 3). We examined phosphoinositides containing phosphates in the following positions: 4, 5; 3, 5; 3; and 3, 4, 5. These head group combinations cover all of the naturally occurring PtdInsP₂ and PtdInsP₃. These experiments were carried out with the synthetic diP acyl chain forms of these lipids, as the purified (AASt) forms are not available. Representative experiments are shown in Fig. 3A, and the data are summarized in Fig. 3B. Even though the 4, 5 and 3, 4, 5 analogs appeared to be slightly more active, all of these forms were able to activate the channel. These analogs also increased MTₐ to a similar extent as diP PtdIns(4,5)P₂ (data not shown). We also examined the effect of diP PtdIns(3,5)P₂ in macropatches, using a protocol similar to that shown on Fig. 2C. Following a 10-min treatment with 2.5 μM PtdIns(3,5)P₂, the Mg²⁺/Na⁺-induced current was 3.87 ± 1.29 times higher than before lipid treatment (n = 4, p < 0.05). This response was similar to that induced by diP PtdIns(4,5)P₂ (see Fig. 2C). Thus, in both small patches and macropatches, activation of GIRK currents by phosphoinositides did not show a dependence on the position of the phosphate group in the inositol ring.

The Effects of Different Phosphoinositides on IRK1 Channel Activity—Because of the high levels of expression of IRK1,
macroscopic currents could easily be obtained from inside-out macropatches. IRK1 channels interact with PtdIns(4,5)P₂ strongly, and as a result, the lipid alone activates the channels without the need for accessory molecules (19). Because macropatch recordings of IRK1 offer the advantage of allowing assessment of the macroscopic kinetics of channel activation by the lipids tested, they were used for most experiments.

First, we examined the effect of the acyl chain using the same analogs as for GIRK1/GIRK4. Fig. 4, A and B, show that diP and AASṭ PtdIns(4,5)P₂ activated the channels with very similar kinetics and to similar extents. The summary of the data is shown in Fig. 4D. Fig. 4C shows the effect of the short chain analog diC₈ PtdIns(4,5)P₂. IRK1 was activated partially by 2.5 μM diC₈ PtdIns(4,5)P₂, whereas 25 μM diC₈ was required for activation similar to that elicited by the longer side chain forms at 2.5 μM. The kinetics of activation were markedly faster than those of the diP or AASṭ forms (Fig. 4D). Also, the effect rapidly reversed upon wash-out of this lipid (Fig. 4C), unlike with the diP and AASṭ analogs. The shortest acyl chain form, diC₄ PtdIns(4,5)P₂, was completely ineffective at 25 μM concentration (not shown).

We next examined whether IRK1 shows stereospecificity toward the position of phosphates on the inositol head group. Fig. 5, A–E, shows representative measurements, and Fig. 5F shows the summary of the data. PtdIns(3,4)P₂ did not activate the channels at concentrations of 2.5 (not shown) and 25 μM (Fig. 5E). PtdIns(3,5)P₂ was inactive at 2.5 μM (Fig. 5A) and was partially active at 25 μM (Fig. 5B). PtdIns(3,4,5)P₃ was partially active at 2.5 μM (Fig. 5C) and almost fully active at 25 μM (Fig. 5D). As a positive control, we applied PtdIns(4,5)P₂ at the end of each experiment. The summary data are expressed as a percentage of the PtdIns(4,5)P₂-induced response (Fig. 5F).

We also examined the effect of the number of phosphates in the head group on IRK1 activation, by applying PtdIns(4)P and PtdIns. PtdIns(4)P was inactive at 2.5 μM (Fig. 6A) and partially active at 25 μM (Fig. 6B). The current induced by 25 μM PtdIns(4)P was 11.5 ± 5.8% of that induced by PtdIns(4,5)P₂ applied at the end of each experiment (n = 4). PtdIns was inactive at 25 μM (Fig. 6C).

Most measurements on GIRK channels were performed on small patches, whereas experiments with IRK1 were done on macropatches. To ensure that the marked differences between the phosphoinositide specificity of the two channels were not because of differences in assay conditions, we repeated some of the measurements on IRK1 with high resistance pipettes (Fig. 7), where clear unitary events could be detected (Fig. 7B). Fig. 7A shows Nₚ analysis of three different measurements. The upper panel shows that diP PtdIns(4,5)P₂ activates the channels, and subsequent application of AASṭ PtdIns(4,5)P₂ does not cause further activation. The middle and bottom panels show that neither PtdIns(3,4)P₂ nor PtdIns(3,5)P₂ activates the channels. The data are summarized on Fig. 7C. Thus, in both macropatches and high resistance patches, activation of IRK1 currents by phosphoinositides, unlike GIRK currents, showed a marked specificity for PtdIns(4,5)P₂ over other lipids, with the phosphate groups at different positions in the inositol ring. In further contrast with GIRK currents, IRK1 activity did not show a dependence on the acyl chain either in macropatches or in single channel measurements.

**Discussion**

The purpose of the present study was to identify the structural elements in PtdIns(4,5)P₂ that are important in activating ion channels. To achieve this goal, we examined the effects of different phosphoinositides on two inwardly rectifying K⁺ channels, IRK1 and GIRK1/GIRK4. We used a sensitive functional assay that provides the final biological response of the protein by virtue of its interaction with the lipid. We found marked differences between the two channels in the specificity of phosphoinositide effects with regard to the position of the phosphates in the inositol head group and the acyl chain composition.

Specific binding of proteins to phospholipids, especially to phosphoinositides, is an emerging new paradigm in signal transduction (38). Numerous protein domains are capable of binding to phosphoinositides, (39) the best studied of which are the PH domains (40). PH domains show remarkably diverse specificity to different phosphoinositides (41). Some PH domains, such as that of Akt/PKB, bind specifically to the products of phosphatidylinositol 3-kinase, mainly PtdIns(3,4)P₂ (42). This specific interaction is thought to play a crucial role in the biological effects of the phosphatidylinositol 3-kinase pathway. The PH domain of phospholipase C binds with quite high specificity to PtdIns(4,5)P₂ (42). PH domains of other proteins (βARK, Ras-GAP, pleckstrin N-terminal), on the other hand, are much less selective with respect to the position of the phosphate groups of phosphoinositides (42).

Although much is known about the stereospecificity of the binding of PH domains to different phosphoinositides (e.g., see Ref. 42), little is known about the stereospecificity of interaction of ion channels with phosphoinositides. In one study,

![Fig. 6. Effect of the number of phosphates in the head group on the efficacy of phosphoinositides on the activation of IRK1 channels.](image-url)
PtdIns(3,4,5)P₃ was shown to have similar activity to PtdIns(4,5)P₂ on K⁺ ATP channels (21), whereas the efficacy of PtdIns(4)P and PtdIns were found to be lower than that of PtdIns(4,5)P₂ (22, 23). PtdIns(4)P showed similar activity to PtdIns(4,5)P₂ on Na⁺-activated nonselective cation channels (16). In one study on endogenous GIRK channels of rat atrial cells, PtdIns(4)P and PtdIns was found to have activity similar to that of PtdIns(4,5)P₂ (43). Surprisingly, however, in this study even neutral phospholipids, e.g. phosphatidycholine, were effective in activating the channels (43).

In the present study, we have found that GIRK1/GIRK4 channels were activated by any of the naturally occurring head group bisphosphate and trisphosphate combinations. Only the number of phosphates in the head group was important, as PtdIns(4)P and PtdIns were found to be lower than that of PtdIns(4,5)P₂ (22, 23). PtdIns(4)P showed similar activity to PtdIns(4,5)P₂ on Na⁺-activated nonselective cation channels (16). In one study on endogenous GIRK channels of rat atrial cells, PtdIns(4)P and PtdIns was found to have activity similar to that of PtdIns(4,5)P₂ (43). Surprisingly, however, in this study even neutral phospholipids, e.g. phosphatidycholine, were effective in activating the channels (43).

In the present study, we have found that GIRK1/GIRK4 channels were activated by any of the naturally occurring head group bisphosphate and trisphosphate combinations. Only the number of phosphates in the head group was important, as PtdIns(4)P and PtdIns were found to be lower than that of PtdIns(4,5)P₂ (22, 23). PtdIns(4)P showed similar activity to PtdIns(4,5)P₂ on Na⁺-activated nonselective cation channels (16). In one study on endogenous GIRK channels of rat atrial cells, PtdIns(4)P and PtdIns was found to have activity similar to that of PtdIns(4,5)P₂ (43). Surprisingly, however, in this study even neutral phospholipids, e.g. phosphatidycholine, were effective in activating the channels (43).

PtdIns(3,4,5)P₃ was shown to have similar activity to PtdIns(4,5)P₂ on K⁺ ATP channels (21), whereas the efficacy of PtdIns(4)P and PtdIns were found to be lower than that of PtdIns(4,5)P₂ (22, 23). PtdIns(4)P showed similar activity to PtdIns(4,5)P₂ on Na⁺-activated nonselective cation channels (16). In one study on endogenous GIRK channels of rat atrial cells, PtdIns(4)P and PtdIns was found to have activity similar to that of PtdIns(4,5)P₂ (43). Surprisingly, however, in this study even neutral phospholipids, e.g. phosphatidycholine, were effective in activating the channels (43).

In the present study, we have found that GIRK1/GIRK4 channels were activated by any of the naturally occurring head group bisphosphate and trisphosphate combinations. Only the number of phosphates in the head group was important, as PtdIns(4)P and PtdIns were found to be lower than that of PtdIns(4,5)P₂ (22, 23). PtdIns(4)P showed similar activity to PtdIns(4,5)P₂ on Na⁺-activated nonselective cation channels (16). In one study on endogenous GIRK channels of rat atrial cells, PtdIns(4)P and PtdIns was found to have activity similar to that of PtdIns(4,5)P₂ (43). Surprisingly, however, in this study even neutral phospholipids, e.g. phosphatidycholine, were effective in activating the channels (43).

Interaction of inwardly rectifying K⁺ channels with PtdIns(4,5)P₂ is thought to take place between the cytoplasmic tails of the channel and the inositol phosphate head group of the lipid. Indeed, direct binding of the C terminus of GIRK1,
terminating channel activation. In contrast to diC8, lipids with decreasing side chain lengths, and therefore their incorporation into phospholipid bilayers is altered. Even in activating IRK1, which showed no difference in sensitivity to the diP analogs were less active. The kinetics of current activation and AASt acyl chains, the shorter side chain PtdIns(4,5)P2 forms have decreasing lipid/water partition coefficients into the membrane in order to activate the channels. The short side chain PtdIns(4,5)P2 on GIRK1/GIRK4 channels may arise from specific interactions of K+ channels with PtdInsP2, which, paired with future structure-function studies, will help us understand the molecular details of channel activation.

With the recent emergence of structural studies on K+ channels, our molecular understanding of these channels is rapidly expanding (47, 48). In view of these structural advances, it is important to understand the nature of interactions of K+ channels with their regulators. Our results provide important information about the interactions of inwardly rectifying K+ channels with PtdInsP2, which, paired with future structure-function studies, will help us understand the molecular details of channel activation.

Acknowledgment—We thank L. Lontsman and X. Yan for oocyte preparation; E. Kobrinsky, S. Kupfer, T. Mirshahi, J. Petit-Jacques, M. Sassaroli, and H. Zhang for critical comments on the manuscript; and M. Fuxreiter for valuable discussions. We also thank J. Peng for the synthesis of PtdIns(3,5)P2, O. Thum for the synthesis of PtdIns(3,4)P2, and L. Gao for phosphate analysis.

REFERENCES
1. Nichols, C. G., and Lopatin, A. N. (1997) Annu. Rev. Physiol. 59, 171–191
2. Jan, L. Y., and Jan, Y. N. (1997) Annu. Rev. Neurosci. 20, 91–123
3. Kuo, Y., Baldwin, T. J., Jan, Y. N., and Jan, L. Y. (1993) Nature 362, 127–133
4. Sui, J. L., Chan, K. W., Langan, M. N., Vivaudou, M., and Logothetis, D. E. (1999) Adv. Second Messenger Phosphoprotein Res. 33, 179–201
5. Yamada, M., Inanobe, A., and Kurachi, Y. (1988) Pharmacol. Rev. 50, 723–757
6. Krapivinsky, G., Gordon, E. A., Wickman, K., Velimirovic, B., Krapivinsky, L., and Clapham, D. E. (1995) Nature 374, 135–141
7. Logothetis, D. E., Kurachi, Y., Galper, J. E., Neer, E. J., and Clapham, D. E. (1987) Nature 325, 321–326
8. Reuveny, E., Slesinger, P. A., Inglese, J., Morales, J. M., Iniguez Lliu, J. A., Lefkowitz, R. J., Bourne, H. R., Jan, Y. N., and Jan, L. Y. (1994) Nature 370, 143–146
9. Krapivinsky, G., Krapivinsky, L., Wickman, K., and Clapham, D. E. (1995) J. Biol. Chem. 270, 29569–29602
10. Sui, J. L., Chan, K. W., and Logothetis, D. E. (1996) J. Gen. Physiol. 108, 381–391
11. Petit Jacques, J., Sui, J., and Logothetis, D. E. (1999) J. Gen. Physiol. 114, 673–684
12. Toker, A. (1998) Curr. Opin. Cell Biol. 10, 254–261
13. Hsuan, J. J., Minogue, S., and dos Santos, M. (1998) Adv. Cancer Res. 74, 167–216
14. Hilgemann, D. W., and Ball, R. (1996) Science 273, 956–959
15. Lupon, V. D., Kuznetsova, E. A., Krishna, U. M., Falek, J. R., and Bezprozvanny, I. (1998) J. Biol. Chem. 273, 14067–14070
16. Zrenner, A. B., and Ache, B. W. (1999) J. Neurosci. 19, 2929–2937
17. Huang, C. L., Feng, S., and Hilgemann, D. W. (1998) Nature 391, 803–806
18. Liu, H. H., Zhou, S.-S., and Huang, C.-L. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 5820–5825
19. Zhang, H., He, C., Yan, X., Mirshahi, T., and Logothetis, D. E. (1999) Nat. Cell Biol. 1, 183–188
20. Sui, J. L., Petit Jacques, J., and Logothetis, D. E. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 1307–1312
21. Shyng SL and Nichols CG (1998) Science 1138–1141
22. Baskirovsky, T., Schulte, U., Oliver, D., Herlitze, S., Krueier, T., Tucker, S. J., Ruppersberg, J. P., and Fakler, B. (1998) Science 1141–1144
23. Fan, Z., and Makielski, J. C. (1999) J. Biol. Chem. 273, 5388–5395
24. Ho, I. H. M., and Murrell-Lagnado, R. (1999) J. Physiol. (Lond.) 520, 645–651
25. Logothetis, D. E., and Zhang, H. (1999) J. Physiol. (Lond.) 520, 630
26. Willars, G. B., Nahirski, R. K., and Challiss, R. A. (1999) J. Biol. Chem. 274, 5037–5046
27. Stauffer, T. P., Ahn, S., and Meyer, T. (1999) Curr. Biol. 8, 343–346
28. Kebir, Y., Cunningham, C. C., Chen, J., Prestwich, G. D., Kosik, K. S., and Janney, P. A. (1999) Biophys. J. 76, A411–A411 (abstr.)
29. Rohacs, T., Chen, J., Prestwich, G. D., and Logothetis, D. E. (1999) Biophys. J. 76, A410 (abstr.)
30. Kubo, Y., Baldwin, T. J., Jan, Y. N., and Jan, L. Y. (1993) Nature 362, 127–133
31. Varnai, P., and Balla, T. (1996) J. Cell Biol. 134, 501–510
32. Kobrinsky, E., Mirshahi, T., and Logothetis, D. E. (1999) Biophys. J. 76, A411–A411 (abstr.)
33. Rameh, L. E., Arvidsson, A., Carraway, K. L., Couvillon, A. D., Rathbun, G., Crompton, A., Van Renterghem, B., Czech, M. P., Ravichandran, K. S., Neher, E., eds) pp. 307–327, Plenum Press, New York
34. Chan, K. W., Langan, M. N., Sui, J. L., Kozak, J. A., Pabon, A., Ladis, J. A., and Logothetis, D. E. (1998) J. Gen. Physiol. 112, 165–175
35. Vivaudou, M., Arnoult, C., and Villaz, M. (1991) J. Membr. Biol. 122, 165–175
36. Hilgemann, D. W. (1995) in Single-channel Recording (Sakmann, B., and Neher, E., eds) pp. 307–327, Plenum Press, New York
37. Alast, D. R., James, S. R., Downes, C. P., Sui, J. L., and Logothetis, D. E. (1996) Nature 381–391
38. Almeida, M. R., Langan, M. N., Vivaudou, M., and Logothetis, D. E. (1996) J. Biol. Chem. 271, 30497–30508
39. Kim, D., and Bang, H. (1999) J. Physiol. (Lond.) 517, 59–74
40. Downes, C. P., Hawkins, P. T., and Stephens, L. (1989) in Inositol Lipids in Cell Signalling (Michell, R. H., Drummond, A. H., and Downes, C. P., eds) pp. 1–38, Academic Press, London
41. Fruman, D. A., Meyers, R. E., and Cantley, L. C. (1998) Annu. Rev. Biochem. 67, 481–507
42. Pike, L. J., and Casey, L. (1996) J. Biol. Chem. 271, 26453–26456
43. Doyle, D. A., Cabrall, J. M., Fuetzner, R. A., Kuo, A., Gulbis, J. M., Cohen, S. L., Chait, B. T., and MacKinnon, R. (1998) Science 280, 69–77
44. Perutz, E., Cortes, D. M., and Cuello, L. G. (1999) Science 285, 73–78