Multiple ion channels have now been shown to be regulated by phosphatidylinositol 4,5-bisphosphate (PIP2) at the cytoplasmic face of the membrane. However, direct evidence for a specific interaction between phosphoinositides and ion channels is critically lacking. We reconstituted pure KirBac1.1 and KcsA protein into liposomes of defined composition (3:1 phosphatidylethanolamine:phosphatidylglycerol) and examined channel activity using an 86Rb+ uptake assay. We demonstrate direct modulation by PIP2 of KirBac1.1 but not KcsA activity. In marked contrast to activation of eukaryotic Kir channels by PIP2, KirBac1.1 is inhibited by PIP2 incorporated in the membrane (K1/2 = 0.3 mol%). The dependence of inhibition on the number of phosphate groups and requirement for a lipid tail matches that for activation of eukaryotic Kir channels, suggesting a fundamentally similar interaction mechanism. The data exclude the possibility of indirect modulation via cytoskeletal or other intermediary elements and establish a direct interaction of the channel with PIP2 in the membrane.

Phosphoinositides constitute a major group of signaling molecules in eukaryotic membranes (1, 2) and modulate an ever growing list of ion channels, whether by application of exogenous phospholipids to the cytoplasmic membrane surface or by manipulation of endogenous phospholipids (3–12). However, the nature of the phosphoinositide-channel interaction remains elusive. For one extensively studied group, the inward rectifier K (Kir) channels, there is an emerging consensus that a direct interaction occurs between cytoplasmic domains of the channel and inositol headgroups, based on electrophysiological analysis (5, 13–16) and biochemical analysis of isolated channel domains (5, 17, 18). Direct interaction of functional channels with phospholipids in the membrane has been difficult to demonstrate unequivocally, and without this, quantification of the dose-response relationships for channel modulation by phospholipids is obtvated, and further mechanistic understanding is limited (19, 20). The recent cloning and crystallization (21), as well as functional analysis of KirBac1.1 channels reconstituted in lipid membranes (22), provides the opportunity to examine channel activity using a highly purified protein preparation in membranes of defined composition and permits direct test of the nature of the channel-phosphoinositide interaction.

EXPERIMENTAL PROCEDURES

Methods are essentially as described previously (22). KcsA and KirBac1.1 in pQE60 vector were expressed in BL21 (DE3) cells after induction with isopropyl β-D-thiogalactopyranoside. Bacteria were lysed by sonication, incubated 2–4 h with 30 mM decylmaltoside (Anatrace), then centrifuged at 30,000 × g for 30 min, and the supernatant was applied to a cobalt affinity column (22). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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family members but decreased or no preference for Kir3.x and Kir6.2 channels. To test the specificity of KirBac1.1 inhibition as a function of phosphate position, we measured channel activity in liposomes with different isomers (Fig. 3). KirBac1.1 shows no preference for headgroup position and as such behaves very similarly to Kir6.x family members (9).

Using highly purified channel protein reconstituted into liposomes of defined composition, the above experiments establish a direct interaction between PIP$_2$ in the membrane and the channel protein: there is no micellar PIP$_2$, and interaction must be with PIP$_2$ incorporated into the liposome membrane. By examining the effects of PIP$_2$ metabolites, we also unequivocally demonstrate that PIP$_2$ itself is the critical interactor and, moreover, that lipid structure specificity is the same as that for activation of eukaryotic channels, when applied exogenously. This is consistent with modulation of eukaryotic Kir channels being through a fundamentally similar direct physical interaction with the channel. Why then is KirBac1.1 inhibited by PIP$_2$, whereas eukaryotic channels are activated? We suggest that subtle differences in the structure of the two groups of channels can give rise to activation in one case and inhibition in the other. Sequence alignments of eukaryotic and bacterial Kir channels (Fig. 4) indicate significant gap-free homologies in transmembrane regions and conserved N- and C-terminal regions that generate the pore and the Kir cytoplasmic domain (21, 28). Notably, however, eukaryotic Kir channels contain two additional 3-amino acid linkers connecting cytoplasmic domains to the slide helix and to the second transmembrane helices. The amino acids in the second linker are generally charged and have repeatedly been invoked as critical interacting charges for PIP$_2$ activation of eukaryotic Kir channels (5, 13, 29–31). We hypothesize that these charged linkers in eukaryotic channels are essential for PIP$_2$ activation; conceivably they appeared only in response to...
evolutionary pressure as the otherwise inhibitory effect of PIP₂ caused loss of Kir channel function in early eukaryotic membranes. While the nature and extent of these linkers is clearly important for channel function, further experiments are warranted to examine whether and how their length and nature are involved in transducing PIP₂ interaction to activation or inhibition of channel activity. It is intriguing to note that opposing effects of PIP₂ are seen in the TRP channel family. For some, e.g., TRPM5, TRPM7, and TRPM8, PIP₂ has an activating effect (32–34). However, PIP₂ inhibits TRPL and TRPV1 channels, and mutation of residues in the same C-terminal “TRP-box” region that is invoked as necessary for PIP₂ activation of TRPM8 channels (34) strengthens or weakens inhibition of TRPV1 by PIP₂ (8).

In studying modulation of channel activity in cell membranes, PIP₂ levels have been indirectly manipulated by hydrolysis (phospholipase) or synthesis (application of MgATP). Such manipulations can cause changes in multiple other membrane constituents and are not quantitative. Although semiquantitation has been cleverly achieved using fluorescent-tagged PIP₂-associating protein domains (35, 36), direct measurement of [PIP₂] is also not possible. PIP₂ has also been added to excised membrane patches in micelles. If the supposed consequence (incorporation of PIP₂ into the membrane leaflet) is achievable, it should also be possible to perform essentially this same experiment in the liposome assay. We incubated liposomes in micellar PIP₂ for 5 min prior to Rb⁺/H⁻ uptake assay (Fig. 5A). KirBac1.1 activity is again inhibited, with no effect on KcsA activity. However, in contrast to the complete inhibition observed when PIP₂ is incorporated into the membrane, inhibition plateaus at ~50%, broadly consistent with the expectation that KirBac1.1 channels can insert into the membrane in either of the two possible orientations and that PIP₂ partitions primarily into the outer leaflet of the membrane.

A further approach to reduce [PIP₂] in native membranes has been to apply poly-L-lysine, which electrostatically shields the anionic headgroups (13, 37). Again, broadly consistent with the hypothesis that PIP₂ headgroups interact with the channel, application of poly-L-lysine reduced the inhibitory effect of membrane PIP₂ on KirBac1.1 fluxes (Fig. 5B).

Multiple ion channel involvements in physiological and disease states, including muscarinic signaling in the brain (10, 38), temperature sensing by TRP channels (8, 34), inward rectifier currents in cardiac arrhythmias (31), and mechanical regulation of 2P-domain potassium channels (12) are critically dependent on phosphoinositide signaling. However, almost all studies of channel-lipid interactions to date are inevitably indirect; in many cases it cannot be established whether the PIP₂ is even acting from within the membrane (19, 20), and although biochemical studies (e.g. Refs. (18 and 39) have shown that peptide fragments of ion channels can directly interact with PIP₂, the possibility of intermediates in physiological regulation cannot be excluded. In the present study, we use a novel approach, purified channel protein, reconstituted into lipid vesicles of fully defined composition, to unambiguously establish three critical principles: (i) an action specifically of PIP₂ in the membrane, (ii) direct interaction between the channel and PIP₂, and (iii) the concentration dependence of this interaction. These are key mechanistic aspects of lipid modulation of channel activity and support the idea that direct interactions underlie eukaryotic Kir channel modulation. This assay provides a model system in
which to study channel-PIP$_2$ interactions; extensive mutagenesis studies, in combination with molecular modeling based on crystal structures (40–42), can now be used to define the detailed structural basis of these interactions.

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REFERENCES

1. Hilgemann, D. W., Feng, S., and Nasuhoglu, C. (2001) Science’s STKE 2001, RE19
2. Fruman, D. A., Meyers, R. E., and Cantley, L. C. (1998) Annu. Rev. Biochem. 67, 481–507
3. Bian, J. S., Kagan, A., and McDonald, T. V. (2004) Pflugers Arch. 449, 507–520
4. Chemin, J., Patel, A. J., Duprat, F., Lauritzen, I., Lazdunski, M., and Honore, E. (2005) EMBO J. 24, 44–53
5. Huang, C. L., Feng, S., and Hilgemann, D. W. (1998) Nature 391, 803–806
6. Himmel, B., and Nagel, G. (2004) EMBO Rep. 5, 85–90
7. Oliver, D., Lien, C. C., Soom, M., Baulkrowitz, T., Jonas, P., and Fakler, B. (2004) Science 304, 265–270
8. Prescott, E. D., and Julius, D. (2003) Science 300, 1284–1288
9. Rohacs, T., Lopes, C. M., Jin, T., Ramdya, P. P., Molnar, Z., and Logothetis, D. E. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 745–750
10. Suh, B. C., and Hille, B. (2002) Neuron 35, 507–520
11. Luo, L., Bauer, C. S., Zhen, X. G., Xie, C., and Yang, J. (2002) Science 295, 243–254
12. Wu, L., Bauer, C. S., Zhen, X. G., Xie, C., and Yang, J. (2002) Science’s STKE 2002, RE5
13. Lopes, C. M., Zhang, H., Rohacs, T., Jin, T., Yang, J., and Logothetis, D. E. (2002) Neuron 35, 933–944
14. Liu, D., and Liman, E. R. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 15160–15165
15. Runnels, L. W., Yue, L., and Clapham, D. E. (2002) Nat. Cell Biol. 4, 329–336
16. Rohacs, T., Lopes, C. M., Michailidis, I., and Logothetis, D. E. (2002) Methods Enzymol. 345, 71–92
17. Deutsch, N., Matsuoka, S., and Weiss, J. N. (1994) J. Gen. Physiol. 104, 773–800
18. Zhang, H., Craciun, L. C., Mirshahi, T., Rohacs, T., Lopes, C. M., Jin, T., and Logothetis, D. E. (2002) Methods Enzymol. 345, 71–92
19. Deutsch, N., Matsuoka, S., and Weiss, J. N. (1994) J. Gen. Physiol. 104, 773–800
20. Zhang, H., Craciun, L. C., Mirshahi, T., Rohacs, T., Lopes, C. M., Jin, T., and Logothetis, D. E. (2002) Methods Enzymol. 345, 71–92
21. Deutsch, N., Matsuoka, S., and Weiss, J. N. (1994) J. Gen. Physiol. 104, 773–800
22. Zhang, H., Craciun, L. C., Mirshahi, T., Rohacs, T., Lopes, C. M., Jin, T., and Logothetis, D. E. (2002) Methods Enzymol. 345, 71–92
23. Deutsch, N., Matsuoka, S., and Weiss, J. N. (1994) J. Gen. Physiol. 104, 773–800
24. Zhang, H., Craciun, L. C., Mirshahi, T., Rohacs, T., Lopes, C. M., Jin, T., and Logothetis, D. E. (2002) Methods Enzymol. 345, 71–92
25. Deutsch, N., Matsuoka, S., and Weiss, J. N. (1994) J. Gen. Physiol. 104, 773–800
26. Zhang, H., Craciun, L. C., Mirshahi, T., Rohacs, T., Lopes, C. M., Jin, T., and Logothetis, D. E. (2002) Methods Enzymol. 345, 71–92
27. Deutsch, N., Matsuoka, S., and Weiss, J. N. (1994) J. Gen. Physiol. 104, 773–800
28. Zhang, H., Craciun, L. C., Mirshahi, T., Rohacs, T., Lopes, C. M., Jin, T., and Logothetis, D. E. (2002) Methods Enzymol. 345, 71–92
29. Deutsch, N., Matsuoka, S., and Weiss, J. N. (1994) J. Gen. Physiol. 104, 773–800
Direct Modulation of Kir Channel Gating by Membrane Phosphatidylinositol 4,5-Bisphosphate
Decha Enkvetchakul, Iana Jeliazkova and Colin G. Nichols

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