Voltage-gated K⁺ (Kᵥ) channels contain a voltage-sensing transmembrane segment that moves in response to changes in membrane potential. A small “gating” current arises from the translocation of positively charged residues within the channel’s voltage sensor. These gating currents occur at the microsecond timescale (Gilly and Armstrong, 1980), indicating a highly ordered translocation (or reorganization) of the voltage sensors within the membrane. These initial events trigger additional, complex conformational changes that open, close, or inactivate the channel, thereby providing distinct kinetics to different types of Kᵥ channels (Benzanilla, 2008). Initial x-ray crystallography data of a mammalian Kᵥ chimeric channel provided the first glimpse of the protein organization of a Kᵥ channel when associated with phospholipid in a mixed phospholipid/detergent environment (Long et al., 2007). However, in vivo, Kᵥ channels are surrounded by a complex sea of lipids. How do these lipids interact with channels, where are critical sites of interaction, and how do they influence voltage sensing and gating? Answers to these important questions are beginning to be provided by x-ray crystal structures of pore regions, voltage sensors, and cytoplasmic domains of several K’ channels, in combination with biophysical, pharmacological and mutagenesis studies (Jiang et al., 2002; Kuo et al., 2003; Long et al., 2005; 2007; Logothetis et al., 2007; Lundbaek, 2008; Tucker and Baukrowitz, 2008).

A consensus is emerging that the anionic phospholipid, phosphatidylinositol-4,5-bisphosphate (PIP₂) has physiologically important interactions with many Kᵥ channels and other transmembrane proteins, including ion transporters and ligand-gated K’ channels. In an explosion of interest for this idea, a rapid succession of studies identified a number of K’ channels that are positively regulated by PIP₂, including all members of the Kᵦ family of inward rectifiers (Sui et al., 1998; Liou et al., 1999; Zhang et al., 1999; Enkvetchakul et al., 2007; Logothetis et al., 2007), as well as voltage-gated KCNQ channels (Suh and Hille, 2002; Loussouarn et al., 2003; Zhang et al., 2003) and delayed rectifier K’ channels (Oliver et al., 2004). Among the reasons for the interest in PIP₂ regulation of channels is that a number of Gₛ coupled receptors that stimulate PIP₂’s breakdown also modulate K’ channel activity, most likely by decreasing the PIP₂ available to associate with channels (Xie et al., 1999; Cho et al., 2001; Suh and Hille, 2002).

Hilgemann and Ball (1996) made the initial observation that PIP₂ positively regulates the cardiac Na’/Ca²⁺ exchanger. Addition of ATP to the cytoplasmic side of inside-out (I/O) giant patches of cardiac myocyte membrane increased the Na’/Ca²⁺ exchange current; in patches pretreated with a bacterial PLC that selectively metabolizes phosphatidylinositol (PI), ATP no longer was able to enhance the current. ATP’s stimulatory action recovered if PI was added to the bath before ATP; PI by itself had no effect, whereas PIP₂ alone stimulated current in patches pretreated with PLC. These seminal findings suggested that phosphorylation of PI produced PIP₂, which then directly interacted with the exchanger to stimulate its activity. To test their model, Hilgemann and Ball (1996) exposed ATP-stimulated patches to a PIP₂-selective PLC, and found that current returned to pre-ATP levels, a result consistent with PIP₂’s actions underlying increased exchanger activity. Using similar approaches, they found an anologous stimulation of K₅ ATP channel activity with conditions that increased endogenous PIP₂ and decreased activity under conditions that decreased PIP₂ levels. Lastly they detected a possible increase in K’ inward rectifier (Kir) current. Soon after, Fan and Makielski (1997) reported for the first time the potentiation of Kir current by PIP₂, a finding confirmed by Hilgemann’s group (Huang et al., 1998). These early studies suggested that the requirement for PIP₂ to elicit channel opening might be a broad principle for a number of K’ channels.

The same approach that implicated PIP₂ as a regulator of Na’/Ca²⁺ exchangers and K₅ ATP channels led to negative outcomes in studies on the large conductance, Ca²⁺-activated K’ channel (also known as slo1, maxi-K’, or BK channel), in skeletal muscle and cardiac myocytes (Hilgemann et al., 2001; Hilgemann, D.W., personal communication). Throughout the body, maxi-K’ channels integrate intracellular Ca²⁺ signals with changes in membrane potential, often providing an important negative feedback mechanism that limits depolarization-driven Ca²⁺ influx. Previous findings demonstrated that metabolic products of PIP₂ and downstream targets

Abbreviations used in this paper: I/O, inside-out; PIP₂, phosphatidylinositol-4,5-bisphosphate.
such as PLC, ryanodine receptors, kinases, and free fatty acids modulate BK channel activity (Dopico et al., 1994; Kirber et al., 1992; Salkoff et al., 2006). From beautifully executed experiments, Alex Dopico and his colleagues (see article by Vaithianathan et al., p. 13) now provide evidence that PIP$_2$ can indeed interact with BK channels, adding another type of K$^+$ channel to the ever-growing list of PIP$_2$-sensitive channels. However, in order to observe enhanced activity, BK channels must co-express with a specific accessory β subunit giving rise to tissue-specific PIP$_2$ effects.

BK channels arise from a single mammalian gene (KCNMA1) with multiple splice sites providing for diverse expression patterns and differing biophysical properties (Salkoff et al., 2006). Not surprisingly, BK channels differ in topological organization from the conventional Kv channels, which are comprised of six transmembrane segments (S1–S6) with both N and C termini located intracellularly. Distinct regions within S1–S4 (S3b and S4 in particular) appear responsible for voltage sensing while a P-loop spans segments S5 and S6 to contribute to the channel’s pore (Long et al., 2005; 2007). Four of these subunits form a functional channel that has a central pore and four voltage sensors. BK channel structure deviates in several ways from this organization. First, the BK channel has an additional S0 segment preceding S1–S6 that results in the N terminus ending extracellularly. S0 may interface with voltage-sensing areas, introducing additional regulation of voltage sensor movements (Liu et al., 2008). Second, each S0 interacts with an accessory β subunit that contains two transmembrane segments. Encoded by distinct genes, expression of different β subunits (β1–β4) in different tissues adds complexity to BK gating, kinetics, and pharmacology. Third, as the name indicates, this Ca$^{2+}$-sensitive maxi-K$^+$ channel opens in response to rises in intracellular Ca$^{2+}$ levels, membrane depolarization, or a combination of the two. BK’s long C terminus tail contains a variety of functional domains involved in divalent sensing, such as the Ca$^{2+}$-binding bowl, and two RCK (regulators of conductance of K$^+$) domains, conferring Ca$^{2+}$ sensitivity to the channel, yet the molecular underpinnings are not fully understood (Salkoff et al., 2006).

To test whether PIP$_2$ may interact directly with BK channels, Dopico and colleagues performed several experiments in the presence of basal levels of Ca$^{2+}$ (300 nM), where exogenous PIP$_2$ robustly and reproducibly increased steady-state activity (NP$_o$) of native BK channels in I/O patches from freshly isolated cerebral vascular myocytes, with no changes in the unitary current amplitude. In contrast, little change in NP$_o$ occurred following extracellular PIP$_2$ application to cell-attached or outside-out patches, indicating that the putative site of interaction is most accessible from the inner leaflet, as would be expected because that is where endogenous PIP$_2$ is found (Laux et al., 2000). The authors then used a dizzying array of pharmacological conditions to examine the effect of endogenous PIP$_2$ on BK current in I/O patch and in perforated-patch recordings where the myocytes remained intact. Every experiment that favored increased endogenous PIP$_2$ levels yielded increased channel activity. Dopico and colleagues then took the investigation a further step. They pressurized endothelium-free cerebral arteries that develop myogenic tone in order to determine whether changes in PIP$_2$ levels could alter vessel diameter. Under conditions that minimized PIP$_2$ metabolism, arterial diameter significantly increased, indicating that elevated levels of PIP$_2$ increase BK channel activity. The resulting increase in BK-driven, outward current then hyperpolarizes myocyte membrane, promoting muscle relaxation and vessel dilation. They confirmed this hypothesis by showing that when BK activity was blocked with paxilline, arteries no longer relaxed under conditions that increased PIP$_2$ levels. This in vivo demonstration is the first report documenting the consequences of a PIP$_2$ interaction with an ion channel on organ function.

Having demonstrated physiological significance for PIP$_2$’s actions on BK channel activity, the authors examined the structural requirements for increased channel activity. They found that more water soluble forms of PIP$_2$ (diC4 and diC8) also increased NP$_o$, but to a lesser degree than the longer-chain form, and reversed with washout more easily than PIP$_2$. This result suggested that the longer fatty acid tails on the one hand facilitate increased partitioning of PIP$_2$ into the lipid environment and hence access to channels and, on the other, slow the off rate from the bilayer to the aqueous phase. Additionally, the authors found that channel activation positively correlated with the number of anionic head group charges (−1 to −4). Moreover, when they included PIP$_2$ antibodies or the anion scavenger poly-L-lysine along with PIP$_2$, the normally robust increase in NP$_o$ was significantly reduced. Lastly, the authors tested whether 1,2-dipalmtoyl-sn-glycero-3-phospho-L-serine (PS) versus 1,2-dipalmitoyl-sn-glycero-3-phosphoinositol (PI) are equally effective in increasing BK activity. Both phospholipids were matched for charge (−1) and “flavor” of fatty acid tails. PI increased NP$_o$ 2.5 times greater than PS. This finding is especially important since it indicates that not only is charge critical, but so too is the structure of the moiety in the sn-3 position of the phospholipid. Taken together, the structural specifications for BK activation, which include the importance of long-chain fatty acid tails, the phospho-moiety in the sn-3 position, and the number of negative charges are consistent with the negatively charged phosphoinositol head group directly interacting with a specific binding site on the channel.

From these findings with native BK channels in cerebral vascular myocytes, Dopico and colleagues turned to the oocyte heterologous expression system to search...
for a potential PIP$_2$ binding site in recombinant BK channels, with surprising results. Following expression of cvb1 (encoded by KCNMA1), a BK splice variant prevalent in cerebral artery myocytes (Liu, J., P. Liu, M. Asuncion-Chin, and A. Dopico. 2005. Soc. Neurosci. Abstr. Online. 960.913), PIP$_2$ robustly increased the current recorded from I/O patches, establishing that cvb1, together with its immediate lipid environment, was sufficient for PIP$_2$ to enhance current. They then identified a three-amino acid sequence (RKK) in the S6–S7 cytosolic linker of cvb1 that, when mutated to AAA, blunted increases in NP$_o$, following PIP$_2$ application, indicating that this sequence may serve as a site of interaction with PIP$_2$. In contrast, mutation of a positively charged amino acid (K239A), located in the S4–S5 cytosolic loop, had no effect on cvb1 sensitivity to PIP$_2$. Taken together, these studies demonstrate that loss of PIP$_2$’s actions does not depend on all positive residues contained within cytoplasmic loops, but rather, on specific positive residues in the S6–S7 segment. Moreover, current enhancement by PIP$_3$ occurred in wt and K239A mutant but not in the RKK to AAA substitution, indicating that PIP$_2$ and PIP$_3$ act at a common site of action.

Cvb1’s RKK sequence is most likely a PIP$_2$ interaction site because it shares certain characteristics with PIP$_2$ binding sites in other K$^+$ channels, which normally contain at least two (though often more than five) positively charged residues (arginine and lysine). At least one of the residues must be arginine. As with cvb1, critical sequences for PIP$_2$ binding have been located to the proximal portion of the C-terminal tail of bacterial and mammalian Kir channels (Huang et al., 1998; Shyng and Nichols, 1998; Logothetis et al., 2007), KCNQ1, the subunit that confers PIP$_2$ sensitivity to KCNQ1/KCNE1 channels (Loussouarn et al., 2003), and KCNQ2, the subunit that when coexpressed with KCNQ3 gives rise to M-current (Zhang et al., 2003). For certain channels, additional positively charged residues in the proximal N terminus also confer channel sensitivity to PIP$_2$. Interestingly, the RKK mutation in cvb1 decreased but did not ablate BK channel sensitivity to PIP$_2$, raising the possibility that additional nearby residues participate in PIP$_2$ binding and/or additional sites of interaction mediate further enhancement; these sites await future discovery.

Analysis of macroscopic currents showed that PIP$_2$ causes a parallel leftward shift in voltage sensitivity of activation without altering the effective valence of BK channels. Thus the negative charge of the PIP$_2$ head group does not appear to alter critical aspects of voltage gating. Further analysis of unitary gating kinetics by the authors revealed increases in both open and closed dwell time distributions caused by PIP$_2$ amplification of Ca$^{2+}$-driven gating. Indeed, when channels were opened with voltage in the absence of Ca$^{2+}$, PIP$_2$ had little effect on BK NP$_o$, whereas in the presence of 300 nM or greater, PIP$_2$ robustly enhanced NP$_o$. Another observation highlights the importance of PIP$_2$ control of Ca$^{2+}$-driven gating. The authors found that increases in cvb1 channel NP$_o$ following PIP$_2$ was markedly less than that observed in I/O patches from cerebral artery myocytes. One notable difference between the two sets of experiments was the absence of B1 subunits in the heterologous expression system, whereas cvb1 tightly associates with B1 subunits in cerebral vascular myocytes (Liu et al., 2005; Salkoff et al., 2006). Therefore, the authors tested whether B1 might potentiate PIP$_2$’s actions when coexpressed with cvb1. As suspected, PIP$_2$’s actions now recapitulated the remarkably large increase in NP$_o$ of vascular myocyte BK channels. In contrast, coexpression of B4 had no potentiating effect. Native BK channels in skeletal myocytes, cells known to barely express B1, responded to PIP$_2$ similarly to homologic slo1 consistent with B1, but not B4, facilitating PIP$_2$’s effects. These findings add a new wrinkle to PIP$_2$ actions on channels where the accessory B1 subunit, known to robustly increase the apparent Ca$^{2+}$ sensitivity of the BK channel complex, confers an additional amplification mechanism that controls the final effect of PIP$_2$.

Though increasing evidence indicates that PIP$_2$ regulates channel availability and P$_o$ of other K$^+$ channels, how this occurs remains ill defined. Nonspecific deformation in lipid packing can alter channel gating (Lundbøk, 2008). However, the first x-ray crystal structures of the initial shell of lipid surrounding a chimeric voltage-sensing paddle yielded evidence that lipids may alter K$^+$ channel conformational changes due to specific electrostatic interactions (Schmidt et al., 2006; Long et al., 2007). In particular, the finding of a phospholipid “wedged” between the voltage sensor and the S4–S5 linker, a domain of the channel that couples voltage sensor movement to S5–S6 channel gating (Long et al., 2007), suggests specific molecular interactions between individual amino acid residues and phospholipid molecules. The specific phospholipids interacting with the channels in mammalian cell membranes remain unidentified. However, molecular modeling of PIP$_2$ with Kir 3.4 (Logothetis et al., 2007) or Kir 6.2 (Haider et al., 2007) find PIP$_2$ localized to a location comparable to the phospholipid “wedge” between the S4–S5 linker in the crystal structures (Long et al., 2007). Similarly, PIP$_2$’s binding to the proximal region of the BK channel’s cytoplasmic tail is consistent with an analogous orientation of the phosphoinositol head, embedded in BK’s large tail region like an anchor; PIP$_2$’s long hydrophobic fatty acid tails serve as tethers by extending into transmembrane regions of the channel to influence gating. Whether additional molecular interactions of the maxi-K$^+$ channel (perhaps via B$_x$ subunit and Ca$^{2+}$) reorient PIP$_2$ within the bilayer and increase coupling between voltage sensors and gating regions within S5–S6 remains to be tested.
In summary, the results of Dopico and colleagues (Vaithianathan et al., 2008) provide an important addition to our understanding of the role of PIP$_2$ in regulating transmembrane proteins. The similar changes occurring across multiple preparations make it unlikely that PIP$_2$’s interaction with the proteolipid environment around the BK channel complex indirectly mediates changes in channel gating. More likely the cbv1 + β1 complex is sufficient to support channel activation by PIP$_2$. The fact that a specific β subunit appears to confer endogenous PIP$_2$ sensitivity to a specific BK channel complex, which exhibits tissue-specific coexpression, strongly suggests that modulation by PIP$_2$ is physiologically significant. In particular, the novel action of PIP$_2$ in increasing channel sensitivity to Ca$^{2+}$-dependent regulation of gating is reminiscent of PIP$_2$’s ability to increase sensitivity to G-protein subunits and Na$^+$ binding observed with other K$^+$ channels (Logothetis et al., 2007). The experimental findings in this report advance the importance of PIP$_2$’s interaction with the cytoplasmic region following S6 in a number of K$^+$ channels.

**REFERENCES**


