Dual regulation of voltage-sensitive ion channels by PIP₂

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INTRODUCTION

Voltage-gated ion channels regulate the flow of different ions across the membrane in response to changes in membrane potential. These channels are composed of four subunits (or four linked domains) symmetrically arranged around a central ion-conducting pore. Voltage-gated ion channels open in response to depolarization or hyperpolarization. The change in membrane potential induces a conformational change in the voltage sensor domain located in the periphery of the ion channel; this conformational change is coupled to the ion-conducting pore, and leads to channel opening (Long et al., 2005; Bezanilla, 2008; Borjesson and Elinder, 2008).

Voltage-gated ion channels can be modulated by numerous factors including free fatty acids (Xiao et al., 2005; Borjesson et al., 2008; Xu et al., 2008a), toxins (Catterall et al., 2007; Swartz, 2007), metal ions (Elinder and Arhem, 2003), glycosylation (Fozzard and Kyle, 2002; Watanabe et al., 2003, 2007), palmitylation (Gubitosi-Klug et al., 2005; Jindal et al., 2008), phosphorylation (Davis et al., 2001; Mohapatra and Trimmer, 2006; Mohapatra et al., 2007; Li et al., 2008), and phospholipids (Ramú et al., 2006; Schmidt et al., 2006; Xu et al., 2008b). Modulation by phospholipids includes phosphatidylinositol 4,5-bisphosphate (PIP₂), the lipid component of the inner membrane leaflet that modulates the activity of most ion channels and transporters tested (Suh and Hille, 2005, 2008; Logothetis et al., 2010).

PIP₂ plays an important role as an intermediate molecule in multiple receptor signaling pathways. PIP₂ hydrolysis by PLC produces 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG; Berridge, 1984). IP₃ mobilizes Ca²⁺ from the endoplasmic reticulum, while DAG activates PKC. However, PIP₂ itself acts as a signaling molecule through direct interactions with target proteins. Our review focuses on the effects of PIP₂ on voltage-dependent ion channels, particularly those showing bidirectional regulation.

VOLTAGE-GATED CALCIUM CHANNELS

Voltage-gated calcium (Cav) channels mediate calcium influx in response to membrane depolarization and regulate intracellular processes such as contraction, secretion, neurotransmission, and gene expression in many different cell types. There are five types of Cav channels: the high voltage-activated L- (Cav1), P/Q- (Cav2.1), N- (Cav2.2), and R- (Cav2.3), and the low voltage-activated T- (Cav3; Catterall, 2011). Cav channels are complexes of α₁, α₂β, β, γ,
and δ subunits. It is in the α1 subunit that the conduction pore and voltage sensor apparatus are located. The α1 subunit is composed of four homologous domains (I–IV), with six transmembrane segments (S1–S6) in each. Similar to Kv and voltage-gated sodium (Nav) channels, the S1–S4 segments serve as the “voltage sensor” and the S5–S6 segments form the “pore domain” (Catterall et al., 2005).

Voltage-gated Ca\(^{2+}\) channels of the Cav2 subfamily (N- and P/Q-type) are regulated by G protein coupled receptors via two distinct pathways in sympathetic neurons (Ikeda and Dunlap, 1999). The first is the faster pathway, voltage-dependent, and membrane delimited, induced by direct interaction of the G protein βγ subunit with the channel protein (Herlitze et al., 1996; Ikeda, 1996; Dolphin, 2003). The second is the slower pathway that is voltage-independent. It uses signals that stimulate the G\(_{q/11}\) type of G proteins to activate phospholipase C (PLC), which hydrolyzes PIP\(_2\) into inositol triphosphate (IP\(_3\)) and diacylglycerol (DAG; Bernheim et al., 1991; Brown et al., 1997). This slower pathway was later attributed to depletion of PIP\(_2\) by the activation of PLC (Wu et al., 2002; Gamper et al., 2004). The faster pathway has also been related to PIP\(_2\). Rousset et al. (2004) reported that decreasing PIP\(_2\) levels suppressed the constitutive inhibition of Cav2.1 channels by endogenous G\(_{q/11}\) subunits. These authors proposed that stabilization of the G\(_{q/11}\) sensitive state of Cav2.1 channels may require direct interaction with PIP\(_2\). However, additional studies are needed to firmly establish the importance of PIP\(_2\) in this pathway.

The first study on Cav channel modulation by PIP\(_2\) reported that this phosphoinositide induces two opposing modulatory effects on Cav2.1 channels (Wu et al., 2002). Rundown of Cav2.1 channels in inside-out patches of *Xenopus* oocytes was greatly slowed and even reversed by the application of exogenous PIP\(_2\) or Mg-ATP to the patch. Conversely, the application of PIP\(_2\)-antibody accelerated rundown, suggesting that PIP\(_2\) stabilizes the activity of Cav2.1 channels and its depletion induces rundown. PIP\(_2\) also exerted a voltage-dependent inhibitory effect by shifting the voltage dependence of activation toward depolarized potentials. This effect was antagonized by activation of protein kinase A (PKA; Wu et al., 2002). Modulation of Cav2.1 channels by PIP\(_2\) has also been reported to occur in neural striatal projection neurons. Stimulation of muscarinic M1 receptors inhibited Cav2.1 channels in these neurons, an effect that could be abolished by inhibition of PLC. Consistent with these results, intracellular application of PIP\(_2\) inhibited all muscarinic modulation of Cav2.1 channels (Perez-Burgos et al., 2010).

In subsequent studies, Cav2.2 modulation by PIP\(_2\) was also reported (Gamper et al., 2004). In inside-out *Xenopus* oocytes patches expressing Cav2.2 channels, PIP\(_2\) significantly slowed or reversed the rundown of these channels. In native Cav2.2 channels from sympathetic neurons, the current inhibition by muscarinic M1 receptor activation was diminished by intracellular application of diC\(_8\)-PIP\(_2\), and the current recovery was abolished when PIP\(_2\) synthesis was blocked. Interestingly, activation of bradykinin receptors, which also activate PLC and induce PIP\(_2\) hydrolysis, failed to inhibit Cav2.2 currents in the same neurons in which muscarinic M1 receptor activation was effective, an effect attributed to a probable concurrent Ca\(^{2+}\)-mediated stimulation of PIP\(_2\) synthesis (Gamper et al., 2004). In a different study with the same type of neurons, bradykinin-induced voltage-independent inhibition of Ca\(^{2+}\) channels was reported and this effect could be abolished by inhibiting PLC, but it was not altered by inhibiting its downstream effectors (Lechner et al., 2005). The discrepancies with the previous study (i.e., Gamper et al., 2004) were attributed by the authors to differences in experimental conditions, such as the use of different cell culture media, differences in the buffering of intracellular Ca\(^{2+}\) concentrations, differences in intracellular Mg\(^{2+}\), and differences in the voltage protocols used.

Another hypothesis for the slow G\(_{q/11}\) mediated inhibition of L-, N-, and P/Q calcium channels involves arachidonic acid (AA). G\(_{q/11}\) coupled receptor stimulation can acutely activate PLA\(_2\) with the subsequent production of AA, which is proposed as the main signal mediating Cav channels inhibition (Roberts-Crowley et al., 2009). Thus, the same receptors that induce PIP\(_2\) depletion can cause concurrent release of AA and modulate Cav channels according to the AA hypothesis.

A recent elegant study used two strategies to deplete PIP\(_2\) without the production of the PLC downstream products (Suh et al., 2010). PIP\(_2\) was depleted by rapamycin-induced translocation of an inositol lipid 5-phosphatase and a voltage-sensitive 5-phosphatase (VSP). These systems convert PIP\(_{4}\) to PIP\(_{3}\) in the plasma membrane of intact cells, without activation of G protein-coupled receptors. Both systems suppressed Cav1.2, Cav1.3, Cav2.1, and Cav2.2 channels. Irreversible depletion of endogenous PIP\(_2\) by rapamycin-induced translocation of INP54p 5-phosphatase to the plasma membrane irreversibly inhibited whole-cell Cav currents. On the other hand, reversible depletion of PIP\(_2\) by the activation of the zebrafish voltage-sensitive phosphatase *Danio rerio* (Dr-VSP) reversibly inhibited Cav channels in whole-cell recordings, suggesting that PIP\(_2\) is a cofactor required for channel activity. These results with intact cells (whole-cell experiments) did not completely recapitulate the effects reported in inside-out patches with Cav2.1 and Cav2.2 channels (Wu et al., 2002; Gamper et al., 2004). While in excised patches currents ran down almost completely, in intact cells PIP\(_2\) depletion inhibited Cav2.1 currents by 29% and Cav2.2 by 55%. In addition, the inhibitory actions of PIP\(_2\) were not observed. These differences as suggested by the authors could potentially reflect preservation in the whole-cell recordings of phosphorylation in some channels or cytoplasmic factors that make channels less PIP\(_2\) sensitive or preserving PIP\(_2\) synthesis that prevents full PIP\(_2\) depletion (Suh et al., 2010).

Recently, it was demonstrated that the β subunits of voltage-gated Ca\(^{2+}\) channels also influence regulation by PIP\(_2\) (Suh et al., 2012). Cav channels co-expressed with the β3 subunit could be partially inhibited by activating a voltage-sensitive lipid phosphatase to deplete PIP\(_2\) (Suh et al., 2010). However, when these channels were co-expressed with the β2a subunit, the inhibition was smaller (Suh et al., 2012). The palmitoylation of two cysteine residues in the N terminus of the β2a subunit was responsible for this decrease in inhibition of Cav channel activity. When the palmitoylation sites were mutated, the β2a subunit behaved more like a β3 subunit. Furthermore, addition of a lipidation motif to β3 subunits reduced the inhibitory effects of Cav channels, similar to the palmitoylated β2a subunit (Suh et al., 2012). Thus, Cav channel...
Voltage-gated potassium (Kv) channels are involved in diverse physiological processes, including action potential repolarization, secretion of hormones and neurotransmitters, contraction of skeletal muscle, and others. Kv channels are homotetrameric, with each subunit containing the S1–S4 voltage sensor domain and the S5–S6 central pore domain (Yellen, 2002). As with Cav channels, the movement of the voltage sensor domain in response to membrane depolarization initiates conformational changes that lead to the pore opening. After channel opening, Kv channels undergo a time-dependent loss of conductivity by a mechanism termed inactivation. Two distinct mechanisms of inactivation have been described, N-type (or "ball and chain") inactivation, in which the N-terminal domain of certain α or β subunits of Kv channels plugs the open channel pore from the cytoplasmic side (Hoshi et al., 1991), and C-type inactivation, which appears to result from constriction of the selectivity filter (Yellen, 1998).

Only a few studies have demonstrated PIP$_2$ involvement in the regulation of voltage-dependent K$^+$ channels. PIP$_2$ shows remarkable effects on the N-type inactivation of certain voltage-gated K$^+$ channels, specifically, Kv1.4 and Kv3.4 (in which the "ball domain" is located in the N terminus of the α pore-forming subunit) and Kv1.1 co-expressed with the "ball domain"-containing Kvβ1.1 accessory subunit (Oliver et al., 2004). Application of exogenous PIP$_2$ to the intracellular side of the membrane expressing these channels removed the rapid N-type inactivation, regardless of whether the "ball domain" resided at the N terminus of the channel α or β subunit. It was proposed that PIP$_2$ insertion into the plasma membrane immobilized the positively charged "ball domain" through its negatively charged head-group and thereby prevented it from accessing the open pore. In this study a cluster of positive residues formed by Arg$^{17}$ and Lys$^{14}$ in the "ball domain" of Kv3.4 were proposed as the place where the electrostatic interaction left-shifted the voltage dependence of activation, increasing current at the same time it decreased the open probability causing an overall decrease in the current level (Decher et al., 2008). Thus, changes in intracellular PIP$_2$ levels might be important for the inactivation of Kv channels and this would profoundly alter electrical signaling.

Shab channels, a prototypical member of the Kv2 channels subfamily (Wei et al., 1990) are also modulated by PIP$_2$. It was shown that standard light stimulation of Drosophila photoreceptors increased Shab currents (Krause et al., 2008). After light stimulation, the voltage dependence of activation of Shab channels was shifted to hyperpolarized potentials about 10 mV, with a small decrease on the current amplitude at depolarized potentials. The mechanism proposed for this modulation of Shab channels involves the activation of PLCβ4 and the resulting hydrolysis of PIP$_2$. Interestingly, a point mutation (R435Q) in the N terminus of the Shab channel abolished the PIP$_2$ hydrolysis effect. The same results were obtained expressing recombinant Shab channels in Drosophila S2 cells and recording currents in inside-out patches. Within a few minutes of patch excision the threshold for activation was left-shifted about 10 mV, an effect that was almost completely reversed by application of diC8-PIP$_2$ to the patch. These results led Krause et al. (2008) to suggest that PIP$_2$ may interact directly with the Shab channel and modulate its activity.

Kv1.3 is another voltage-gated K$^+$ channel reported to be modulated by PIP$_2$. The Kv1.3 channel is important in the activation and function of effector memory T cells (Gilhar et al., 2011). Recently, it was reported that PIP$_2$ applied through patch pipettes in whole-cell recordings significantly reduced Kv1.3 currents in Jurkat T cells and this regulation may be significant for the maintenance of T lymphocyte activation in immune responses (Matsushita et al., 2009). However, the mechanisms of this modulation need to be further explored.

Contrary to previous reports on Kv channel modulation by PIP$_2$, Krause et al. (2012) recently tested a long list of Kv channels and found most of them insensitive to PIP$_2$; this list includes Kv1.1/Kvβ1.1, Kv1.3, Kv1.4, Kv1.5/Kvβ1.3, Kv2.1, Kv3.4, Kv4.2, and Kv4.3 (with different KCn channels). To test the effect of PIP$_2$ on Kv channels this group used three different strategies to deplete PIP$_2$ in intact cells, activation of the G protein-coupled muscarinic receptor M1, a zebrafish voltage-sensitive lipid 5-phosphatase (Dr-VSP), or an engineered fusion protein carrying both lipid 4-phosphatase and 5-phosphatase activity (pseudojovanin). Krause and colleagues offer some explanations for the discrepancy between previous experiments and their results, principally relying on the different strategies used to modulate the PIP$_2$ concentrations in the membrane. While previous groups applied exogenous PIP$_2$ to inside-out excised patches risking to increase PIP$_2$ concentration to supramaximal levels, this group transiently depleted PIP$_2$ in the membrane of intact cells maintaining all the constituents of the cell near normal conditions (Hilgemann, 2012; Krause et al., 2012).

Recently, we also examined the effects of PIP$_2$ depletion on the voltage-dependent Kv1.2 channel using different approaches not only in inside-out patches but also in intact cells, using the voltage-sensitive phosphatase Ci-VSP. We obtained similar results to the Cav channels, namely a bidirectional regulation, where PIP$_2$ depletion left-shifted the voltage dependence of activation, increasing current at the same time it decreased the open probability causing an overall decrease in the current level (Figure 1C, Rodriguez-Menchaca et al., 2012). These two effects were kinetically distinct and exhibited distinct molecular determinants and sensitivities to PIP$_2$. The effect on the voltage dependence of activation proceeded through interactions of the S4–S5 linker that links the voltage sensor to the channel pore with PIP$_2$ (Figure 1A). Gating current measurements revealed that PIP$_2$ constrains the movement of the sensor via specific interactions of basic residues with PIP$_2$ in the closed state. In summary, using a similar strategy as Krause et al. (2012) to transiently deplete PIP$_2$ in intact cells namely a voltage-sensitive phosphatase, we obtained contrasting results showing a dual effect on Kv1.2 and Shaker channels after PIP$_2$ depletion. In contrast, Krause et al. did not observe any effect on the current
amplitude of several Kv channels after PIP$_2$ depletion and did not test for changes in voltage dependency.

Consistent with our results, Abderemane-Ali et al. (2012) also reported a dual effect of PIP$_2$ on Shaker potassium channels; PIP$_2$ exerts a gain-of-function effect on the maximal current amplitude and a positive shift in the voltage dependence of activation, through an effect on the voltage sensor movement.

**HYPERPOLARIZATION-ACTIVATED HCN CHANNELS**

Hyperpolarization-activated Cyclic nucleotide-gated (HCN) channels unlike most voltage-gated channels open only in response to membrane hyperpolarization (Gauss et al., 1998; Ludwig et al., 1998; Santoro et al., 1998). The S1–S4 domain constitutes the voltage sensor again but with HCN channels the S4 segment moves inward upon membrane hyperpolarization (Manniiko et al., 2002; Bell et al., 2004). The coupling mechanism between this inward movement of the S4 and the opening of the gate, which is presumably located near the intracellular end of S6, is not clear. The cytoplasmic domain of HCN channels possesses a canonical cyclic nucleotide binding domain (CNBD). The CNBD is connected to the S6 segment through a 90-aa sequence called the C-linker (CL). Direct binding of cAMP or cGMP (cNMP) to the CNBD facilitates channel opening. At the macroscopic current level, the cNMP-dependent gating right shifts the voltage dependence of activation and increases the current amplitude (Robinson and Siegelbaum, 2003; Craven and Zagotta, 2006; Biel et al., 2009). The CL plays a dominant role in the coupling of cNMP binding to the channel opening.

Hyperpolarization-activated cyclic nucleotide-gated channel activity is also under the control of PIP$_2$. PIP$_2$ right shifts the hyperpolarization-dependent HCN channel activation, making the channel easier to open (Pian et al., 2006; Zolles et al., 2006; Flynn and Zagotta, 2011; Ying et al., 2011). This effect is remarkable – about 20 mV for mammalian HCN channels (HCN1, 2, and 4) and independent from the regulation by cAMP and cGMP. Direct evidence supporting these conclusions came from the experiments on inside-out membrane patches. Applying either native PIP$_2$ or the soluble diC$_8$-PIP$_2$ produced a depolarizing shift in HCN channel activation (Pian et al., 2006; Zolles et al., 2006; Flynn and Zagotta, 2011). The right shift in the I–V relationship could be reproduced separately under conditions of saturating concentrations of cAMP; in mutant channels that do not bind to cNMP, or in the HCN AC channels, in which the CL-CNBD has been deleted. Therefore, the interaction between PIP$_2$ and the transmembrane domains with their connecting loops of HCN channels are likely to be responsible for the voltage-dependent effect rather than the CL and CNBD, which are essential for cAMP-dependent gating. It has been suggested that most likely, PIP$_2$ exerts its effect on voltage dependence by stabilizing the activated state of the voltage sensor, which could be through either a generalized effect on the local electrostatic environment by PIP$_2$ or a specific interaction between the negatively charged head-group of PIP$_2$ with positively charged residues in the S4 or its surroundings (Flynn and Zagotta, 2011).

SpIH, a HCN channel cloned from Sea Urchin, can be fully activated by cAMP but only partially by cGMP (Flynn et al., 2007). Unlike the mammalian HCN channels, SpIH shows a bidirectional regulation by PIP$_2$ (Flynn and Zagotta, 2011). Like mammalian HCN channels it shows a right shift of the voltage dependence of activation (~10 mV) in the presence or PIP$_2$, albeit half the magnitude of that in the mammalian channels (Figure 1D). Unlike the mammalian HCN channels, SpIH inactivates quickly in response...
to a hyperpolarizing voltage step (Gaus et al., 1998). Binding of cAMP or cGMP relieves this voltage-dependent inactivation and markedly increases the macroscopic current amplitude. Independent from the positive effect on the voltage-dependent activation, PIP$_2$ seems to have an inhibitory effect on the cGMP-induced current (Figure 1D). This inhibitory effect is clearly related with the efficacy of the agonists. The maximal current under saturating concentration of cGMP is only about half of that of cAMP. Consistently, PIP$_2$ strongly inhibits the cGMP-dependent current but has a minimal effect on the cAMP-induced current.

Noticeably, as reported more than a decade ago, PIP$_2$ has a strong inhibitory effect on CNG channels, which is homologous to HCN channel and the cNMP binding is obligatory for its opening (Womack et al., 2000; Kaupp and Seifert, 2002). Several studies have produced atomic resolution structures of the HCN cytoplasmic C-terminal domains including that from SpnH channel (Zagotta et al., 2003; Flynn et al., 2007; Xu et al., 2010). Mutagenesis studies of the SpnH channel have identified several positively charged residues in the CL that contribute to the inhibitory effect by PIP$_2$ (Figure 1B). It has been suggested that through these specific electrostatic interactions, PIP$_2$ inhibits the channel opening by stabilizing the conformation of the CL that underlies the closed channel state in the absence of agonists (Flynn and Zagotta, 2011).

**CONCLUDING REMARKS**

The bidirectional effects of PIP$_2$ in three very different voltage-sensitive channels is quite remarkable. In the depolarization-activated Cav2.1 (Wu et al., 2002) and Kv1.2 (Rodríguez-Menchaca et al., 2012) studies, PIP$_2$ right-shifted the voltage dependence of activation (inhibitory) while it prevented rundown by stabilizing the open probability of the channel (stimulatory). In the hyperpolarization-activated HCN channel from the sea urchin (SpnH) PIP$_2$ right-shifted the voltage dependence of activation (stimulatory), while it inhibited the cGMP-induced activation (inhibitory). In Kv1.2, PIP$_2$ stabilized the inactive state of the voltage sensor by binding basic residues in the S4–S5 linker and the N terminus that could only coordinate PIP$_2$ in the closed state of the channel. In the SpnH HCN channel it was CL basic residues that accounted for the non-voltage-dependent effects of PIP$_2$. In both cases these molecular determinants affected only one or the other of the dual effects of PIP$_2$. These results from these very different channels (Cav, Kv, and HCN) suggest that the mechanism by which PIP$_2$ regulates channels sensitive to voltage may be conserved.

What might be the physiological significance of the dual regulation of certain voltage-gated ion channels that we have highlighted in this review? We speculate that in channels that are dependent on voltage but also to other modulatory intracellular signals, a balance needs to be achieved to regulate gating in a coordinated manner. PIP$_2$ and the S4–S5 linker are both perfectly positioned at the interface of the membrane to the cytosol to integrate cytosolic signals (e.g., cyclic nucleotides, Ca$^{2+}$, etc.) with the movement of the transmembrane voltage sensor.
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