The where and how of PIP regulation of cone photoreceptor CNG channels

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In this issue, Dai et al. present an investigation of the biophysics of phosphatidylinositide (PIP) regulation of the cyclic-nucleotide gated (CNG) channels expressed in cone photoreceptors. Although PIPs facilitate or stabilize the activation of many other types of ion channels, they strongly inhibit the response to cAMP or cGMP (collectively, cNMP) of CNG channels. Previous studies have addressed the molecular basis for PIP regulation of the olfactory CNG channel but not for that of rod and cone CNG channels. From a myriad of potential mechanisms, the present study elegantly teased out two regulatory structural elements that are located in the N- and C-terminal regions of the cone CNG channel and identified a modulatory mechanism that involves complex intersubunit communications. In conjunction with earlier studies on PIP regulation of the olfactory CNG channel and related hyperpolarization-activated CNG (HCN) and HERG channels, the present study provides insights into the molecular mechanism and the physiological role of PIP regulation of CNG channels.

Cyclic nucleotides act as second messengers inside of cells. In the olfactory and visual systems, exogenous stimuli, odorants or photons, respectively, activate G-protein coupled receptors (GPCRs), triggering signal transduction pathways that result in an increase in cAMP concentration in olfactory neurons or a decrease in cGMP concentration in photoreceptors. In both of these sensory systems, CNG channels transduce the signal encoded by the changes in intracellular cNMP into changes in the membrane excitability of the primary neurons. Molecular cloning has identified six major types of CNG channels, including three α subunits (A1, A2, and A3) that can themselves form functional channels and three β subunits (A4, B1, and B3) that share similar topology with the α subunits but which form functional channels only when coassembled with α subunits (Zagotta and Siegelbaum, 1996; Kaupp and Seifert, 2002). Native CNG channels are composed of A2, A4, and B1b in a 2:1:1 ratio (olfactory neurons); A1 and B1a (3:1, rods); and A3 and B3 (2:2, cones), respectively (Weitz et al., 2002; Zheng et al., 2002; Zhong et al., 2002; Peng et al., 2004; Zheng and Zagotta, 2004). By coassembling with the α subunits, the β subunits promote the proper trafficking of α subunits to their subcellular destination and fine tune channel sensitivity to cNMP and other regulators, including membrane potential, calmodulin, and PIPs. In both olfactory neurons and photoreceptors, homomeric (α subunit) channels that have been well-characterized in *Xenopus laevis* oocytes and HEK293 cells fail to traffic to the appropriate areas of cilia and outer segments, highlighting the importance of the modulatory β subunits as molecular chaperons in polarized membrane trafficking (Biel and Michalakis, 2007).

Studies over the past 15 years have revealed that PIPs, which are low-abundance lipid molecules mainly distributed on the inner leaflet of the membrane, associate with and modify the function of virtually all membrane proteins, especially ion channels and transporters (Hilgemann et al., 2001; Suh and Hille, 2008; Logothetis et al., 2010). PIPs act as signaling molecules through various mechanisms. PIPs carry a substantial number of negative charges in their headgroups, up to five for phosphatidylinositol 4,5-biphosphate (PIP_2) and seven for phosphatidylinositol 3,4,5-triphosphate (PIP_3). Thus, PIPs directly interact with positively charged residues in integral membrane proteins and in membrane-associated proteins to modulate their structures and functions. Two models are thought to mediate PIP binding: (1) a structured binding pocket that provides high binding affinity and PIP selectivity and potentially a fixed binding stoichiometry; and (2) a less-organized form that enables diffusive and dynamic electrostatic contacts with PIPs (Suh and Hille, 2008). The crystal structure of the Kir 2.2 channel in complex with PIP_2 provides an atomic view, especially in the interfacial region between the transmembrane domain (TMD) and the cytoplasmic domain (CTD), where PIP_2 binds to a well-defined pocket in the channel (model 1) and exerts its regulatory function. PIP_2 binding leads to a 6-Å translational movement that brings the TMD and CTD closer, which is predicted to be a key molecular motion mediating PIP-dependent gating (Hansen et al., 2011). Moreover, because the activity of most channels depends on the interactions with

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PIPs, hydrolysis or dephosphorylation of PIPs regulates protein activity. Membrane-associated, receptor-activated PLC catalyzes the hydrolysis of PIP_2 to produce inositol 1,4,5 triphosphate (IP_3) and diacylglycerol (DAG), two second messengers that mobilize intracellular Ca^{2+} and activate PKC, respectively. Still, many details of the biophysics and physiology of PIP-dependent channel regulation remain to be clarified. Depletion of physiological PIP_2 through the activation of GPCRs or membrane-affiliated enzymes strongly inhibits the activity of Kir and M-type (Kv7) potassium channels, but for many other types of Kv channels, it has been unclear whether, under physiological conditions, PIP_2 dynamically regulates channel function or acts constitutively (Hilgemann, 2012; Kruse et al., 2012).

Patch-clamp recording of heterologously expressed A1/B1 heteromers and native rod CNG channels showed that PIP_2 application to the intracellular side of excised patches reduced maximal current and the apparent affinity for cGMP (reflected in an increase in $K_{1/2}$ or EC50, the concentration of cGMP that produces half maximal opening; Womack et al., 2000). PIP_3 inhibits heterologously expressed olfactory CNG channels (Brady et al., 2006). PIP_3 reduces the sensitivity to cAMP ~30-fold for the homomeric CNGA2 channel or eightfold for the heteromeric A2/A4/B1b channel, and reduces the maximal current by half for both. Truncation of a 30-aa structural element located in the N terminus of CNGA2 that contains multiple positively charged residues abolished PIP_3's inhibitory effect. This region also harbors a binding site for calmodulin, which has inhibitory effects on CNGA2. PIP_3 treatment occludes the inhibition by calmodulin, indicating a competition between the PIP_3 and calmodulin regulatory pathways. Possibly, because of their nonselective and omnipresent nature, PIPs can directly interfere with and compete with other modulatory factors, such as calmodulin, and act through the same or closely coupled gating apparatus. This feature has been recognized in other channels such as the G protein–coupled inwardly rectifying potassium (GIRK) channels, in which PIP_2 and G proteins act synergistically on the cytoplasmic G loop gate and the inner helix gate to open the channel (Petit-Jacques et al., 1999; Whorton and MacKinnon, 2011).

The research group led by M.D. Varnum reported in 2007 that PIP_3 inhibits the A3/B3 heteromer and decreases its sensitivity to cGMP (Bright et al., 2007). Here, the same research group tackled the question of where and how PIPs interact with and regulate cone CNG channels. They first characterized two opposite regulatory effects by PIPs: (1) in homomeric A3 channels, PIPs actually increased the maximal current elicited by saturating concentrations of cAMP, which is a partial agonist for the A3 channel and elicits only minimal channel opening at saturating concentrations (Po: 0.14, cAMP; 0.97, cGMP); (2) in heteromeric A3/B3 channels, PIPs decreased the apparent affinity for cGMP by 60% and thus the sensitivity to the physiological agonist. Subsequently, the authors identified two structural elements, each containing seven positively charged residues within a stretch of 30 aa, that mediated responses to PIPs. One of these elements was located in the N terminus, preceding the TMD, and the other was located in the C terminus, immediately after the cyclic nucleotide binding domain (CNBD; Fig. 1 A).

Next, the authors mutated these two structural elements to investigate the mechanisms of PIP regulation. The C-terminal element played a major role in mediating the potentiation of cAMP gating of the A3 homomeric channel by PIPs. Truncation of the C-terminal element downstream of CNBD abolished the PIP-dependent increase in cAMP-gating efficacy but, at the same time, uncovered a PIP-dependent inhibition of sensitivity to cGMP, as had been observed in A3/B3 heteromers. This inhibition could be abolished by ablating both N- and C-terminal elements. Finally, the authors showed that both elements synergistically contribute to PIP suppression of cGMP gating in A3/B3 heteromers. Removing either of the structural elements only partially reduced the regulation by PIPs.

The location of the C-terminal element, in conjunction with independent biochemical evidence supporting a direct interaction with PIP_3, suggests that the C-terminal region of CNGA3 downstream from CNBD might be in close contact with the lipid membrane. This picture is at odds with the current view of the CNG channel C-terminal structure, which is based on the homologous structures of HCN channels and ELK potassium channels in the KCNH family, two types of K channels that most likely share a common ancestor with CNG channels (Zagotta et al., 2003; Xu et al., 2010; Brelidze et al., 2012). In the HCN channel structure, the C-terminal end of the C-helix, the last structured element in CNBD, is located ~50 Å away from the lipid membrane (Fig. 1 B, left). In the ELK channel, the C-helix becomes shorter and the immediate downstream sequence forms a short β strand that occupies the canonical cNMP binding site and potentially functions as an intrinsic ligand (Fig. 1 B, right). Thus, both structures position the C-terminal PIP interacting element in CNGA3 potentially at a distance from the lipid membrane, which makes any direct contacts with PIPs on the lipid membrane less likely. However, consistent with the current study, the comparable region of the HERG channel, also downstream from the CNBD, was proposed to underlie PIP regulation, and based on functional data, was pictured to be in close contact with the membrane (Bian et al., 2004). The recently reported structure of the mosquito ERG channel C-terminal fragment might provide a clue for this puzzle (Brelidze et al., 2012). In the ERG channel, the C-linker, which couples the CNBD with the TMD and critically determines cNMP-dependent gating efficacy,

adopts a drastically different conformation from those of HCN and ELK channels, which indicates the intrinsic flexibility of this region. It might be possible that during cNMP-dependent ligand gating, the C-terminal fragment undergoes a substantial conformational change to promote a dynamic contact between PIPs in the lipid membrane and the distal C-terminal element. Indeed, significant conformational changes including a rearrangement of subunit assembly and thus a change in channel symmetry have been proposed for the ligand-dependent gating in CNG and HCN channels (Liu et al., 1996; Richards and Gordon, 2000; Ulens and Siegelbaum, 2003; Taraska and Zagotta, 2007). Another possibility, favored by the authors, is that the distal C-terminal structure element is not in direct contact with the lipid membrane, but mediates the response to PIPs by interacting with other channel-interacting proteins that bind PIPs (Dai et al., 2013). Growth factor receptor–bound protein 14 is a potential candidate because it contains a pleckstrin homology domain that binds to PIPs and has been found to interact with the C terminus of CNGA1 (Gupta et al., 2010).

Noticeably, both of the PIP binding elements are located distally from the "core" of the cone CNG channel, the transmembrane domain that harbors the ion-conducting pore. In contrast, PIPs closely associate with the pore of many other types of ion channels and form intimate contacts with the surrounding structural elements, including the intracellular end of S4, the S4–S5 linker, the intracellular end of S6 where the inner activation gate is located, etc. In the C-linker of the SpIH HCN channel, positively charged residues are found to mediate the regulatory effects by PIP_2 (Flynn and Zagotta, 2011). Potential PIP-interacting sites located in those regions in cone CNG channels remain to be identified. Nevertheless, the distributed localization of the PIP response elements is consistent with the variety of the modulatory effects of the PIPs potentially move multiple parts of the gating machinery, as has been indicated for other channels, including the inward rectifying K channel, the depolarization-activated Kv channel and calcium channel, and the hyperpolarization-activated HCN channel (Wu et al., 2002; Pian et al., 2006; Zolles et al., 2006; Flynn and Zagotta, 2011; Abderemane-Ali et al., 2012; Rodríguez-Menchaca et al., 2012b). For Kv channels, PIPs exert a dual effect on channel gating: they stabilize the open state, reflected in the increase in maximal current amplitude, and constrain the movement of the voltage sensor, reflected in the right-shift of voltage-dependent activation (Rodriguez-Menchaca et al., 2012a). In the case of HCN channels, PIP_2 shifts the I-V curve in a depolarized direction by ~20 mV and thus enhances channel gating; at the same time, it suppresses the HCN maximal current. It remains to be clarified for each case whether different groups of PIPs interact with the corresponding structural elements or if the same group of PIP molecules produces diverse effects by interacting with different structural elements in a state-dependent manner. The low selectivity between PIP_2 and PIP_3 by CNGA3 is consistent with the notion that the N- and

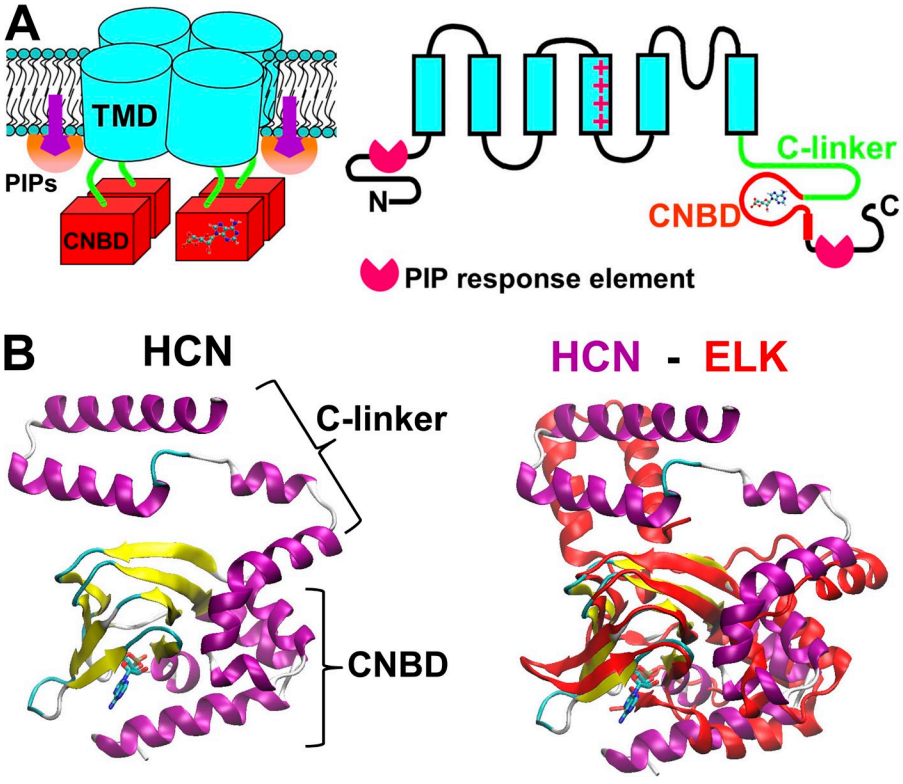

Figure 1. CNG channel topology and homologous C-terminal structures. (A) Schematic drawing of CNG channel topology. Two structural elements underlying the response to PIPs, located on the inner leaflet on lipid membrane, have been identified in the N and C terminus of CNG channels, respectively. (B) Left, crystal structure of human HCN4 channel C-terminal fragment (Xu et al., 2010). Right, overlay of HCN and zebrafish ELK C-terminal structures (Brelidze, T.I., F. DiMaio, M.C. Trudeau, and W.N. Zagotta. 2013. Biophysical Society 57th Annual Meeting. Abstr. 1826-plat). The CNBDs can be aligned well but the C-linkers cannot.

C-terminal structural elements do not form specific and structured binding pockets for PIPs and might dynamically interact with PIPs on the membrane as proposed in model 2 (as discussed earlier) for PIP binding (Suh and Hille, 2008).

All told, the study by Dai et al. (2013) provides a detailed analysis of the structural basis of PIP regulation of cone CNG channels heterologously expressed in *Xenopus* oocytes. Most likely, the two structural elements identified in the N- and C-terminal regions from different subunits function cooperatively to produce the PIP-dependent inhibition of the native channel that contains both A3 and B3 subunits. Notably, the B3 subunit, which diversifies the channel's response to external stimuli, including sensitivity to calmodulin and membrane potential, and regulates native channel biogenesis, does not seem to contain PIP response elements. It remains to be determined whether other than the response to cNMP and gating properties, PIPs modulate the folding, assembly, and trafficking of these CNG channels in the native membranes of cone photoreceptors, and how such regulation is exploited by cones in their light responses or other cell biological functions.

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