Putting G protein–coupled receptor-mediated activation of phospholipase C in the limelight

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Summary

Phospholipase C (PLC) activation by cell surface receptors has been recognized as a fundamental early transmembrane signaling event that triggers a wide variety of cellular responses. These range from egg fertilization through immune cell activation, hormone secretion, and synaptic transmission to invertebrate photoreception. In each case, ligand binding to cell surface receptors initiates a chain of similar molecular events that involve heterotrimeric G nucleotide–binding proteins and PLC enzymes, ultimately leading to the hydrolysis of the plasma membrane regulatory lipid, phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2). Each of the individual molecular steps along this cascade has been scrutinized separately in populations of cells or purified membrane preparations, or using reconstituted recombinant proteins by in vitro biochemical analysis, providing invaluable information about their inner molecular workings. However, recent progress in fluorescence technology has now allowed detection and kinetic analysis of each of these biochemical events in single living cells. For the first time, these molecular steps have been put in a sequence after a thorough systematic analysis of each of them with true rate constants measured in intact cells. This accomplishment, combined with mathematical modeling, has created a novel framework in which the individual molecular steps could be analyzed and predictions be tested about their regulatory features. These new developments will help us better understand the question of what made this pathway such a suitable instrument for the detection of a plethora of signal modalities in eukaryotic cells.

The universal PLC signaling system

It is paramount in cell–cell communication that cells be able to receive and decode environmental signals to respond appropriately. How cells detect external cues and transmit information to the cell interior has been the subject of signal transduction research for half a century. It was in the 1960's and 70's that researchers in pioneering studies demonstrated the presence of high affinity and specific hormone binding sites, called receptors, on the cell surface and postulated that GTP-binding proteins were important in the signal transmission process across the plasma membrane (Gilman, 1987). With the discovery of cAMP, the first "second messenger" was introduced (not counting Ca^{2+} ions), and hence, the basic principles of transmembrane signaling have been outlined (Grahame-Smith et al., 1967). The cAMP system notwithstanding, one of the most widely used signal transmission processes in all eukaryotic cells is the receptor-mediated stimulation of PLC enzymes that is coupled to cytoplasmic Ca^{2+} increases and to the activation of several protein kinase cascades (Berridge, 1984). The universality of this system is best showcased by its fundamental role in both relatively simple processes, such as egg fertilization or invertebrate photosensing, as well as in those with the highest complexity, such as cellular communication between neurons in the brain.

The individual elements in the PLC activation cascade vary between the different cellular systems. For example, receptors coupled to PLC activation could be the heptahelical, G protein–coupled kind, such as the angiotensin II, the V2 vasopressin, or the m1, m3 muscarinic, and the α1-adrenergic receptors, to name a few. However, receptor tyrosine kinases are also able to activate the PLC signaling system by using different PLC isoforms and bypassing the heterotrimeric G proteins. Similarly, there is more than one class of heterotrimeric G proteins that activates PLC enzymes; the most frequently used is the Gq/11 family members or those belonging to the pertussis toxin–sensitive Gi/Go family. PLC enzymes also show a great variety (Rebecchi and Pentyala, 2000). The best-known ones are the PLC-β forms that are activated by heterotrimeric G proteins (either their α or βγ subunits) and the PLC-γ forms that are regulated by receptor tyrosine kinases. PLC-ε is primarily controlled by small GTP-binding proteins, whereas less is known about the regulatory features of additional PLC isoforms, although all PLCs are stimulated by Ca^{2+} ions (Harden and Sondek, 2006).

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For a relatively long period of time, PLC activation was thought to serve the sole purpose of generating two messenger molecules, inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) and diacylglycerol by hydrolyzing the small pool of $PtdIns(4,5)P_2$ in the plasma membrane. $Ins(1,4,5)P_3$ liberates Ca^{2+} from internal Ca^{2+} stores and hence indirectly controls Ca^{2+} entry via store-operated Ca^{2+} entry pathway(s), and diacylglycerol stimulates the activity of protein kinase C enzymes, thereby initiating a cascade of downstream signaling responses (Berridge, 1984). However, an increasing body of evidence suggested that plasma membrane $PtdIns(4,5)P_2$ is also a regulatory molecule and that changing its level influences cytoskeletal dynamics, endocytosis, and exocytosis, and the activity of enzymes, ion channels, and transporters (Hilgemann et al., 2001; Di Paolo and De Camilli, 2006). One of the earliest of these was the discovery that plasma membrane $PtdIns(4,5)P_2$ is a regulator of certain forms of potassium channels (Hilgemann and Ball, 1996).

How to study $PtdIns(4,5)P_2$ changes in intact living cells

The importance of $PtdIns(4,5)P_2$ as a regulatory lipid required that its localization and dynamic changes be followed in intact living cells. Several groups have worked independently to achieve this goal (Balla et al., 2000). They all used the pleckstrin homology (PH) domain of the PLCδ1 enzyme that had been known to bind with high affinity and specificity to $PtdIns(4,5)P_2$ (Ferguson et al., 1995). This module, fused to green fluorescent protein (GFP) and expressed in cells, was able to show where $PtdIns(4,5)P_2$ was located in intact cells and how its level was changing after PLC activation (Stauffer et al., 1998; Várnai and Balla, 1998). These and other studies detecting other forms of inositides initiated a new era of phosphoinositide research, with the added benefit of spatial information and dynamic time-lapse analysis. However, the new approach also had its own caveats. Expression of a $PtdIns(4,5)P_2$ binding reporter occupies the lipid $PtdIns(4,5)P_2$, hence interfering with the lipid's ability to regulate downstream effectors and forcing the cells to make more of it as a way of compensation. Moreover, the soluble $Ins(1,4,5)P_3$, having a high affinity to the PH domain reporter, competes with its $PtdIns(4,5)P_2$ binding, complicating the interpretations of how to relate changing probe distribution between the membrane and cytosol to $PtdIns(4,5)P_2$ changes in the plasma membrane. A molecular modeling study that offered experimentally testable predictions was instrumental in understanding the behavior of the PH probe and the impact of its expression on lipid changes (Xu et al., 2003).

The high fidelity by which certain K^+ channels (Kir and the KCNQ) followed $PtdIns(4,5)P_2$ changes has offered an alternative approach to follow $PtdIns(4,5)P_2$ changes in patch clamp studies (Horowitz et al., 2005). The advantage of this method was that it could monitor $PtdIns(4,5)P_2$ changes without significant distortion, as the number of channels required for these analyses was negligible compared with the PH domains, causing significantly less distortion in the system. However, it took some time to prove that the channel behavior in the cells indeed reflected $PtdIns(4,5)P_2$ changes and not the action of some of the messengers generated by PLC activation. For this, one needed to find a way to rapidly change $PtdIns(4,5)P_2$ levels in the membrane separately from PLC activation within the intact cells. Previous attempts to do this used overexpression of PIP 5-kinase or $PtdIns(4,5)P_2$ 5-phosphatase enzymes and determined channel activities 16–24 h later. Unfortunately, such long-term manipulations with these enzymes can completely rework the trafficking pattern of the cells, introducing several secondary changes that could lead to altered channel activities. To overcome this problem, two groups independently developed a method by which an active $PtdIns(4,5)P_2$ 5-phosphatase domain could be rapidly recruited from the cytosol to the plasma membrane to quickly deplete membrane $PtdIns(4,5)P_2$ (Suh et al., 2006; Varnai et al., 2006). This method was based on the rapamycin-inducible heterodimerization of the FKBP12 protein and the FRB fragment of mTOR previously described and characterized by Belshaw et al. (1996). In these studies, the targeting of one of the partners (FRB) to the plasma membrane and fusing the 5-phosphatase (stripped of its own localization sequences) to FKBP12 allowed a drug-regulated acute depletion of $PtdIns(4,5)P_2$ in the membranes. With these elegant studies, Suh et al. (2006) unequivocally showed that KCNQ2/3 channels indeed follow $PtdIns(4,5)P_2$ changes without the generation of any other messengers.

Using Förster resonance energy transfer (FRET) to probe conformational transitions

Rapid advances in GFP technology yielded spectral variants such as CFP and YFP (followed by further improved versions; see Shaner et al., 2005) that could be used as donor-acceptor pairs for FRET analysis (Miyawaki et al., 1997). FRET occurs when an excited CFP molecule can directly transfer its energy to an adjacent YFP molecule without radiation causing the latter to emit light with its own characteristic emission spectrum. However, FRET only occurs when the donor and acceptor molecules are in close proximity and proper dipole orientation. Because FRET efficiency decreases with the sixth power of the distance between the two partners, this principle has great potential in determining molecular proximity (in the 1–10-nm range) or a change in dipole orientation (for more on FRET, see Wouters and Bastiaens, 2001).

This principle was used to follow the ligand-induced conformational change of G protein–coupled receptors by placing CFP and YFP (or other appropriate fluorescent molecule pairs) within the third intracellular loop

and the end of the C-terminal tail of the receptor (Vilardaga et al., 2003). Using a similar principle, the activation state of heterotrimeric G proteins was monitored with FRET after the insertion of fluorescent molecules into the α subunit and into the βγ subunits, respectively (Janetopoulos et al., 2001). FRET was also used to follow PLC activation simply by expressing both the CFP- and YFP-fused versions of the PLCδ1 PH domain. Here, FRET occurs when the PH domains are at close proximity as they bind to PtdIns(4,5)P_2 in the membrane, and the release of the probes from the membrane due to PLC activation is reflected in a FRET decrease (van der Wal et al., 2001). These important advances in live cell monitoring of the individual signaling steps (see Balla, 2009) made it possible to follow the whole sequence in real time and determine the kinetic parameters of these reactions.

Connecting all of the pieces together

In two papers in this issue (see Falkenburger et al. [Kinetics of M_1 muscarinic receptor...] and Falkenburger et al. [Kinetics of PIP_2 metabolism...]), the authors used all of the above-listed advances and performed a systematic analysis of the signal transmission process starting with M1 muscarinic receptors and mediated via Gq proteins to activation of PLCβ1 yielding to PtdIns(4,5)P2 hydrolysis and altered KCNQ channel activity. In a previous paper (Jensen et al., 2009), the authors measured the kinetic parameters of M1R activation, Gq conformational transition, PLC activation, PtdIns(4,5)P2 hydrolysis, and M current suppression after the application of 10 μM of the muscarinic agonist, oxotremorin, using the FRET approach for the individual steps outlined above. These studies measured activation and recovery rates (after removal of the stimulus) for each of the steps in this series of reactions and concluded that the rate-limiting step was the hydrolysis of PtdIns(4,5)P_2 in the membrane. However, all of these measurements were done in cells expressing the appropriate fluorescent proteins for FRET analysis, and the amounts of these expressed reporters had significant impact on the kinetic parameters. Therefore, in the first of the two studies, the authors took a serious look at the expression level of these molecules and estimated in each case the number of fluorescent molecules by a series of thorough calibration measurements based on fluorescence. These measurements gave them a good estimate of the number of molecules that were overexpressed in the cells they studied in the kinetic analysis. With this information in hand, the authors constructed a kinetic model using their previously measured kinetic rate constants. They used the publicly available Virtual Cell Model framework (http://www.nrcam.uchc.edu) that was the basis of the study modeling the behavior of PH domains (Xu et al., 2003). The current model described the experimental observations remarkably well for all of the components, with kinetic parameters that were in line with published values determined by similar or alternative methods by previous studies. Importantly, the model allowed extrapolation of the kinetic parameters to "naive" cells expressing only endogenous levels of receptors, G proteins, and PLC enzymes. This analysis revealed that most of the delay observed in PLC activation stems from relatively slow activation of G proteins by receptors, and that PLC speeds up this process by increasing the GTP exchange and hydrolysis on the GTP-binding protein.

To extend the model to the steps that link PtdIns(4,5)P_2 changes to altered KCNQ channel activity, in the second study the authors used a recently described, very elegant tool to manipulate PtdIns(4,5)P_2 levels in the cells. They used the voltage-sensitive PtdIns(4,5)P_2 5-phosphatase enzyme (Murata et al., 2005) that allowed extremely rapid and reversible depletion of PtdIns(4,5)P_2. This approach has significant advantages over the above-discussed rapamycin-inducible system in terms of speed and reversibility of changes. With these manipulations, the authors showed that KCNQ2/3 channels follow a cooperative square law relative to PtdIns(4,5)P_2 changes. They also measured that the resident time of the lipid on the channels is comparable to that on the reporter PH domains. An important novel conclusion of these studies is that the rate-limiting step in the replenishment of the PtdIns(4,5)P_2 pools is the formation of PtdIns4P in the plasma membrane. The authors extended the kinetic model to these steps in the signaling cascade now covering the whole process from receptors to the KCNQ2/3 channels. These two studies have significantly advanced our understanding of the molecular steps in the PLC signaling cascade and clearly pinpointed the areas where further research is needed to fill the gaps. These studies exemplify the best in experimental design and precision, aiming for the quantitative description of a biochemical cascade that is widely used in eukaryotic cell regulation.

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