

A mechanism for different receptors coupled to the same G protein to generate different responses mediated by different second messengers

Don-On Daniel Mak

Physiology Department, University of Pennsylvania, Philadelphia, PA 19104

To respond to changes in the extracellular environment and maintain effective intercellular communication, a signal transduction system of astonishing complexity has evolved in eukaryotic cells. A cell can express a menagerie of literally thousands of different types of cell surface receptors, with each type binding its own set of agonist(s) with high specificity, and up to subnanomolar affinity. However, despite the diversity of the receptors, stimulation of receptors by extracellular agonists is transduced through only a relatively small number of second messenger systems (Downes and Macphee, 1990; Hartl and Wolfe, 1990; McKnight, 1991; Berridge, 1993; Vaandrager and de Jonge, 1996; Guse, 1999; Santella, 2005) to elicit stimulus-specific cellular responses. Thus, the fascinating question remains of how different cell surface receptors coupled to the same G protein, when stimulated by their individual agonists, can generate different kinds of responses mediated by different second messengers (Delmas and Brown, 2002; Zaika et al., 2011). Localizing intracellular signaling machinery into distinct compartmentalized microdomains is one mechanism to achieve differential regulation of cellular responses (Delmas and Brown, 2002; Bornfeldt, 2006; Zaccolo et al., 2006). A paper from the Hille laboratory in this issue of the Journal elucidates, with remarkably thorough and meticulous experimental work, another such mechanism: one that combines quantitative difference in cell surface receptor abundance with different sensitivity of various cellular responses to activation.

With this paper (see [Dickson et al.](#) in this issue), the Hille laboratory ventured into investigating the inositol 1,4,5-trisphosphate (IP₃)-cytoplasmic free Ca²⁺ (Ca²⁺) branch of the phospholipase C (PLC)-mediated signaling cascade (see Fig. 1) (Rhee, 2001) in their long-standing effort (Suh and Hille, 2002, 2006, 2007; Suh et al., 2004, 2006; Horowitz et al., 2005; Jensen et al., 2009; Falkenburger et al., 2010a,b) to study receptor modulation of the KCNQ2/3 potassium channel that generates the M current (Shapiro et al., 2000), which plays a critical role in the regulation of neuronal excitability (Hamilton et al., 1997). Intracellular signals related

to IP₃ production by PLC activity resulting from maximal activation of a purinergic or a muscarinic G_q protein-coupled receptor (G_qPCR) (Smrcka et al., 1991; Rhee, 2001) in cultured tsA201 cells were quantified and compared. Rise in cytoplasmic free Ca²⁺ concentration ([Ca²⁺]_i) caused by IP₃ activation of endoplasmic reticulum (ER)-localized IP₃ receptor (IP₃R) channels (Berridge and Irvine, 1989) was followed using Ca²⁺ imaging with Fura-4F dye, whereas the depletion of inositol 4,5-bisphosphate (PIP₂) due to hydrolysis by PLC was followed either by directly monitoring PIP₂ level using Förster resonance energy transfer (FRET) (van der Wal et al., 2001), or by measuring the decrease in KCNQ2/3 K⁺ current resulting from PIP₂ depletion using patch-clamp electrophysiology in perforated-patch configuration. The purinergic G_qPCR investigated was an endogenous receptor determined to be the P2Y₂R (Abbracchio et al., 2006) by its activation by uridine 5'-triphosphate (UTP) to generate IP₃ without increasing cytoplasmic [cAMP] (Nikolaev et al., 2004). This identification was further confirmed pharmacologically by the suppression of UTP-evoked [Ca²⁺]_i rise with P2Y₂R-specific antagonist suramin; and by changing the amplitude of UTP-evoked [Ca²⁺]_i rise through manipulation of P2Y₂R abundance, especially the elimination of UTP-evoked [Ca²⁺]_i rise with siRNA knockdown of P2Y₂R expression. Recombinant M₁ muscarinic receptor (M₁R) was transiently transfected into the tsA201 cells, which do not express endogenous muscarinic G_qPCR, with expression level several orders of magnitude higher than that of the endogenous P2Y₂R (Falkenburger et al., 2010a).

Whereas maximal UTP (100 μM) stimulation of the endogenous P2Y₂R and maximal oxotremorine-M (Oxo-M; 10 μM) stimulation of overexpressed M₁R generated similar [Ca²⁺]_i increases, maximal UTP stimulation of the endogenous P2Y₂R failed to cause any detectable suppression of the KCNQ2/3 current, even though maximal Oxo-M stimulation of overexpressed M₁R suppressed KCNQ2/3 current significantly. Inability of the

Correspondence to Don-On Daniel Mak: dmak@mail.med.upenn.edu

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maximally activated endogenous P2Y₂R to reduce PIP₂ abundance appreciably while maximally activated over-expressed M₁R depleted PIP₂ substantially was also confirmed by FRET measurement.

Given the huge difference in the abundance of the endogenous P2Y₂R and the transiently transfected M₁R, it is reasonable to surmise that the observed inability of the endogenous P2Y₂R to affect PIP₂ levels even in the presence of a saturating level of UTP is caused by its low expression level, whereas the overexpressed recombinant M₁R, when stimulated, has no problem depleting the PIP₂, thereby reducing the KCNQ2/3 current measurably. In that case, the comparable [Ca²⁺]_i rises elicited by both maximally activated endogenous P2Y₂R and overexpressed M₁R suggest that Ca²⁺ release from ER through the IP₃R is much more sensitive to activation by G_qPCR than PIP₂ depletion and KCNQ2/3 current reduction, so the number of ligand-bound G_qPCRs required to stimulate Ca²⁺ release is significantly smaller than that required to deplete PIP₂ and suppress KCNQ2/3 current. Therefore, even P2Y₂R expressed at low endogenous levels is sufficient to generate a maximal IP₃R-mediated Ca²⁺ response. In other words, the receptor reserve (spare receptors) for Ca²⁺ release is much larger than that for PIP₂ depletion.

To validate this hypothesis, the Hille laboratory modified the receptor density and agonist concentration independently. When tsA201 cells were transiently transfected with recombinant P2Y₂R to boost the density of P2Y₂R by ~100-fold, saturating UTP was able to generate PIP₂ depletion and suppression of KCNQ2/3 current, at levels comparable to those caused by maximal levels of Oxo-M. This demonstrated that the inability of UTP stimulation of endogenous P2Y₂R to affect PIP₂ level and KCNQ2/3 current is caused by the low

quantity of P2Y₂R present. The application of Oxo-M at a concentration (1 nM) shown to be too low to elicit observable PIP₂ depletion or KCNQ2/3 current suppression (Jensen et al., 2009) was nevertheless sufficient to cause substantial rise in [Ca²⁺]_i, demonstrating that IP₃R-mediated [Ca²⁺]_i rise is more sensitive to G_qPCR activation than PIP₂ depletion and KCNQ2/3 current suppression. Collectively, these observations indicate that the different responses generated by equally maximally stimulated P2Y₂R and M₁R are caused by the combination of differences in the densities of the two receptors and in receptor reserves for the responses (IP₃R-mediated [Ca²⁺]_i rise vs. PIP₂ depletion and KCNQ2/3 current suppression), and not a result of intrinsic, qualitative differences in the P2Y₂R- and M₁R-mediated stimulation.

To investigate at which point in the G_q signaling pathway the exquisite sensitivity of the IP₃R-mediated Ca²⁺ release arises, IP₃ production was monitored with a FRET reporter, LIBRAVIII, based on the ligand-binding domain of IP₃R (Tanimura et al., 2009). Calibration of the LIBRAVIII probe by dialyzing different [IP₃] into tsA201 cells via a patch pipette indicated that the probe is not sensitive to [IP₃] < 1 μM and is mostly saturated at [IP₃] > 10 μM. An Oxo-M concentration (0.1 μM) sufficient to stimulate a maximal rise in [Ca²⁺]_i was also able to elicit a maximal change in LIBRAVIII FRET signal, suggesting that saturation of IP₃R-mediated [Ca²⁺]_i rise is largely caused by saturation of production of IP₃. This also indicates that saturating G_qPCR stimulation can raise cytoplasmic [IP₃] to ~10 μM. However, the low level (1 nM) of Oxo-M sufficient to generate a robust though submaximal [Ca²⁺]_i rise failed to change the LIBRAVIII FRET signal detectably. Thus, the sensitivity of the IP₃R-mediated Ca²⁺ signal does not arise from IP₃ production.

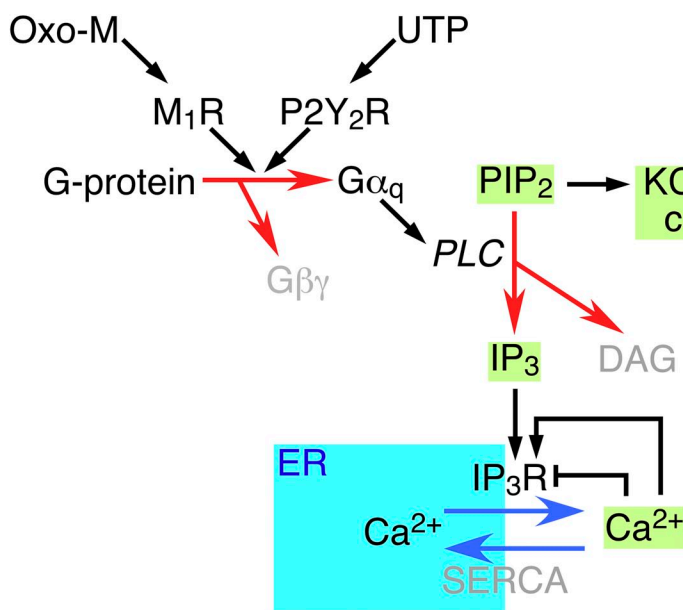


Figure 1. A schematic diagram of the IP₃ cytoplasmic Ca²⁺ branch of the PLC-mediated G_qPCR signaling pathway studied in Dickson et al. (2013) (modified from Fig. 5 A in Falkenburger et al., 2013). Elements highlighted in green were monitored in experiments described in Dickson et al. (2013), and those in gray are not directly described in Dickson et al. (2013).

This leaves the sensitivity of the IP₃R Ca²⁺ release channel to IP₃ stimulation as the main source of the large receptor reserve for G_qPCR-mediated [Ca²⁺]_i rise. This is not surprising as high sensitivity and positive cooperativity in IP₃ activation of IP₃R channel have been observed in electrophysiological studies of InsP₃R channels (Foskett et al., 2007). Furthermore, with saturating [IP₃], positive cooperativity in Ca²⁺_i activation of IP₃R channels and positive feedback activation of IP₃R by Ca²⁺ released through the channels can generate robust Ca²⁺ signals that arise more rapidly and peak sooner than the IP₃ signals, as observed when Fura-4 fluorescence signals and LIBRAVIII FRET signals were compared (Dickson et al., 2013).

The large receptor reserve for IP₃R-mediated Ca²⁺ signal was confirmed by the insensitivity of the Ca²⁺ signal to reduction in IP₃ production caused by severe but not total depletion of PIP₂ by prolonged activation of overexpressed PIP₂ 5-phosphatase localized to the plasma membrane (Suh et al., 2006) or of voltage-sensitive phosphatase (Falkenburger et al., 2010a).

This paper is complemented by a companion (see Falkenburger et al. in this issue), which focuses on the other signals—diacylglycerol (DAG) production and protein kinase C (PKC) activation—that originate from PLC activation by G_qPCR. Although a detailed discussion of that companion paper is beyond the scope of this commentary, a brief summary of its contents is provided here. The high sensitivity of PKC to DAG activation was shown to be the chief reason why the endogenous P2Y₂R expressed at low levels in tsA201 cells was able to activate PKC maximally, just like the overexpressed recombinant M₁R, even though P2Y₂R generated substantially less DAG than M₁R. That paper also presents evidences to show that KCNQ2/3 current is mainly regulated by PIP₂ levels and not significantly affected by Ca²⁺-calmodulin or AKAP/PKC signaling, and that PLC can hydrolyze phosphatidylinositol 4-phosphate (PI(4)P) into DAG and inositol 1,4-bisphosphate. Experimental observations presented in these back-to-back papers (Dickson et al., 2013; Falkenburger et al., 2013) were incorporated into a dynamic model developed in Falkenburger et al. (2010b). The resulting extended model presented in Falkenburger et al. (2013) takes into consideration many relevant elements involved in the PLC-mediated intracellular signaling cascade, and was able to generate numerical simulations that agree quantitatively with many experimental results and account for other results qualitatively. This comprehensive model provides valuable insights into the complex interactions of the various elements in the PLC-mediated signals and reveals new features in the signaling cascade, like the existence of bound PIP₂ that dissociates the production of DAG and InsP₃ from PIP₂ depletion, and hydrolysis of PI(4)P by PLC that dissociates DAG production from InsP₃ production.

Together, these two papers (Dickson et al., 2013; Falkenburger et al., 2013) represent a worthy addition to the literature on the PLC-mediated signal transduction pathway. Multiple techniques using a broad range of probes were applied to investigate various processes involved in the signaling pathway. The model derived from these and previous experimental efforts from the Hille laboratory and other investigators is, of course, very much a work in progress and will no doubt be further expanded and improved as future studies provide more data to account for. This is especially true for the novel addition describing the IP₃R-mediated Ca²⁺ signals, from which many relevant processes have been omitted (as pointed out by the authors), including the buffering of cytoplasmic Ca²⁺ by Ca²⁺-binding proteins and organelles, depletion of ER Ca²⁺ store, and the resulting store-operated Ca²⁺ entry, Ca²⁺ release by ryanodine receptors, and Ca²⁺ removal by plasma membrane Ca²⁺ pumps. Furthermore, whereas using the relatively simple De Young–Keizer model (De Young and Keizer, 1992) to evaluate the Ca²⁺ released by IP₃R channels under various [Ca²⁺]_i and [IP₃] may be sufficient to simulate Ca²⁺ signals in a whole-cell context, only one set of functional affinities for Ca²⁺_i activation and Ca²⁺_i inhibition (K_{Ca} and k_{Ca}) of the IP₃R channel was considered in the model, even though a broad range of values has been reported in the literature for different IP₃R isoforms from different cell types investigated by different methods (Foskett et al., 2007). Nevertheless, the remarkable ability of the model to numerically reproduce a multitude of experimental observations with reasonable resemblance suggests that the modeling effort is proceeding in the right direction, and I look forward to seeing future installments of this investigative effort.

Edward N. Pugh Jr. served as editor.

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