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

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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 (IP_3)-cytoplasmic free Ca^{2+} (Ca^{2+}_i) 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 IP_3 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 Ca^{2+} concentration ($[Ca^{2+}]_i$) caused by IP_3 activation of endoplasmic reticulum (ER)-localized IP_3 receptor (IP_3R) channels (Berridge and Irvine, 1989) was followed using Ca^{2+} imaging with Fura-4F dye, whereas the depletion of inositol 4,5-bisphosphate (PIP_2) due to hydrolysis by PLC was followed either by directly monitoring PIP_2 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 PIP_2 depletion using patch-clamp electrophysiology in perforated-patch configuration. The purinergic G_qPCR investigated was an endogenous receptor determined to be the $P2Y_2R$ (Abbracchio et al., 2006) by its activation by uridine 5′-trisphosphate (UTP) to generate IP_3 without increasing cytoplasmic [cAMP] (Nikolaev et al., 2004). This identification was further confirmed pharmacologically by the suppression of UTP-evoked $[Ca^{2+}]_i$ rise with $P2Y_2R$-specific antagonist suramin; and by changing the amplitude of UTP-evoked $[Ca^{2+}]_i$ rise through manipulation of $P2Y_2R$ abundance, especially the elimination of UTP-evoked $[Ca^{2+}]_i$ rise with siRNA knockdown of $P2Y_2R$ expression. Recombinant M_1 muscarinic receptor (M_1R) 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 $P2Y_2R$ (Falkenburger et al., 2010a).

Whereas maximal UTP (100 µM) stimulation of the endogenous $P2Y_2R$ and maximal oxotremorine-M (Oxo-M; 10 µM) stimulation of overexpressed M_1R generated similar $[Ca^{2+}]_i$ increases, maximal UTP stimulation of the endogenous $P2Y_2R$ failed to cause any detectable suppression of the KCNQ2/3 current, even though maximal Oxo-M stimulation of overexpressed M_1R suppressed KCNQ2/3 current significantly. Inability of the

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maximally activated endogenous $P2Y_2R$ to reduce PIP_2 abundance appreciably while maximally activated overexpressed M_1R depleted PIP_2 substantially was also confirmed by FRET measurement.

Given the huge difference in the abundance of the endogenous $P2Y_2R$ and the transiently transfected M_1R, it is reasonable to surmise that the observed inability of the endogenous $P2Y_2R$ to affect PIP_2 levels even in the presence of a saturating level of UTP is caused by its low expression level, whereas the overexpressed recombinant M_1R, when stimulated, has no problem depleting the PIP_2, thereby reducing the KCNQ2/3 current measurably. In that case, the comparable $[Ca^{2+}]_i$ rises elicited by both maximally activated endogenous $P2Y_2R$ and overexpressed M_1R suggest that Ca^{2+} release from ER through the IP_3R is much more sensitive to activation by G_qPCR than PIP_2 depletion and KCNQ2/3 current reduction, so the number of ligand-bound G_qPCRs required to stimulate Ca^{2+} release is significantly smaller than that required to deplete PIP_2 and suppress KCNQ2/3 current. Therefore, even $P2Y_2R$ expressed at low endogenous levels is sufficient to generate a maximal IP_3R-mediated Ca^{2+} response. In other words, the receptor reserve (spare receptors) for Ca^{2+} release is much larger than that for PIP_2 depletion.

To validate this hypothesis, the Hille laboratory modified the receptor density and agonist concentration independently. When tsA201 cells were transiently transfected with recombinant $P2Y_2R$ to boost the density of $P2Y_2R$ by ~100-fold, saturating UTP was able to generate PIP_2 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 $P2Y_2R$ to affect PIP_2 level and KCNQ2/3 current is caused by the low quantity of $P2Y_2R$ present. The application of Oxo-M at a concentration (1 nM) shown to be too low to elicit observable PIP_2 depletion or KCNQ2/3 current suppression (Jensen et al., 2009) was nevertheless sufficient to cause substantial rise in $[Ca^{2+}]_i$, demonstrating that IP_3R-mediated $[Ca^{2+}]_i$ rise is more sensitive to G_qPCR activation than PIP_2 depletion and KCNQ2/3 current suppression. Collectively, these observations indicate that the different responses generated by equally maximally stimulated $P2Y_2R$ and M_1R are caused by the combination of differences in the densities of the two receptors and in receptor reserves for the responses (IP_3R-mediated $[Ca^{2+}]_i$ rise vs. PIP_2 depletion and KCNQ2/3 current suppression), and not a result of intrinsic, qualitative differences in the $P2Y_2R$- and M_1R-mediated stimulation.

To investigate at which point in the G_q signaling pathway the exquisite sensitivity of the IP_3R-mediated Ca^{2+} release arises, IP_3 production was monitored with a FRET reporter, LIBRAvIII, based on the ligand-binding domain of IP_3R (Tanimura et al., 2009). Calibration of the LIBRAvIII probe by dialyzing different $[IP_3]$ into tsA201 cells via a patch pipette indicated that the probe is not sensitive to $[IP_3] < 1$ µM and is mostly saturated at $[IP_3] > 10$ µM. An Oxo-M concentration (0.1 µM) sufficient to stimulate a maximal rise in $[Ca^{2+}]_i$ was also able to elicit a maximal change in LIBRAvIII FRET signal, suggesting that saturation of IP_3R-mediated $[Ca^{2+}]_i$ rise is largely caused by saturation of production of IP_3. This also indicates that saturating G_qPCR stimulation can raise cytoplasmic $[IP_3]$ to ~10 µM. However, the low level (1 nM) of Oxo-M sufficient to generate a robust though submaximal $[Ca^{2+}]_i$ rise failed to change the LIBRAvIII FRET signal detectably. Thus, the sensitivity of the IP_3R-mediated Ca^{2+} signal does not arise from IP_3 production.

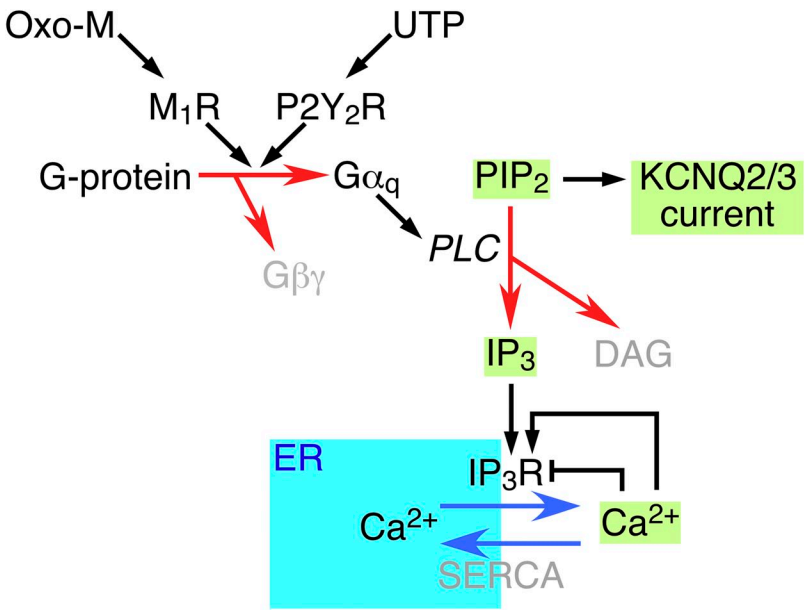

Figure 1. A schematic diagram of the IP_3 cytoplasmic Ca^{2+} branch of the PLC-mediated G_qPCR signaling pathway studied in Dickson et al. (2013) (modified from Fig. 5 A in Falkenburger et al., 2013). Elements highlighted in green were monitored in experiments described in Dickson et al. (2013), and those in gray are not directly described in Dickson et al. (2013).

This leaves the sensitivity of the IP_3R Ca^{2+} release channel to IP_3 stimulation as the main source of the large receptor reserve for G_qPCR-mediated $[Ca^{2+}]_i$ rise. This is not surprising as high sensitivity and positive cooperativity in IP_3 activation of IP_3R channel have been observed in electrophysiological studies of $InsP_3R$ channels (Foskett et al., 2007). Furthermore, with saturating $[IP_3]$, positive cooperativity in $Ca^{2+}{}_i$ activation of IP_3R channels and positive feedback activation of IP_3R by Ca^{2+} released through the channels can generate robust Ca^{2+} signals that arise more rapidly and peak sooner than the IP_3 signals, as observed when Fura-4 fluorescence signals and LIBRAvIII FRET signals were compared (Dickson et al., 2013).

The large receptor reserve for IP_3R-mediated Ca^{2+} signal was confirmed by the insensitivity of the Ca^{2+} signal to reduction in IP_3 production caused by severe but not total depletion of PIP_2 by prolonged activation of overexpressed PIP_2 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 $P2Y_2R$ expressed at low levels in tsA201 cells was able to activate PKC maximally, just like the overexpressed recombinant M_1R, even though $P2Y_2R$ generated substantially less DAG than M_1R. That paper also presents evidences to show that KCNQ2/3 current is mainly regulated by PIP_2 levels and not significantly affected by Ca^{2+}-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 PIP_2 that dissociates the production of DAG and $InsP_3$ from PIP_2 depletion, and hydrolysis of PI(4)P by PLC that dissociates DAG production from $InsP_3$ 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 IP_3R-mediated Ca^{2+} signals, from which many relevant processes have been omitted (as pointed out by the authors), including the buffering of cytoplasmic Ca^{2+} by Ca^{2+}-binding proteins and organelles, depletion of ER Ca^{2+} store, and the resulting store-operated Ca^{2+} entry, Ca^{2+} release by ryanodine receptors, and Ca^{2+} removal by plasma membrane Ca^{2+} pumps. Furthermore, whereas using the relatively simple De Young–Keizer model (De Young and Keizer, 1992) to evaluate the Ca^{2+} released by IP_3R channels under various $[Ca^{2+}]_i$ and $[IP_3]$ may be sufficient to simulate Ca^{2+} signals in a whole-cell context, only one set of functional affinities for $Ca^{2+}{}_i$ activation and $Ca^{2+}{}_i$ inhibition (K_{Ca} and k_{Ca}) of the IP_3R channel was considered in the model, even though a broad range of values has been reported in the literature for different IP_3R 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.

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