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PERSPECTIVE

Probing the Roles of Protein Kinases in G-Protein-Coupled Receptor Desensitization

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For decades we have understood that G-protein-coupled receptors (GPCRs) are crucial links in the relay of information from extracellular stimuli to intracellular responses. Their importance is highlighted by the wide variety of GPCRs encoded in the human genome (Fredriksson et al., 2003; Perez, 2003) and the multiple mechanisms by which they are regulated (Clark, 1986; Palczewski and Benovic, 1991; Kohout and Lefkowitz, 2003). The potential for pharmacological intervention in GPCR function has led to an enormous effort to determine the mechanisms of GPCR activation and desensitization after stimulation by hormones and drugs. In this issue of *Molecular Pharmacology*, Willets et al. (2003) investigate the mechanisms of M_3 muscarinic acetylcholine receptor (mAChR) desensitization in intact cells after agonist stimulation. These authors have confronted a number of the problems associated with the identification of protein kinases that mediate desensitization of GPCRs (see Table 1). It is refreshing to read such a considered discussion of the types of problems that have hindered efforts to understand protein kinase regulation of GPCRs.

Perhaps the most well understood protein kinase-meditated desensitization of a GPCR is that of rhodopsin. The large concentrations of rhodopsin present in rod outer segments (~3 mM) have allowed detailed analysis of its activation, regulation, and structure (Molday, 1998; Filipek et al., 2003) and led to the early recognition of the roles of GRKs and arrestin in GPCR desensitization. Studies of the rod outer segment have also led the way in understanding the regulation of GRKs by recoverin (Polans et al., 1996), and the role of RGS proteins in the desensitization process (Wensel, 2002). However, despite the ease of analysis of this system, it has not been immune to the problems listed in Table 1. For example, early studies based upon cell-free preparations with purified components, not intact rod outer segments, demonstrated that seven or eight residues in the carboxyl terminal tail were phosphorylated by rhodopsin kinase (Wilden and Kuhn, 1982). More recently, Palczewski and colleagues (Ohguro et al., 1995; Maeda et al., 2003) have shown that, in vivo, only one residue is initially phosphorylated by rhodopsin kinase and that with more prolonged light stimulation, two additional residues are phosphorylated.

The elegant body of work on rhodopsin desensitization illustrates how crucial the synthesis of in vitro and in vivo approaches is to the illumination of complex mechanisms of regulation, and it has guided the study of protein kinase regulation of GPCRs in a variety of cellular systems. An example of that has been the study of the role of protein kinases in the desensitization of the β_2-adrenergic receptor (Clark, 1986; Palczewski and Benovic, 1991; Premont et al., 1995; Kohout and Lefkowitz, 2003). All of the problems outlined in Table 1 have been encountered in studies of the role of β_2-adrenergic receptor kinase (GRK2) in desensitization. As with the rod outer segment system, it has taken the merger of in vitro work that was crucial to the discovery of GRK2 with in vivo studies to approach a consensus. To illustrate this point, it was initially thought that as many as six to nine sites were phosphorylated by GRKs on the distal C-tail of the receptor based on cell-free studies with purified components (Kim et al., 1993; Fredericks et al., 1996). However, more recent evidence from studies of intact cells indicates that the number of sites affecting desensitization is considerably less and that their location is more proximal on the C-tail than originally thought (Seibold et al., 2000; Friedman et al., 2003).

In the work by Willets et al. (2003), it is noted that cell-free studies identified GRK2 and GRK3 as the protein kinases that phosphorylate the M_3 mAChR (DebBurman et al., 1995) whereas GRK6 was ineffective. Using dominant negative GRK2, GRK3, and GRK6, Willets et al. (2003) now show that muscarinic agonist-induced phosphorylation of endogenous

ABBREVIATIONS: GPCR, G-protein-coupled receptor; mAChR, muscarinic acetylcholine receptor; GRK, G-protein coupled receptor kinase; RGS, regulator of G protein signaling; eGFP-$PH_{PLC\delta1}$; pleckstrin homology domain of PLCδ1 labeled with the green fluorescent protein; IP_3, inositol 1,4,5-triphosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; dn, dominant negative.

M_3 mAChR in SH-SY5Y cells does not involve GRK2 or GRK3, but rather GRK6. This regulation is not to be confused with the phosphorylation-independent regulation by GRK2 and GRK3 previously demonstrated by several groups (Carman et al., 1999; Sallese et al., 2000) and further documented in the study by Willets et al. (2003). The mechanism of the phosphorylation-independent regulation seems to be the formation of complexes between the RGS domain of GRK2 and Gq. The recent crystal structure of the GRK2/G protein $\beta\gamma$ subunit elegantly illustrates how this complex of the RGS domain and Gq could occur (Lodowski et al., 2003). The mechanism by which GRK6 recognizes and phosphorylates the agonist-occupied receptor, but GRK2 or GRK3 (both of which are present in these SH-SY5Y cells) do not, remains to be shown. Importantly, opposite conclusions were derived from studies of endogenous M_3 mAChR versus studies of overexpressed receptors and cell-free preparations, which dramatically illustrate the pitfalls of discriminating the roles of GRKs in intact cells.

In the current (Willets et al., 2003) and prior studies (Willets et al., 2001, 2002), the authors found that dnGRK6 caused only a 50% inhibition of agonist-stimulated phosphorylation of the M_3 mAChR. This illustrates another of the problems outlined in Table 1, namely that dnGRKs rarely cause complete inhibition, and one cannot be certain whether full inhibition of endogenous GRKs was achieved. This prompted the authors to attempt to determine whether another protein kinase (or some other mechanism) was responsible for the residual 50% phosphorylation. To that end, they searched unsuccessfully for a role of other protein kinases. They found that heterologous activation of protein kinase C by phorbol 12,13-dibutyrate or phorbol 12-myristate 13-acetate indeed caused phosphorylation of the M_3 mAChR; however, it was not stimulated by agonist. They also found that dominant negative-casein kinase α was without effect on the phosphorylation of this receptor, although it had previously been reported that overexpressed receptor in Chinese hamster ovary cells was phosphorylated by this kinase in response to agonist (Tobin et al., 1997; Budd et al., 2000), ". . . highlight[ing] the possible differential regulation of overexpressed and endogenous M_3 mAChR[s]" (Willets et al., 2003). Furthermore, they explored possible regulation of GRKs by protein kinase A, protein kinase C, calmodulin kinase, and Src (Cong et al., 2001; Fan et al., 2001; Kohout and Lefkowitz, 2003); however, no effects were observed.

A novel aspect of the work by Willets et al. (2003) was their assessment of receptor desensitization in single cells using the pleckstrin homology domain of PLCδ1 tagged with the green fluorescent protein (eGFP-$PH_{PLC\delta1}$) as a monitor of agonist-induced inositol 1,4,5-trisphosphate (IP_3) production. This approach was pioneered by Klarlund et al. (1997) and has since been used to monitor changes in the intracellular concentration of several lipid messengers (Varnai and Balla, 1998; Hirose et al., 1999; Oatey et al., 1999; Balla et al., 2000; Nash et al., 2001; Tengholm and Meyer, 2002). The basis of this technique is that the PH-domain translocates from the plasma membrane to the cytosol in response to agonist stimulation of M_3 mAChR and G_q/PLC. The redistribution of eGFP-$PH_{PLC\delta1}$ can be monitored using confocal or evanescent wave imaging techniques. It was initially thought that the redistribution was caused by reductions of phosphatidylinositol 4,5-bisphosphate (PIP_2) concentration (Stauffer et al., 1998). In a more recent in vitro study, it was shown that eGFP-$PH_{PLC\delta1}$ has $\approx$20-fold higher affinity for the product, IP_3, relative to the substrate, PIP_2, indicating that the translocation is primarily caused by increases in cytosolic IP_3 concentration rather than reductions in PIP_2 concentration (Hirose et al., 1999). However, depletion of PIP_2 may still contribute to the translocation of eGFP-$PH_{PLC\delta1}$ in vivo. Careful quantitation of IP_3-induced translocation in intact cells is required to correctly interpret these responses. Such quantitation of IP_3 can potentially be achieved using calibrated photolysis of caged-IP_3. Other difficulties in interpreting the time course of translocation data are more subtle, including our lack of knowledge concerning either the concentration or distribution of (heterologously expressed) eGFP-$PH_{PLC\delta1}$ within the plasma membrane and the impact of buffering by eGFP-$PH_{PLC\delta1}$ on IP_3 signals. The problems associated with buffering of second messenger signals (e.g., Ca^{2+} and cAMP) by other 'real-time' biosensors have been examined in detail (Neher and Augustine, 1992; Pape et al., 1993; Rich and Karpen, 2002), and it is likely that future studies will address these concerns for eGFP-PH-domain biosensors.

Despite these difficulties, Willets et al. (2003) were able to assess desensitization of muscarinic agonist responses in single cells by monitoring translocation of eGFP-$PH_{PLC\delta1}$ throughout the following three step protocol: 1) treatment with a submaximal concentration ($\approx EC_{50}$) of methacholine; 2) wash-in of high agonist concentration; and 3) a second measurement at the submaximal concentration. The level of desensitization could then be estimated as the ratio of response magnitudes during steps 1 and 3. Interestingly, with this approach, the estimated level of desensitization is largely independent of whether eGFP-$PH_{PLC\delta1}$ translocation was primarily triggered by a decrease in PIP_2 concentration (assuming that cellular PIP_2 levels were not significantly depleted during the first two steps) or an increase in IP_3

TABLE 1
Difficulties encountered in the study of phosphorylation-induced GPCR desensitization

1.	Regulation of overexpressed receptors can differ from that of endogenous receptors
2.	Results from cell-free preparations (using purified protein kinases and receptors) are often inconsistent with results from intact cells
3.	It is difficult to identify sites phosphorylated by specific protein kinases and to identify the resulting effects on GPCR activity
4.	The lack of phospho-specific antibodies to protein kinase sites precludes quantitative interpretation of phosphorylation kinetics
5	Overexpressing dominant negative protein kinases, or knocking out or down specific kinases seldom gives 100% inhibition, and specificity is difficult to demonstrate
6.	Protein kinase regulation of GPCRs is likely to vary between cell- and tissue-types
7.	Effects of subcellular localization and trafficking of receptors and protein kinases have not been adequately addressed
8.	Receptor phosphorylation may occur in a hierarchical manner
9.	Protein kinases are often regulated by multiple signaling pathways
10.	Lack of specific protein kinase inhibitors
11.	Generally poor understanding of GPCR dephosphorylation

concentration. Thus, the authors were able to show that transfection with GRK6 increased desensitization by ≈80%, and dnGRK6 inhibited desensitization by ≈30%, in good agreement with their phosphorylation data. Overexpression of either dnGRK2 or dnGRK3 caused 75% suppression of the agonist-stimulated IP_3 responses, consistent with the phosphorylation-independent regulation by these GRKs.

To summarize, this study by Willets et al. (2003) demonstrates the dual mechanism of suppression of agonist responses: agonist- and phosphorylation-dependent (GRK6) and agonist- and phosphorylation-independent (GRK2 and GRK3). It also addresses the pitfalls of depending on either cell-free analysis of GRK function or on heterologous transfection systems. Clearly, it is important to consider that whenever possible experiments might best be performed with endogenous receptors. Ironically, this study depended in part on ≈30-fold overexpression of wild-type or dominant-negative GRKs and therefore could not completely avoid the issues of overexpression. Finally, in a closing statement, the authors note that "gene silencing by means of antisense or RNA interference should identify relevant GRKs in the physiological regulation of muscarinic receptors". We agree and would add only that all of the problems discussed here (Table 1) and by the authors must be addressed to resolve the molecular mechanisms of protein kinase-mediated GPCR desensitization.

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