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PERSPECTIVE

Double Feature at the Signalplex

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ABSTRACT

In this issue of *Molecular Pharmacology*, Oliveras-Reyes et al. (p. 356) describe the agonist-stimulated formation of a caveolin-dependent signalplex that includes both the angiotensin AT_1 receptor and the epidermal growth factor receptor, and probably also a number of other signal transduction intermediates. The signalplex is thought to facilitate the action of protein kinases that mediate angiotensin II-induced transactivation of the epidermal growth factor receptor and activation of extracellular signal-regulated kinase, and epidermal growth factor-induced inositol phosphate accumulation and phosphorylation/desensitization of the AT_1 receptor. This work contributes to an emerging view of the complexity and nonlinearity of signaling via G protein-coupled receptors and receptor tyrosine kinases, and of the importance of membrane compartmentalization to signal transduction.

Many responses to G protein-coupled receptors (GPCRs) are mediated by transactivation of receptor tyrosine kinases (RTKs), a process by which GPCRs recruit classic RTK-activated effectors such as phosphatidylinositol 3-kinase and mitogen-activated protein kinases. RTK transactivation may contribute to clinically significant phenomena such as proliferation of malignant cells and cardiac hypertrophy. Three general mechanisms of transactivation that are commonly observed are shedding of latent ligands by protein tyrosine kinase-induced activation of proteinases and heparanases, direct phosphorylation of the RTK by protein tyrosine kinases, and participation of GPCR and RTK in a signalplex, either by direct receptor/receptor interaction or the binding of both receptors to the same scaffolding protein (Wetzker and Böhmer, 2003; Shah and Catt, 2004). A fourth potential mechanism of transactivation is prevention of dephosphorylation by protein tyrosine phosphatases (Wetzker and Böhmer, 2003). For example, protein tyrosine phosphatases can be reversibly inactivated by reactive oxygen species such as H_2O_2. The AT_1 receptor is an example of a GPCR whose activation leads to the intracellular accumulation of reactive oxygen species (de Gasparo et al., 2000), and AT_1 receptor transactivation of the EGF receptor in vascular smooth muscle cells requires the generation of reactive oxygen species (Ushio-Fukai et al., 2001). These mechanisms are not mutually exclusive; indeed, one of the interesting aspects of the article discussed below is that it is one of a series of articles that have now identified three of these mechanisms in the transactivation of one RTK by one GPCR in one cell line.

It is also becoming increasingly clear that to speak only of GPCR transactivation of RTKs is to oversimplify. GPCR-induced inhibition of RTK activity, or trans*in*activation, has also been observed (Lin et al., 2003; Nouet et al., 2004). Activation of RTKs can also increase phosphorylation, desensitization, and internalization of GPCRs (Medina et al., 2000; Doronin et al., 2002; Ullian et al., 2004) and there are many examples of G protein-dependent signaling by RTKs (Alderton et al., 2001; Rakhit et al., 2001; Kreuzer et al., 2004; Lyons-Darden and Daaka, 2004). In some cases, the activation of heterotrimeric G proteins is a direct consequence of tyrosine phosphorylation that is facilitated by the presence of both GPCR (with G protein) and RTK in the signalplex (Alderton et al., 2001), but it seems likely that in other cases, the

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ABBREVIATIONS: GPCR, G protein-coupled receptor; ERK, extracellular signal-regulated kinase; Ang II, angiotensin II; RTK, receptor tyrosine kinase; EGF, epidermal growth factor; HB, heparin-binding; AG1478, 4-(3′-chloroanilino)-6,7-dimethoxy-quinazoline; PKC, protein kinase C; PLC, phospholipase C.

requirement for G protein will be found to reflect transactivation of the GPCR and its associated G protein.

Caveolae are flask-shaped invaginations in the membrane that are enriched in cholesterol and sphingolipids and also contain caveolin (Hnasko and Lisanti, 2003). Caveolins are a family of three 18- to 24-kDa proteins that form oligomeric structures composed of 14 to 16 monomers. The oligomerization domain of caveolin-1 is residues 61 to 101, just to the N-terminal side of the hydrophobic membrane-inserted segment. A host of signaling proteins interact with caveolin, many by binding directly to the caveolin-scaffolding domain, which is a subregion (residues 82–101) of the oligomerization domain (Ostrom and Insel, 2004; Williams and Lisanti, 2004). Caveolin regulates signal transduction in a cell-specific manner that depends in part on the complement of signaling proteins within that cell. Binding to caveolin causes some proteins to be inhibited or sequestered in caveolae but facilitates signaling by other proteins by concentrating them in this membrane compartment along with other components of the appropriate signaling cascade (Ostrom and Insel, 2004).

In this issue of *Molecular Pharmacology*, Olivares-Reyes et al. (2005) describe reciprocal interactions between the angiotensin AT_1 receptor, a GPCR, and the EGF receptor, a receptor tyrosine kinase. The AT_1 receptor is a $G\alpha_{q/11}$ and $G\alpha_{i/o}$-coupled receptor that mediates contractile, secretory, and growth-promoting actions of angiotensin II on smooth muscle and other cells. Activation of the AT_1 receptor transactivates a number of RTKs, including the EGF and insulin-like growth factor 1 receptors, and RTK transactivation contributes to AT_1 receptor stimulation of the activity of ERK mitogen-activated protein kinases (de Gasparo et al., 2000). Olivares-Reyes et al. (2005) first confirm that Ang II stimulates the activity of ERK1/2 in rat hepatic C9 cells via the EGF receptor. Ang II stimulation of ERK1/2 is prevented by an EGF receptor-selective tyrosine kinase inhibitor and is associated with tyrosine phosphorylation and internalization of the EGF receptor. In other work, this research group has identified a bifurcating pathway by which the AT_1 receptor transactivates the EGF receptor and stimulates ERK1/2 in these cells. This pathway consists of sequential activation of the AT_1 receptor, $G\alpha_q$, phospholipase C-β (PLCβ), PKCδ, and the protein tyrosine kinase Src. The pathway then splits, with Src activating both a matrix metalloproteinase and the proline-rich protein tyrosine kinase Pyk. The matrix metalloproteinase catalyzes cleavage and shedding of HB-EGF, which binds to and activates the EGF receptor (Shah et al., 2004), whereas activated Src and Pyk bind directly to the EGF receptor (Shah and Catt, 2002). The current article demonstrates that binding of agonist to either receptor also induces the formation of a complex that includes both the EGF and AT_1 receptors. Determination of whether this receptor/receptor interaction is required for transactivation awaits further studies in which the interaction is prevented, but it suggests the possibility that full transactivation requires activation of the EGF receptor by HB-EGF, tyrosine phosphorylation of the receptor by protein tyrosine kinases, and the establishment of a large signalplex that includes both receptors, at least two protein tyrosine kinases, and at least one scaffolding protein (Fig. 1).

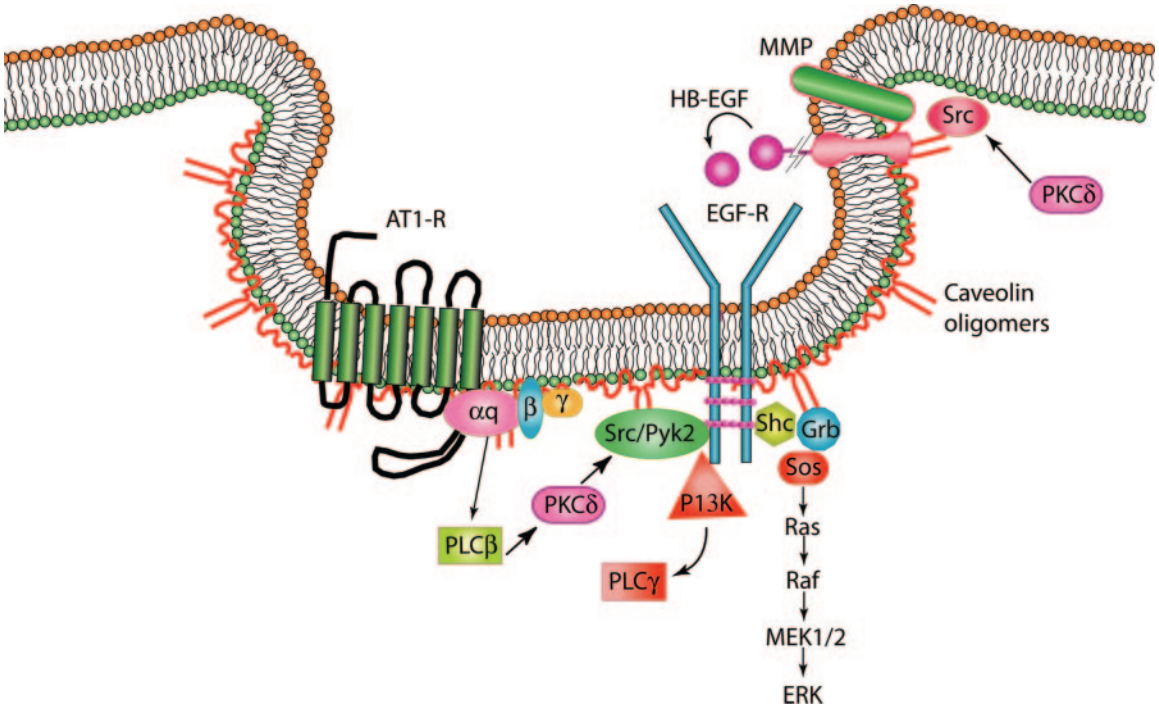

Fig. 1. EGF receptor transactivation and hypothetical composition of the signalplex. Pathways described in the text for EGF receptor transactivation in C9 hepatocytes are depicted schematically. One pathway includes sequential activation of the AT_1 receptor (AT1-R), $G\alpha_q$, (α_q) phospholipase C-β (PLCβ), protein kinase C-δ, (PKCδ), the protein tyrosine kinase Src, and a matrix metalloproteinase (MMP), which liberates the EGF receptor ligand heparin-binding EGF (HB-EGF). Src also activates the proline-rich protein tyrosine kinase Pyk2, promoting binding of both activated kinases to the EGF receptor. Two canonical RTK pathways leading to activation of PLCγ by phosphatidylinositol 3-kinase and activation of the GTP-binding protein Ras by Shc, Grb, and the guanine nucleotide-exchange factor Sos are also shown, along with the protein kinase cascade leading from the MAP kinase kinase kinase Raf to extracellular signal-regulated kinase (ERK). A hypothetical signalplex anchored in a caveola by caveolin oligomers may include both receptors and, at a minimum, most of the signaling intermediates depicted as colored shapes.

It is interesting that treating C9 cells with EGF stimulates inositol phosphate accumulation and also results in phosphorylation of the AT_1 receptor, which is mostly prevented by treatment with tyrphostin AG1478. The inositol phosphate accumulation is assumed to be caused by a G protein-independent, tyrosine kinase-dependent activation of PLC-γ1 (Todderud et al., 1990), although it may actually be a manifestation of AT_1 receptor transactivation by the EGF receptor. As demonstrated previously for EGF-induced phosphorylation of the α_{1b}-adrenoceptor, which leads to desensitization of that receptor (Medina et al., 2000), both phosphatidylinositol 3-kinase and PKC contribute to this response. It is puzzling that the EGF receptor kinase inhibitor AG1478 did not completely prevent AT_1 receptor phosphorylation at a concentration that almost completely inhibited activation of ERK; the residual EGF-induced AT_1 receptor phosphorylation implies the existence of a second mechanism that does not involve EGF receptor tyrosine kinase activity. The presence of both receptors in one signaling complex creates the possibility that the conformational changes in the EGF receptor induced by binding of EGF might cause corresponding changes in the AT_1 receptor that enhance accessibility for the protein kinase, perhaps PKC, that catalyzes phosphorylation of the AT_1 receptor. Enhanced receptor internalization and modest desensitization of Ang II-stimulated inositol phosphate accumulation accompany the EGF-induced phosphorylation of the AT_1 receptor. Thus, activation of either receptor enhances phosphorylation and internalization of the other, but transactivation may go only one way: the GPCR ligand enhances RTK signaling, whereas canonical GPCR signaling may be unchanged or decreased by the RTK ligand. As noted above, it is also possible that EGF stimulation of inositol phosphate accumulation reflects EGF receptor transactivation of the AT_1 receptor and $G\alpha_q$.

Perhaps the most novel aspect of the work by Olivares-Reyes et al. (2005) is the exploration of the role of caveolin in AT_1 receptor function. Cholesterol depletion, which disrupts lipid rafts, including the subset of rafts that include caveolin, prevents AT_1 receptor signaling, including both EGF receptor-dependent and -independent signaling pathways and receptor internalization. Interpretation of the effects of cholesterol depletion is complicated because the treatment has effects that extend beyond caveolae, including nonspecifically preventing clathrin-mediated internalization, but other data in this article provide stronger evidence of a specific requirement for caveolin. Treating cells with either Ang II or EGF causes phosphorylation of caveolin-1 and association of the integral membrane protein with the AT_1 receptor. It is interesting that $G\alpha_q$ also binds caveolin and is concentrated in caveolae (Oh and Schnitzer, 2001). Together, these results indicate that caveolin-1 is a necessary part of the signalplex, which may also include $G\alpha_q$ (Fig. 1).

The contribution of caveolin to signaling is a rapidly evolving story in which few general rules have been identified and in which results vary from one cell type to another. This article suggests that, in C9 cells, caveolin-1 is a receptor-activated scaffold for the formation of a large signalplex that supports reciprocal interactions of AT_1 and EGF receptors. One might ask why this complicated multipathway mechanism exists for receptor transactivation when, for example, simply stimulating the shedding of HB-EGF should be sufficient for the AT_1 receptor to activate the EGF receptor. As noted by Downward (2003), the term transactivation implies a linear process in which activation of one receptor leads to signaling via another, and even when perceived as a reciprocal process in which either receptor can transactivate the other, this scheme may be as oversimplified, as is the linear model in which a ligand-activated GPCR stimulates a heterotrimeric G protein, which, in turn, modulates effector activity. That heterotrimeric G proteins (and perhaps GPCR transactivation) are required for growth factor stimulation of canonical RTK signaling pathways such as ERK (Alderton et al., 2001; Lyons-Darden and Daaka, 2004), and even required for a process as fundamental to RTK activation as growth factor-dependent receptor autophosphorylation (Kreuzer et al., 2004), implies interplay between the components of the signalplex that is much more intricate than linear transactivation of one receptor by another.

The well-characterized model system described in this article is ideal for further analysis of this signalplex, and determination of the extent to which the more complicated, nonlinear model alluded to previously is needed to explain its function. Is the signalplex required for receptor transactivation in either direction? Is the signalplex required for canonical GPCR or RTK signaling? Does EGF transactivate the AT1 receptor, so that AT_1 receptor signaling is enhanced before the receptor is phosphorylated and internalized? What is the role of the binding of activated Src/Pyk to the EGF receptor? How many of these signaling proteins are simultaneously present in the signalplex? How many bind directly to caveolin? Addressing questions such as these will no doubt continue to expand our view of signaling mechanisms for GPCRs and RTKs.

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