

## REVIEW

# The evolving role of lipid rafts and caveolae in G protein-coupled receptor signaling: implications for molecular pharmacology

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The many components of G-protein-coupled receptor (GPCR) signal transduction provide cells with numerous combinations with which to customize their responses to hormones, neurotransmitters, and pharmacologic agonists. GPCRs function as guanine nucleotide exchange factors for heterotrimeric ( $\alpha$ ,  $\beta$ ,  $\gamma$ ) G proteins, thereby promoting exchange of GTP for GDP and, in turn, the activation of 'downstream' signaling components. Recent data indicate that individual cells express mRNA for perhaps over 100 different GPCRs (out of a total of nearly a thousand GPCR genes), several different combinations of G-protein subunits, multiple regulators of G-protein signaling proteins (which function as GTPase activating proteins), and various isoforms of downstream effector molecules. The differential expression of such protein combinations allows for modulation of signals that are customized for a specific cell type, perhaps at different states of maturation or differentiation. In addition, in the linear arrangement of molecular interactions involved in a given GPCR–G-protein–effector pathway, one needs to consider the localization of receptors and post-receptor components in subcellular compartments, microdomains, and molecular complexes, and to understand the movement of proteins between these compartments. Co-localization of signaling components, many of which are expressed at low overall concentrations, allows cells to tailor their responses by arranging, or spatially organizing in unique and kinetically favorable ways, the molecules involved in GPCR signal transduction. This review focuses on the role of lipid rafts and a subpopulation of such rafts, caveolae, as a key spatial compartment enriched in components of GPCR signal transduction. Recent data suggest cell-specific patterns for expression of those components in lipid rafts and caveolae. Such domains likely define functionally important, cell-specific regions of signaling by GPCRs and drugs active at those GPCRs.

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**Keywords:** Caveolae; lipid rafts;  $\beta$ -adrenergic receptors; adenylyl cyclase; compartmentation; G protein-coupled receptors; G proteins

**Abbreviations:** AC, adenylyl cyclase; AKAP, A kinase anchoring protein;  $\beta$ AR, beta-adrenergic receptor; cAMP, 3',5'-cyclic adenosine monophosphate; cGMP, 3',5'-cyclic guanosine monophosphate; eNOS, endothelial nitric oxide synthase; GPCR, G-protein-coupled receptor; GRK, G protein receptor kinase; NO, nitric oxide; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C

## Introduction

Ever since ideas developed by Ehrlich, Langley, Dale, and others in the early 20th century, pharmacologists have recognized the key role of receptors as entities for extracellular agonists to regulate target cells. The emergence of new data led to the general concept of signal transduction, in particular across the lipid bilayer of the plasma membrane that separates the predominantly hydrophilic extracellular and intracellular environments. With the initial completion of the human genome project and the deciphering of the genomes of other species, it has become clear that signal transduction molecules are highly represented in numerous genomes. For example, membrane-bound G-protein-coupled receptors (GPCRs) are

one of the largest superfamilies, comprising approximately 3% of human genes. Recent studies suggest that there are 750–800 human GPCRs (Fredriksson *et al.*, 2003; Vassilatis *et al.*, 2003) and that individual cells express >100 different GPCR, a substantial number of different subunits for heterotrimeric ( $\alpha$ ,  $\beta$ ,  $\gamma$ ) G proteins and multiple G-protein-linked effectors (Hakak *et al.*, 2003; Ostrom *et al.*, 2003; Tang & Insel, 2004). Expression of such large numbers of the critical components involved in GPCR signaling leads to many questions, some of which we will address in this review: What are the absolute and relative levels of protein expression of the different components? How do cells organize such components in a kinetically favorable way so as to promote rapid changes in second messenger formation? Do development, differentiation, aging, and disease states alter such organization? Does such organization play an important role in influencing pharmacologic responses?

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## Overview of GPCR signaling and questions

Although individual GPCRs can couple to multiple heterotrimeric G proteins, many GPCRs appear to couple preferentially to particular G proteins. This interaction, primarily driven by intrinsic affinity between the GPCR and G protein, is favored by agonist occupancy of the GPCR. Certain GPCRs can couple to more than one G protein. Examples of such 'promiscuous' receptors include P2Y<sub>11</sub>,  $\beta_2$ AR,  $\beta_3$ AR, 5HT<sub>2</sub>C, and several dopamine receptor subtypes (Hermans, 2003). In addition, different agonists that bind to the same receptor appear able to direct the G-protein pathway that is activated by the receptor, a process termed ligand-selective agonism; such patterns of response differ among different cell types that express the same receptor (Kenakin, 2001; 2003). What are the critical cellular and molecular determinants of such patterns of GPCR–G protein coupling? Might the coupling, which was termed in early work, 'collision coupling' (Levitzki & Bar-Sinai, 1991), of these receptors to one of several potential pathways be governed by compartmentation (i.e., co-localization of components)? Although it is tempting to speculate that the answer is 'yes', no unequivocal evidence has thus far been shown to support this view.

Each of the four principal subtypes of heterotrimeric G proteins regulate particular effector systems: G<sub>s</sub> stimulates adenylyl cyclase (AC) activity and regulates certain Ca<sup>2+</sup> channels; G<sub>i</sub> inhibits AC activity and regulates K<sup>+</sup> and Ca<sup>2+</sup> channels, G<sub>q/11</sub> stimulates phospholipase C (PLC) activity, and G<sub>12/13</sub> regulates GTP exchange factors (GEF) of rho, a low-molecular-weight G protein. Thus,  $\alpha$  subunits of heterotrimeric G proteins likely possess intrinsic affinity for a particular effector; in addition, regulation of the activity of such effectors occurs *via* actions of beta–gamma subunits (Clapham & Neer, 1997). One can readily imagine how compartmentation could contribute to this step in the pathways, as the short activation cycle of G $\alpha$  proteins likely limits their effective radius of activity. The kinetics of G protein activation is determined by the intrinsic GTPase activity of G $\alpha$  and by enhancement of this activity by effector molecules and RGS proteins, which serve as GTPase-activating proteins, or GAPs (Dohlman & Thorner, 1997; Zheng *et al.*, 1999; 2001). RGS proteins display inherent affinity for terminating the activation of particular G $\alpha$  proteins, an action that has been termed 'kinetic scaffolding' or 'spatial focusing' (Ross & Wilkie, 2000; Zhong *et al.*, 2003). The G $\beta\gamma$  heterodimer functions as a single entity and regulates effector molecules and other proteins involved in GPCR signaling, in particular when not interacting with G $\alpha$  subunits in their GDP-bound (inactive) state.

Is the signaling by G $\alpha$  subunits dependent upon co-localization with effectors? The G-protein-regulated enzymes AC and PLC generate key second messengers following activation of certain GPCRs. AC catalyzes the synthesis from ATP of cyclic adenosine 3',5' monophosphate (cAMP), which, in turn, regulates cell function primarily *via* activation of cyclic AMP-dependent protein kinase (PKA). PKA phosphorylates serine and threonine residues on targets to initiate cellular actions of cAMP; this phosphorylation is targeted to specific substrates *via* A kinase anchoring proteins (AKAPs) (Michel & Scott, 2002). Recent data indicate that cAMP can also act *via* PKA-independent mechanisms, including the GEF of Rap-1, a low-molecular-weight G protein, and certain cAMP-gated ion channels (Kawasaki *et al.*, 1998; Yatani *et al.*, 1999; Kopperud

*et al.*, 2003). PLC hydrolyzes phosphatidylinositol bisphosphate (PIP<sub>2</sub>) into inositol trisphosphate (IP<sub>3</sub>) and diacylglycerol (DAG). IP<sub>3</sub> causes release of calcium from intracellular stores while DAG activates protein kinase C (PKC), second messengers that have rapid and delayed effects on cell metabolism and function.

In addition to the ability of RGS proteins to accelerate GTP hydrolysis and contribute to termination of GPCR signaling, other mechanisms contribute to homologous (receptor-specific) and heterologous (receptor-nonspecific) desensitization of GPCRs. A key mechanism for homologous desensitization involves G-protein receptor kinases (GRKs), which are recruited to active GPCR, in part, at least for some GRKs, *via* their affinity for free G $\beta\gamma$  subunits (Krupnick & Benovic, 1998; Lefkowitz, 1998). GRKs phosphorylate the associated GPCR, causing the recruitment of  $\beta$ -arrestin, which impairs receptor interaction with G proteins and can act as an adapter between the receptors and clathrin-coated pits (Luttrell & Lefkowitz, 2002; Kohout & Lefkowitz, 2003). In many cases, association of a GPCR with clathrin-coated pits leads to internalization of the GPCR in clathrin-coated vesicles and eventual degradation of the receptor *via* lysosomes (or proteasomes (von Zastrow, 2003)) or recycling of the receptor back to the plasma membrane (Ferguson, 2001). Clathrin-coated pits thus function as a membrane microdomain involved in signal termination, but, in addition, in the activation of certain intracellular events such as activation of mitogen-activated protein kinase cascades (Hall *et al.*, 1999; Pierce *et al.*, 2001).

A basic tenet of signal transduction by GPCRs is that high-affinity protein–protein interactions determine the G-protein heterotrimer and, in turn, the signal transduction pathway that a particular GPCR activates. However, different receptors that couple to the same G protein can elicit different biochemical, cellular or physiological responses (Steinberg & Brunton, 2001). A 'classical' one-dimensional view of GPCR signal transduction (i.e., that a given GPCR activates a single G-protein and single effector) cannot readily account for such observations. Moreover, GPCRs, G proteins, and effector enzymes are expressed at relatively low concentrations in mammalian cells. This is particularly the case for GPCRs and effectors (Alousi *et al.*, 1991; Milligan, 1996; Ostrom *et al.*, 2000a), yet GPCR–G-protein–effector systems display rapid, high-fidelity signaling characteristics. Such observations make the idea that the components of GPCR signal transduction are 'pre-arranged' or 'selectively compartmentalized' quite attractive.

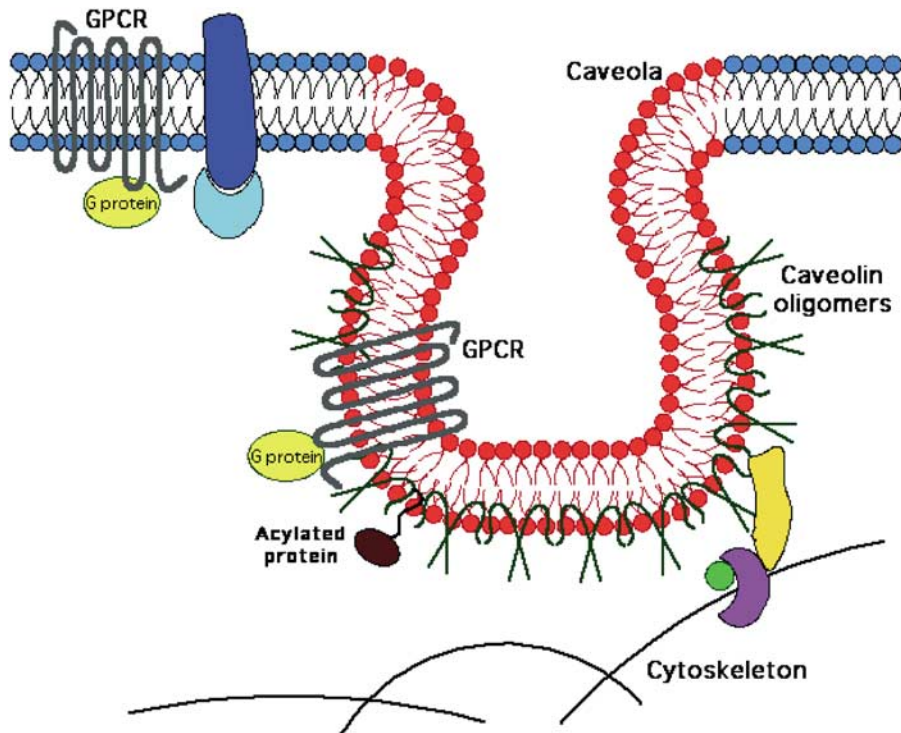
Signaling proteins must physically interact in order to transmit information. It is presumed that, once activated, most signaling molecules possess inherent high affinity for binding to their partners, but data on the affinity of such interactions are limited. However, even high-affinity interactions require effective concentrations of the reactants in order to thermodynamically favor rapid conformational changes (in the case of proteins) and information exchange between molecules. Therefore, mechanisms likely exist for rapid and efficient signal transduction, since bulk concentrations of the reactants are relatively low. Typical receptor concentrations are  $< \sim 10,000$ /cell while certain effectors, such as ACs, appear to be expressed at similar orders of magnitude (Alousi *et al.*, 1991; Post *et al.*, 1995; Milligan, 1996). In GPCR signaling, receptors, G proteins, effector enzymes, and key accessory

proteins, the latter of which may be quite numerous (Bockaert *et al.*, 2003), are generally thought to be membrane-associated. Given the overall low abundance of key signaling molecules in cells, uniform distribution within the plasma membrane seems unlikely to provide sufficient enrichment to achieve the rapid signaling responses characteristic of GPCR activation. Since target cells have relatively low concentrations of signaling components, one way to account for rapid response is that cells concentrate signaling molecules in membrane microdomains.

One such microdomain may be lipid rafts in the plasma membrane that are formed by the coalescence of sphingolipid and cholesterol. Caveolae are 50–100 nm flask-like indentations, or 'little caves' of the plasma membrane that have a lipid composition similar to rafts but that also contain caveolin proteins localized on the inner leaflet of the membrane bilayer (Anderson, 1998) (Figure 1). Based on their similar lipid composition, caveolae are generally considered subsets of lipid rafts but these entities may have other differences (Sowa *et al.*, 2001; Williams & Lisanti, 2004). While only cells expressing caveolins express caveolae, all mammalian cells express lipid rafts (Hooper, 1999); leukocytes, for example, have lipid rafts but not caveolae. There are three isoforms of caveolins: caveolin-1, caveolin-2, and caveolin-3. Caveolae generally form if cells express either caveolin-1, the predominant isoform, or caveolin-3, the striated muscle-specific isoform (Song *et al.*, 1996a; Tang *et al.*, 1996). Caveolin-2 is found in hetero-oligomers with caveolin-1 and caveolin-3; it is not clear if caveolin-2 can induce caveolae biosynthesis on its own

(Scherer *et al.*, 1996; 1997; Razani *et al.*, 2002b; Lahtinen *et al.*, 2003; Rybin *et al.*, 2003). Thus, enrichment of GPCR signaling components in lipid rafts or caveolae may be a universal mechanism for increasing the effective concentration of these proteins by restricting their movement, thereby favoring interaction of components in the signal transduction pathway.

Methodologically, lipid rafts and caveolae are most often studied by disrupting cells, extracting these domains based upon their insolubility in certain detergent or nondetergent conditions, then isolating them by centrifugation based upon their differential buoyancy in a gradient (Anderson, 1998; Ostrom *et al.*, 2000a; Pike, 2003). Such methodologies cannot distinguish between caveolae and lipid rafts since they rely upon properties common to both these domains. While lipid rafts were initially described as detergent-resistant membrane (DRM) fractions, nondetergent approaches are now generally preferred, based, at least in part, upon their ability to exclude nonmembrane markers and to include certain loosely associated membrane proteins (Smart *et al.*, 1995; Shaul *et al.*, 1996; Song *et al.*, 1996b; Rybin *et al.*, 1999). Caveolae can be preferentially isolated from lipid rafts using immunological approaches to trap caveolin-rich membrane domains (Oh & Schnitzer, 1999; Ostrom *et al.*, 2001). Use of these approaches has led to the idea that lipid rafts and caveolae share common qualities but can differ in terms of the nature of the signaling proteins they contain (Oh & Schnitzer, 1999). The role of caveolins as scaffolding proteins has also been assessed using immunoprecipitation of caveolin or by expressing peptides that interfere with the caveolin-binding motif, a domain on



**Figure 1** Schematic representation of the lipid and protein organization of a caveola. Sphingolipid- and cholesterol-rich domain is shown in red and nonraft lipid domains are shown in blue. Caveolae contain a coat of oligomeric caveolin molecules inserted into the cytoplasmic leaflet of the membrane. Some proteins, including certain GPCR (shown as heptahelical structures with associated G protein), partition to caveolar domains due to either acylation, binding to caveolin or formation of a sphingolipid 'shell' around the protein (or by a combination of these, and/or yet unknown, mechanisms). Also shown are undefined cytoskeletal interacting proteins (orange, green, purple) and noncaveolar membrane proteins (blue) and partners (light blue).

caveolin-1 and caveolin-3 that preferentially serves as a docking site for binding (and inhibiting) signaling molecules (Okamoto *et al.*, 1998). The function of lipid rafts and caveolae in signaling or other cellular processes can also be inferred from studies using cholesterol depletion. Methyl- $\beta$ -cyclodextrin, a cholesterol-binding agent, can remove cholesterol from cells in culture and disrupt lipid rafts and caveolae. Filipin, a polyene antibiotic and sterol-binding agent, is generally more cytotoxic, but can also be used to disrupt lipid rafts and caveolae, albeit with less efficiency in certain cells (Schnitzer *et al.*, 1994; Orlandi & Fishman, 1998; Awasthi-Kalia *et al.*, 2001). Both methods of cholesterol depletion are associated with nonspecific effects that must be controlled for, usually by adding back cholesterol to cells in order to show reversibility and specificity of action of the agent.

## Compartmentation of GPCR signaling

The concept of compartmentation (compartmentalization) as a means to achieve selective responses to certain GPCR agonists is not new, but the identification of caveolins has provided a molecular 'tag' to assist in biochemically defining caveolae as a subset of lipid rafts, and to show that signal transduction proteins are enriched in lipid rafts and/or caveolae (Anderson, 1998; Razani *et al.*, 2002c; Pike, 2003). Mitogen-activated protein kinases and receptor tyrosine kinases were first recognized as residing in caveolin-rich microdomains; certain GPCRs and associated molecules were subsequently shown to be enriched in these domains (Chun *et al.*, 1994; de Weerd & Leeb-Lundberg, 1997; Feron *et al.*, 1997; Schwencke *et al.*, 1999b). The bulk of the work to date has relied upon biochemical isolation of lipid rafts and caveolae, typically using sucrose density centrifugation, an approach that cannot distinguish between these two domains. We will use this operational definition in our discussion.

Compartmentation of GPCR signaling proteins in caveolae or lipid rafts appears to be a major determinant of receptor-effector coupling.  $\beta_1$ AR and  $\beta_2$ AR are enriched with AC type 6 (AC6) in caveolae or lipid rafts (buoyant, caveolin-rich membrane fractions) of cardiac myocytes, together with a portion of the membrane  $G_s$ , and these receptors couple efficiently to the activation of AC6. (Schwencke *et al.*, 1999a, b; Ostrom *et al.*, 2000b; 2001; Rybin *et al.*, 2000; Xiang *et al.*, 2002b). However, not all GPCRs (or G proteins) are found in these lipid raft fractions. For example, in cardiac myocytes, prostanoid EP<sub>2</sub> receptors are excluded from lipid raft fractions and cannot activate AC6 despite their ability to activate  $G_s$  (Ostrom *et al.*, 2001).  $G_s$  is found in both lipid rafts and nonraft fractions (Rybin *et al.*, 2000; Ostrom *et al.*, 2001). In addition, cardiac myocyte  $\beta_2$ AR activate AC6 less efficiently than do  $\beta_1$ AR, and this appears attributable to rapid agonist-promoted translocation of  $\beta_2$ AR into clathrin-coated pits (Goodman *et al.*, 1996; Gagnon *et al.*, 1998) and out-of-lipid rafts and caveolae compared to  $\beta_1$ AR, which, at least initially, remain localized with AC6 in caveolae upon agonist activation (Rybin *et al.*, 2000; Ostrom *et al.*, 2001). Therefore, the degree to which a given GPCR couples to a signaling pathway appears to depend upon a close physical association between an activated R-G complex and a suitable effector enzyme.

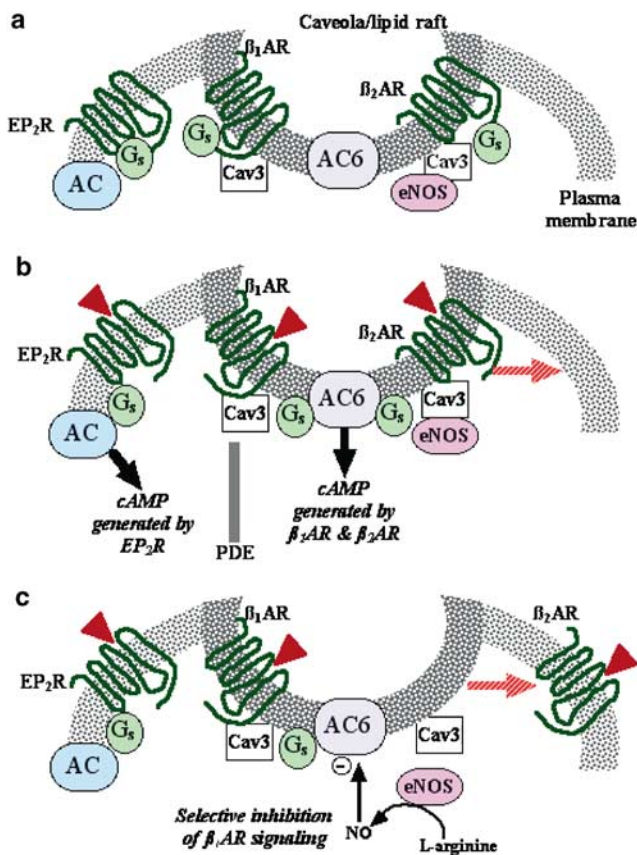
Such intimate association of signaling proteins in lipid rafts or caveolae also appears to be important for crosstalk between

different signaling pathways. We have recently shown that, in cardiac myocytes, nitric oxide (NO) production by endothelial nitric oxide synthase (e.g., NOS3, eNOS) inhibits  $\beta$ AR-stimulated cAMP production, but has little effect on prostanoid-stimulated cAMP accumulation (Ostrom *et al.*, 2004). This selective effect of eNOS activity appears attributable to two factors: (1) activity of AC6 (and AC5 but not other AC isoforms) is inhibited *via* direct nitrosylation by NO (McVey *et al.*, 1999; Hill *et al.*, 2000), and (2)  $\beta$ AR, but not prostanoid receptors, couple to AC6 due to  $\beta$ AR-AC6 localization in lipid rafts or caveolae. Disruption of lipid rafts with methyl- $\beta$ -cyclodextrin treatment uncovers an NO-mediated inhibition of the prostanoid response in conditions of high eNOS activity, indicating that the organization provided by lipid rafts or caveolae normally compartmentalizes the NO signal with the components of the  $\beta$ AR signal transduction pathway. Figure 2 illustrates this concept.

New data shed some light on the different behaviors of  $\beta_1$ AR and  $\beta_2$ AR with respect to association with lipid rafts. Using phosphorylation-deficient mutants of the  $\beta_1$ AR, Rappaciolo *et al.* (2003) have suggested that PKA-mediated phosphorylation directs internalization of the receptor *via* caveolae, while GRK-promoted phosphorylation directs internalization *via* clathrin-coated pits. Although such results are of interest, it is unclear whether  $\beta_1$ AR are primarily phosphorylated by GRK in native cell settings and whether such results with the  $\beta_1$ AR can be applied to  $\beta_2$ AR or other GPCRs.  $\beta_1$ AR and  $\beta_2$ AR are known to signal differently in cardiac myocytes, where they are co-expressed, due to  $\beta_2$ AR coupling sequentially to  $G_s$  and then  $G_i$  (Xiao *et al.*, 1994; 1999). Intact lipid rafts are necessary for the  $\beta_2$ AR to couple to  $G_i$  (Xiang *et al.*, 2002b); both  $\beta_1$ AR and  $\beta_2$ AR require a carboxy-terminal PDZ-binding motif and interaction with PDZ domain-containing proteins for their signaling and trafficking behaviors (Xiang *et al.*, 2002a; Xiang & Kobilka, 2003). Such data suggest that interaction of  $\beta$ AR with PDZ-containing proteins is related to their co-localization in lipid rafts; we speculate that this is the case, but further studies are needed to test this idea.

Studies of the oxytocin receptor in MDCK cells provide another example of localization determining receptor signaling. In those cells, this  $G_q$ -coupled receptor is predominantly expressed in nonraft domains and its activation inhibits cell growth (Guzzi *et al.*, 2002). However, fusion of the oxytocin receptor with caveolin-2 localizes it to lipid rafts and 'switches' receptor activation to stimulation of cell proliferation. While this latter effect might result from altered desensitization of the oxytocin receptor fused to caveolin (the chimeric receptor does not internalize upon agonist exposure), an alternative possibility is that localization of the receptor in lipid rafts leads to its coupling to effector molecules that elicit a different cellular response.

Recent evidence indicates that, of the nine isoforms of the transmembrane ACs, only certain isoforms localize to lipid rafts. Different tissues express different patterns of expression of these AC isoforms, which are subject to various types of regulation by intracellular factors (Hanoune & Defer, 2001; Ludwig & Seuwen, 2002). Using immunoblot analysis of lipid raft fractions, we have analyzed the AC isoform expression and localization in several cell types. Adult rat cardiac fibroblasts express AC2, AC3, AC4, AC5/6, and AC7, but only AC3 and AC 5/6 proteins (AC5 and AC6 are detected by



**Figure 2** Schematic representation of GPCR- $G_s$ -AC signaling in lipid rafts and caveolae of cardiac myocytes and the effects of eNOS. (a) Caveolar/lipid raft domains contain  $\beta_1AR$ ,  $\beta_2AR$ , AC6,  $G_s$ , eNOS, and caveolin-3 (Cav3), but exclude prostanoid EP<sub>2</sub>R (Ostrom *et al.*, 2000b). (b) Catecholamines stimulate  $\beta$ AR and the generation of cAMP in the caveolar/lipid raft domain. Prostanoids activate receptors located outside lipid rafts and, as a result of compartmentation by phosphodiesterases (PDE), a separate pool of cAMP is generated.  $\beta_2AR$  translocate out of caveolae upon agonist activation, presumably to internalize via clathrin-coated pits, thus generating a more transient activation of the caveolar/lipid raft pool of cAMP (Ostrom *et al.*, 2001). (c) Activation of eNOS activity leads to the generation of high NO concentrations in the caveolar/lipid raft domain. Due to the localization of eNOS in caveolae and the sensitivity of AC6 to NO-mediated nitrosylation, eNOS activity selectively inhibits the caveolar/lipid raft pool of cAMP (i.e., that stimulated by  $\beta$ AR) (Ostrom *et al.*, 2004).

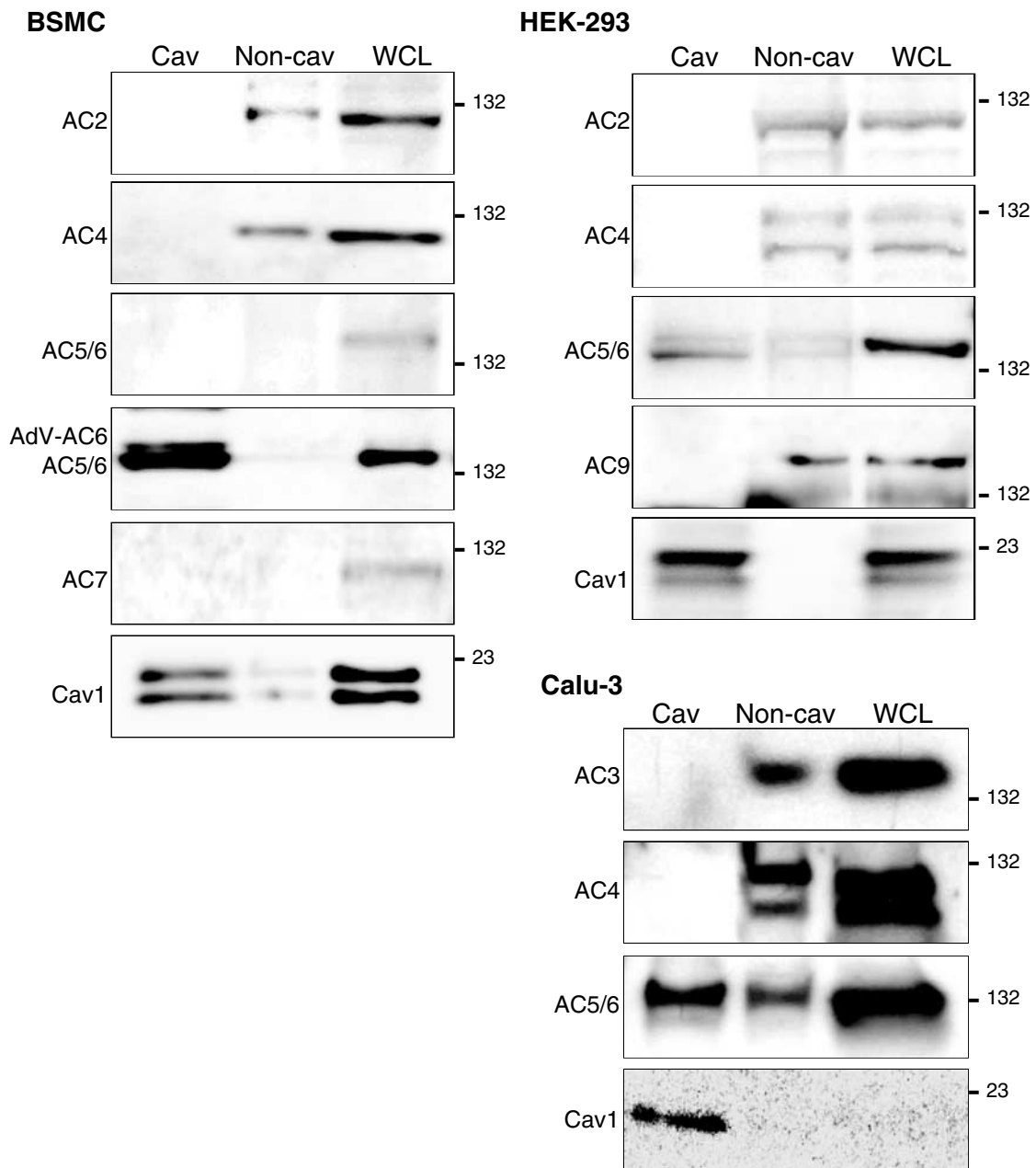
a single antibody) are enriched in lipid raft fractions (Ostrom *et al.*, 2003). Fractionation and immunoblot analysis of AC isoforms expressed in cultured human bronchial smooth muscle cells (BSMC) indicate that AC2 and AC4 are the most readily detected AC isoforms, but these isoforms are not detected in caveolin-rich, lipid raft fractions (Figure 3). Results from similar studies with HEK-293 cells, airway epithelial cells, and vascular smooth muscle cells are consistent with the idea that AC3, AC5 and AC6, but perhaps not all other AC isoforms, preferentially localize to lipid rafts (Figure 3; Ostrom *et al.*, 2002). We have not detected endogenous AC8 expression in any of the cells we have examined, but data from others indicate that this isoform, too, localizes in lipid rafts (Fagan *et al.*, 2000; Smith *et al.*, 2002).

The localization of AC isoforms in lipid raft or caveolar microdomains is likely to be important for the regulation

of the enzyme. Overexpression of AC6, which is subject to inhibition by multiple regulators (Ostrom *et al.*, 2000a, b), does not increase basal cAMP production in cardiac myocytes. By contrast, expression of AC6 in RASMIC can lead to non-raft localization of the enzyme and a concomitant increase in basal cAMP production (Ostrom *et al.*, 2002). Thus, localization of AC6 (and perhaps other isoforms) in lipid rafts may serve to maintain a low basal activity of the enzyme, perhaps, in the case of AC6 which is inhibited by calcium, via co-localization with sites of calcium entry (Fagan *et al.*, 2000; Lohn *et al.*, 2000) and/or NO generation (Ostrom *et al.*, 2004). Expression of AC8 in HEK-293 cells leads to localization of this calcium-stimulable isoform in lipid rafts and imparts an activation of cAMP production by capacitive calcium entry (Smith *et al.*, 2002; Cooper, 2003), which is likely mediated by lipid raft-localized Trp1 channel (Lockwich *et al.*, 2000; Clapham *et al.*, 2001). Thus, the localization of calcium-sensitive AC isoforms appears critical for determining the type of calcium signal that regulates cAMP production.

Different types of G proteins appear to segregate differently with respect to lipid rafts versus caveolae, an observation suggesting that differences exist between the two domains. Although this issue has not been extensively studied, certain data have shown that  $G_q$  preferentially localizes in caveolae, while  $G_s$  and  $G_i$  localize in lipid rafts (Oh & Schnitzer, 2001). Consistent with the idea that expression of caveolin is a major distinguishing feature between lipid rafts and caveolae, Oh & Schnitzer (2001) found that  $G_q$ , but not  $G_s$  and  $G_i$ , could be immunoprecipitated with caveolin-1, but if a cell lacked caveolin expression  $G_q$  would localize in lipid rafts. Thus, while all three G proteins can be found in lipid rafts,  $G_q$  'prefers' caveolae in cells that express caveolin (and contain morphologic caveolae), presumably due to its ability to bind to the caveolin scaffolding domain. These data highlight a fundamental question: What causes a protein to localize to lipid rafts or caveolae? Some proteins require interaction with caveolin, implying that such proteins will preferentially localize in caveolae (relative to lipid rafts, such as  $G_s$  and  $G_i$ , in the study of Oh & Schnitzer, 2001), while other proteins do not interact with caveolin and thus would be found in the lipid environment common to both lipid rafts and caveolae. As will be discussed below, the cell type in which a given signaling protein is expressed may also be a critical determinant of lipid raft or caveolar localization.

Another G protein, transducin, has been shown to translocate to lipid rafts upon activation by its cognate GPCR, rhodopsin, and its agonist, light, as part of the activation pathway of cGMP phosphodiesterase, and in turn cGMP-gated cation channels (Nair *et al.*, 2002). Interestingly, the translocation of transducin appears to occur in a complex with RGS9,  $G\beta\gamma$ , and arrestin. Given that rhodopsin and the cGMP phosphodiesterase were found in both lipid raft and nonraft fractions but that guanylyl cyclase was found only in lipid rafts of vertebrate retina, translocation of the transducin complex appears to favor interaction between the activated G protein and the pools of effector (cGMP phosphodiesterase) that are localized with the site of second messenger synthesis (guanylyl cyclase in the lipid raft). It is not known whether the cGMP-gated cation channels are co-localized in lipid rafts of vertebrate photoreceptor cells. These data in the retinal system strongly suggest that large multi-protein complexes can



**Figure 3** Immunoblot analyses of AC isoform expression in caveolae/lipid raft fractions isolated from human BSMC, HEK-293 cells or human airway epithelial cells (Calu-3). Buoyant lipid raft fractions were isolated from the indicated cells using a nondetergent method followed by centrifugation on a sucrose density gradient, as described previously (Ostrom *et al.*, 2004). The buoyant fractions (cav) and the nonbuoyant fractions (non-cav) were collected from the gradient and, along with whole-cell lysates (WCL), were subjected to SDS-PAGE and immunoblot analyses. The data and others discussed in the text are consistent with the idea that AC5, AC6, and AC3 (except in Calu-3 cells) localize to caveolae/lipid raft fractions, while other AC isoforms localize to nonraft fractions. AC8 has also been shown to localize to lipid raft fractions (Fagan *et al.*, 2000; Smith *et al.*, 2002).

translocate in a rapid manner to facilitate interaction of components of GPCR signaling.

Agonist activation of  $\beta$ AR recruits  $\beta$ -arrestin to the plasma membrane, where it interacts with the activated receptor and, in addition, can recruit cyclic nucleotide phosphodiesterase (in particular, the PDE4 isoform), which degrades cAMP (Perry *et al.*, 2002; Baillie *et al.*, 2003). Therefore,  $\beta$ -arrestin translocation to the plasma membrane enhances the local degradation of cAMP and terminates the stimulus by desensitizing the receptor. Furthermore,  $\beta$ -arrestin-PDE4 recruitment to

activated  $\beta_2$ AR appears critical for regulating a G protein switching (from  $G_s$  to  $G_i$ ) of this receptor by limiting its phosphorylation by PKA (the key step in causing the G protein switch (Baillie *et al.*, 2003)). Given that  $\beta_2$ AR and AC are expressed in lipid rafts and/or caveolae (Rybin *et al.*, 2000; Ostrom *et al.*, 2001) and that pools of cAMP localize in cardiac T-tubules where the bulk of caveolae can be found (Zaccolo & Pozzan, 2002), it is evident that lipid rafts and caveolae are likely to be key sites for both cAMP generation and cAMP response (Insel, 2003). Thus, cAMP-PKA signaling is highly

localized in and targeted to multiple subcellular compartments, increasing its specificity for certain biological effects (Tasken & Aandahl, 2004).

Numerous ion channel proteins or subunits have been described as associating with lipid rafts, including  $K_v1.4$ ,  $K_v1.5$ ,  $K_v4.2$ , L-type  $Ca^{2+}$  channel, the plasma membrane  $Ca^{2+}$  pump, voltage-gated  $Na^+$  channel, Aquaporin-1, Trp4, and Trp1 (Schnitzer & Oh, 1996; Page *et al.*, 1998; Darby *et al.*, 2000; Martens *et al.*, 2000; 2004; Torihashi *et al.*, 2002; Yarbrough *et al.*, 2002; Bergdahl *et al.*, 2003; Brady *et al.*, 2004; Wong & Schlichter, 2004). Of these, however, only a few have thus far been shown to display direct regulation by GPCR in a lipid raft-dependent manner. Voltage-gated  $Na^+$  channels expressed in the heart associate with lipid rafts and can be activated by  $\beta AR-G_s$  in a cAMP-independent manner (Yarbrough *et al.*, 2002). As the introduction of a caveolin-3 antibody inhibits the activation of voltage-gated  $Na^+$  channel activity by a  $\beta AR$  agonist, the pathway appears to depend upon interaction of a co-localized receptor, G protein, and the channel that is facilitated by the caveolin scaffold. Other data indicate that endothelin-induced contraction of endothelium-denuded arteries is partially dependent upon activation of Trp1 channels, which localize with  $ET_A$  receptors in lipid rafts (Bergdahl *et al.*, 2003). In that system, depletion of cellular cholesterol (a treatment that disrupts caveolae and lipid rafts) causes both the loss of Trp1-caveolin-1 co-localization and a diminution of the endothelin-mediated contractile response. Thus, regulation of vascular reactivity by endothelin appears dependent, at least in part, on the co-localization of  $ET$  receptors with key effector molecules, Trp1 channels. Cyclic nucleotide-gated ion channels, effector molecules of light and olfactory receptors, localize to lipid rafts in olfactory epithelium and, when heterologously expressed, in HEK-293 cells (Brady *et al.*, 2004). Cholesterol depletion of HEK-293 cells expressing a cyclic nucleotide-gated ion channel abolishes the stimulation of channel activity by prostaglandin, but this effect is likely attributable to diminished affinity of the channel for cAMP rather than a loss of juxtaposition of the GPCR and the channel.

While much work on the compartmentation of cAMP generation focuses on differentiated responses (such as regulation of contractility), other data imply that cAMP regulation of certain metabolic pathways may also show compartmentation in lipid raft *versus* nonraft domains. Disruption of caveolae and lipid rafts by cholesterol depletion inhibits glycolysis but stimulates gluconeogenesis in vascular smooth muscle cells (Lloyd & Hardin, 2001), likely reflective of the fact that a major glycolytic enzyme, phosphofruktokinase, is expressed in lipid rafts in those cells (Vallejo & Hardin, 2004). Thus, cAMP generated in lipid rafts in response to activation of raft-localized  $\beta AR$  (or other GPCRs) may selectively regulate metabolic enzymes in the same compartment.

### Determinants of protein localization to lipid rafts and caveolae

Little is known about how proteins localize to different lipid domains. Three mechanisms for lipid raft targeting have been proposed or described: (1) Proteins may bind to caveolin *via* a scaffolding domain located near the N-terminus of caveolin-1

and caveolin-3 (Song *et al.*, 1996b; Okamoto *et al.*, 1998). Many proteins that bind to caveolin contain a putative caveolin-binding motif (a loosely defined pattern of aromatic and nonaromatic residues) (Couet *et al.*, 1997). (2) N-linked myristoylation (of a glycine residue following the initial methionine) or thio-acylation with palmitate (palmitoylation of cysteine residues) causes partitioning into the lipid-ordered phase of lipid rafts (Milligan *et al.*, 1995; Shaul *et al.*, 1996; Mumby, 1997; Song *et al.*, 1997; Galbiati *et al.*, 2001; Zacharias *et al.*, 2002). Caveolins, G proteins, and other proteins rely upon these types of post-translational modifications for their interaction with membranes and lipid rafts. Indeed, coupling of the  $5HT_{1A}$  receptor to  $G_i$  and the inhibition of AC activity depends upon the palmitoylation of two cysteine residues in the C-terminal region of the receptor, which presumably serves to retain the receptor in lipid rafts together with  $G_i$  and AC (Papoucheva *et al.*, 2004). (3) Structural components of the transmembrane-spanning region of proteins, in particular hydrophobic residues, cause proteins to 'prefer' the slightly thicker membrane of the lipid raft (Anderson & Jacobson, 2002; Yamabhai & Anderson, 2002). As this mechanism depends upon the protein containing at least one transmembrane-spanning domain, it cannot readily account for targeting of lipid raft-associated proteins that lack such domains. It is important to note that these three mechanisms are not mutually exclusive and that it is not currently possible to predict, based upon amino-acid sequence, the localization of a given membrane-associated protein. Localization of different proteins may rely upon different mechanisms, or a combination thereof.

Caveolins function not only as scaffolds that localize signaling proteins, but, in addition, can inhibit numerous enzymes, including AC, eNOS, and several kinases and serine/threonine phosphatases (García-Cardeña *et al.*, 1997; Oka *et al.*, 1997; Engelman *et al.*, 1998; Feron *et al.*, 1998; Toya *et al.*, 1998; Carman *et al.*, 1999; Razani *et al.*, 1999; Razani & Lisanti, 2001; Hnasko & Lisanti, 2003). Consistent with the latter findings, data from studies with knockout animal models and from overexpression protocols suggest that caveolins play central roles in regulating signal transduction by various systems (Razani *et al.*, 2002a,b; Schubert *et al.*, 2002; Woodman *et al.*, 2002; Hnasko & Lisanti, 2003). Such roles imply that caveolins are not just organizers, but regulators of signal transduction. Therefore, approaches to increase or decrease caveolin expression, including expression of caveolin peptides corresponding to the scaffolding domain, cannot be viewed as pure 'probes' of lipid raft localization or compartmentation.

It has also become apparent that some molecules localize to lipid rafts and caveolae in a cell-dependent manner. Low levels of AC6 overexpression in vascular smooth muscle cells lead to localization of AC6 in lipid rafts (its native location), but with higher levels of expression the enzyme is found in nonraft fractions, where the bulk of both  $\beta_1AR$  and  $\beta_2AR$  were detected in these cells (Ostrom *et al.*, 2002). Such results imply that lipid raft domains in some cells may contain a limited, saturable pool of signaling molecules, such as AC. The results with vascular smooth muscle cells contrast with those from cardiac myocytes, where overexpression of a large range of levels of AC6 leads to co-localization with both  $\beta AR$  subtypes in caveolin-rich fractions (Ostrom *et al.*, 2000b; 2001). Therefore, different cells can localize the same protein differently,

**Table 1** Summary of cell-specific localization of GPCR and AC in caveolae/lipid rafts

Cell type	Caveolae/lipid rafts	Non-cav/non-raft
Cardiac myocyte	$\beta_1$ AR, $\beta_2$ AR, $G_s$ , AC5/6, PKA, AKAP	EP <sub>2</sub> R, EP <sub>4</sub> R, $G_s$ , (H <sub>2</sub> R, A <sub>2</sub> R, Glucagon-R)
Cardiac fibroblast	$\beta_2$ AR, $G_s$ , AC5/6, AC3, $G_s$ (IPR)	EP <sub>2</sub> R, $G_s$ , AC2, AC4, AC7 (A <sub>2</sub> R)
Aortic smooth muscle	AC5/6, AC3, $G_s$	$\beta_1$ AR, $\beta_2$ AR, AdV-AC6, EP <sub>2</sub> R, $G_s$
Airway epithelia	AC5/6, $G_s$	$\beta_1$ AR, $\beta_2$ AR, AC3, AC4, $G_s$
Airway smooth muscle	$\beta_1$ AR, $\beta_2$ AR, $G_s$ , AC5/6 (CGRP-R)	EP <sub>2</sub> R, EP <sub>4</sub> R, $G_s$ (IPR)

implying that the mechanisms governing lipid raft localization are not wholly dependent upon protein sequence, but instead appear to be cell type-dependent. Perhaps this is the result of lipid modifications that occur to a greater extent in one cell *versus* another or from some cells containing limited quantities of lipid rafts, but clearly other explanations are also possible. Data from several cell types on the localization of various GPCR and AC isoforms are summarized in Table 1. Taken together, these results add uncertainty to the conclusions reached from studies with only a single cell type, especially data that involve heterologous expression. At a minimum, the findings add complexity to our understanding of GPCR signaling to include the idea that differentiated cells can tailor their arrangement of signaling proteins, perhaps to attain customized response characteristics.

## Conclusions and future directions

The present techniques for studying caveolae and lipid rafts are rife with methodological pitfalls and limitations (Pike, 2003). As noted above, most approaches utilize cell fractionation procedures that break cells apart and destroy cell morphology before analysis by biochemical assays or immunological reagents. Yet compartmentation is, by nature, a morphological phenomenon best addressed with microscopic techniques. Unfortunately, light microscopy (including fluorescence) lacks the resolution required for detecting lipid rafts and caveolae, while electron microscopic approaches are limited by the effectiveness of antibodies in detecting low-abundance membrane-associated proteins. Newer approaches, such as fluorescent and bioluminescent resonance energy transfer (FRET and BRET, Zacharias *et al.*, 2002), are powerful but largely limited to expression of exogenous proteins and the associated pitfalls of not studying native proteins. New methodologies for studying lipid rafts are needed. Such new approaches may emerge as the mechanisms for protein targeting to lipid rafts become clearer. In the meantime, the combined use of experimental methods that yield confirmatory results with one another appears to be the best approach for drawing conclusions regarding lipid rafts and caveolar microdomains.

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Lipid rafts and caveolae have sparked great interest among investigators interested in signal transduction, especially because these entities define microdomains likely involved in compartmentation and scaffolding of signaling proteins. Current evidence implies that compartmentation plays an important role in cell signaling by creating intimate juxtaposition between and among signaling molecules, thereby facilitating efficient and rapid information flow in a given signal transduction pathway as well as contributing to crosstalk among pathways. A cell's ability to express different proteins with different patterns of localization allows it to tailor its signaling to match the needs of its differentiated state. Understanding the three-dimensional nature of signal transduction in the context of cell structure, be it lipid rafts, caveolae or other domains, is likely to be critical for building complete circuit maps of signaling.

From a pharmacological perspective, the recognition of signaling microdomains in the membrane – in lipid rafts/caveolae, clathrin-coated pits, or perhaps other specialized regions – provides an important advancement, albeit one that adds complexity. Such complexity mirrors that of other aspects of GPCR signaling: for example, identification of a large number of known and orphan GPCRs in genomes and individual cells, homo- and heterodimerization of receptors, combinatorial assembly of different subunits of G $\alpha$ , G $\beta$ , and G $\gamma$ , expression of multiple isoforms of key effector molecule regions, ligand-specific and inverse agonism, etc. The challenge will be to develop ways to integrate the evolving data from these newly recognized complexities to further our understanding of drug action and to lay the groundwork for the discovery of new types of drugs. Tissue-specific differences in expression of signaling components in microdomains (Table 1) may provide an opportunity to target microdomains and differentially influence regions of different receptors in different cell types. Thus, just as in life itself, adversity and complexity provide both a challenge and an opportunity.

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