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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 (α, β, γ) 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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Abbreviations: AC, adenylyl cyclase; AKAP, A kinase anchoring protein; βAR, 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 (α, β, γ) 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 $P2Y_{11}$, β_2AR, β_3AR, $5HT_2C$, 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_s stimulates adenylyl cyclase (AC) activity and regulates certain Ca^{2+} channels; G_i inhibits AC activity and regulates K^+ and Ca^{2+} channels, $G_{q/11}$ stimulates phospholipase C (PLC) activity, and $G_{12/13}$ regulates GTP exchange factors (GEF) of rho, a low-molecular-weight G protein. Thus, α 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 Gα proteins likely limits their effective radius of activity. The kinetics of G protein activation is determined by the intrinsic GTPase activity of Gα 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 Gα 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 Gα subunits in their GDP-bound (inactive) state.

Is the signaling by Gα 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_2) into inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 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 β-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

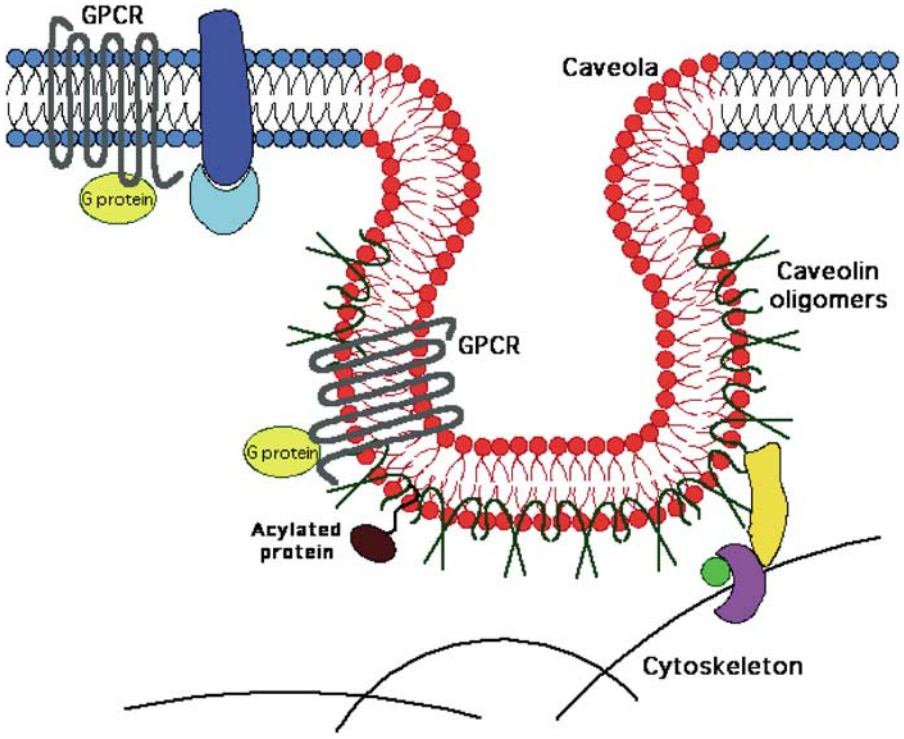

Figure 1 Schematic representation of the lipid and protein organization of a caveola. Sphingolipid- and cholesterol-rich domain is shown in red and nonraft lipid domains are shown in blue. Caveolae contain a coat of oligomeric caveolin molecules inserted into the cytoplasmic leaflet of the membrane. 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-β-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. β_1AR and β_2AR 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_2 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 β_2AR activate AC6 less efficiently than do β_1AR, and this appears attributable to rapid agonist-promoted translocation of β_2AR into clathrin-coated pits (Goodman *et al*., 1996; Gagnon *et al*., 1998) and out-of-lipid rafts and caveolae compared to β_1AR, 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 βAR-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) βAR, but not prostanoid receptors, couple to AC6 due to βAR-AC6 localization in lipid rafts or caveolae. Disruption of lipid rafts with methyl-β-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 βAR signal transduction pathway. Figure 2 illustrates this concept.

New data shed some light on the different behaviors of β_1AR and β_2AR with respect to association with lipid rafts. Using phosphorylation-deficient mutants of the β_1AR, Rapacciuolo *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 β_1AR are primarily phosphorylated by GRK in native cell settings and whether such results with the β_1AR can be applied to β_2AR or other GPCRs. β_1AR and β_2AR are known to signal differently in cardiac myocytes, where they are co-expressed, due to β_2AR coupling sequentially to G_s and then G_i (Xiao *et al*., 1994; 1999). Intact lipid rafts are necessary for the β_2AR to couple to G_i (Xiang *et al*., 2002b); both β_1AR and β_2AR 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 βAR 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

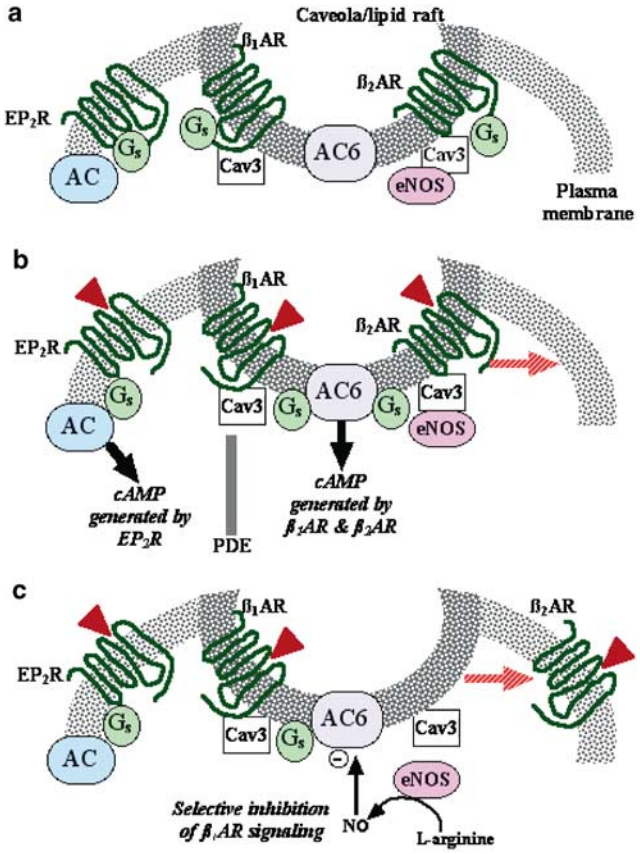

Figure 2 Schematic representation of GPCR-G_s-AC signaling in lipid rafts and caveolae of cardiac myocytes and the effects of eNOS. (a) Caveolar/lipid raft domains contain β_1AR, β_2AR, AC6, G_s, eNOS, and caveolin-3 (Cav3), but exclude prostanoid EP_2R (Ostrom *et al*., 2000b). (b) Catecholamines stimulate βAR 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. β_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 βAR) (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 RASMC 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

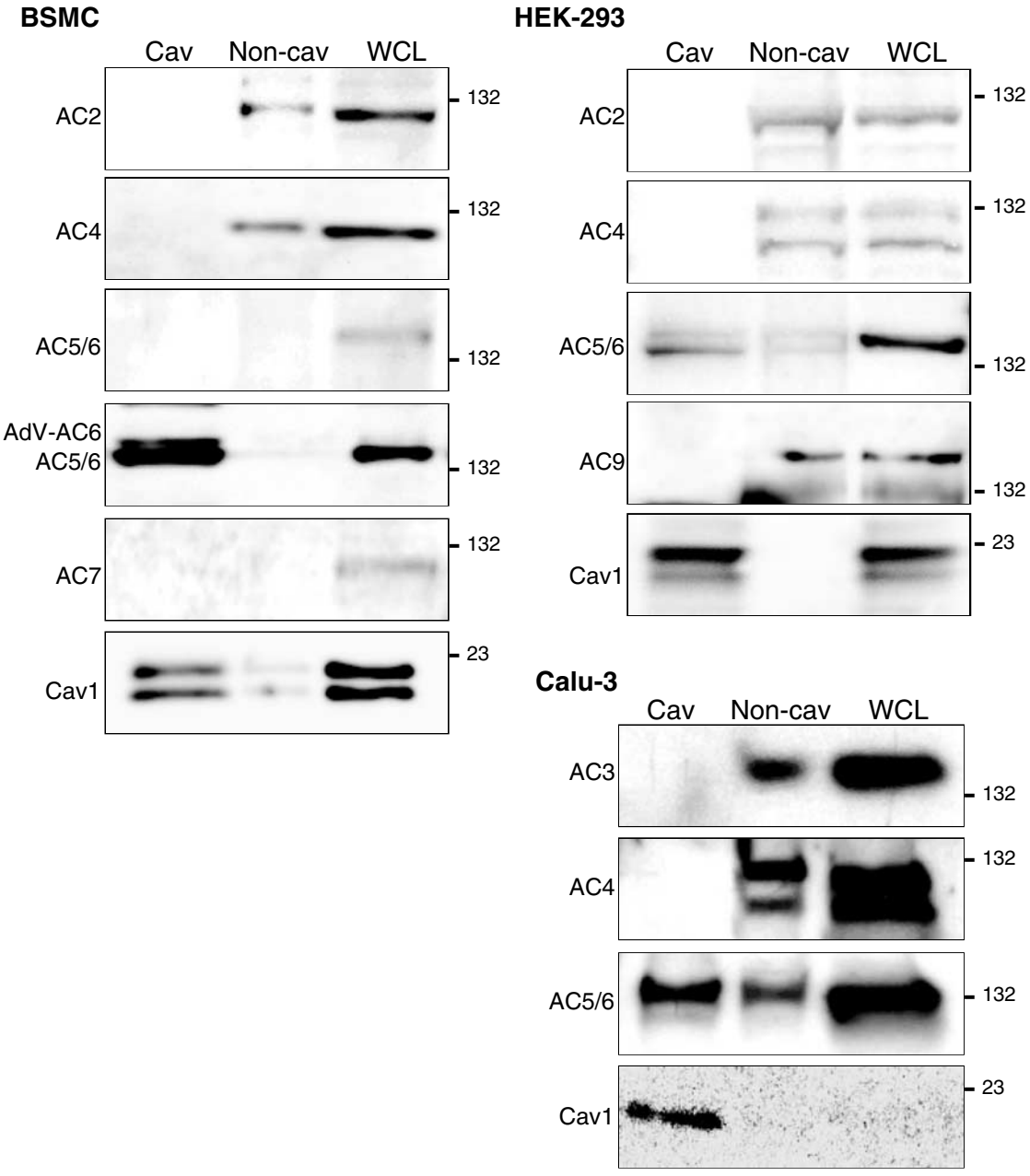

Figure 3 Immunoblot analyses of AC isoform expression in caveolae/lipid raft fractions isolated from human BSMC, HEK-293 cells or human airway epithelial cells (Calu-3). Buoyant lipid raft fractions were isolated from the indicated cells using a nondetergent method followed by centrifugation on a sucrose 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 βAR recruits β-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, β-arrestin translocation to the plasma membrane enhances the local degradation of cAMP and terminates the stimulus by desensitizing the receptor. Furthermore, β-arrestin-PDE4 recruitment to activated β_2AR 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 β_2AR 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 βAR-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 βAR 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, phosphofructokinase, is expressed in lipid rafts in those cells (Vallejo & Hardin, 2004). Thus, cAMP generated in lipid rafts in response to activation of raft-localized βAR (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 β_1AR and β_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 βAR 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	β_1AR, β_2AR, G_s, AC5/6, PKA, AKAP	EP_2R, EP_4R, G_s, (H_2R, A_2R, Glucagon-R)
Cardiac fibroblast	β_2AR, G_s, AC5/6, AC3, G_s (IPR)	EP_2R, G_s, AC2, AC4, AC7 (A_2R)
Aortic smooth muscle	AC5/6, AC3, G_s	β_1AR, β_2AR, AdV-AC6, EP_2R, G_s
Airway epithelia	AC5/6, G_s	β_1AR, β_2AR, AC3, AC4, G_s
Airway smooth muscle	β_1AR, β_2AR, G_s, AC5/6 (CGRP-R)	EP_2R, EP_4R, 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.

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 Gα, Gβ, and Gγ, 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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References

ALOUSI, A.A., JASPER, J.R., INSEL, P.A. & MOTULSKY, H.J. (1991). Stoichiometry of receptor-Gs-adenylate cyclase interactions. *FASEB J.,* **5,** 2300–2303.

ANDERSON, R.G. (1998). The caveolae membrane system. *Annu. Rev. Biochem.,* **67,** 199–225.

ANDERSON, R.G. & JACOBSON, K. (2002). A role for lipid shells in targeting proteins to caveolae, rafts, and other lipid domains. *Science,* **296,** 1821–1825.

AWASTHI-KALIA, M., SCHNETKAMP, P.P. & DEANS, J.P. (2001). Differential effects of filipin and methyl-beta-cyclodextrin on B cell receptor signaling. *Biochem. Biophys. Res. Commun.,* **287,** 77–82.

BAILLIE, G.S., SOOD, A., MCPHEE, I., GALL, I., PERRY, S.J., LEFKOWITZ, R.J. & HOUSLAY, M.D. (2003). beta-Arrestin-mediated PDE4 cAMP phosphodiesterase recruitment regulates beta-adrenoceptor switching from Gs to Gi. *Proc. Natl. Acad. Sci. U.S.A.,* **100,** 940–945.

BERGDAHL, A., GOMEZ, M.F., DREJA, K., XU, S.Z., ADNER, M., BEECH, D.J., BROMAN, J., HELLSTRAND, P. & SWARD, K. (2003). Cholesterol depletion impairs vascular reactivity to endothelin-1 by reducing store-operated Ca2+ entry dependent on TRPC1. *Circ. Res.,* **93,** 839–847.

BOCKAERT, J., MARIN, P., DUMUIS, A. & FAGNI, L. (2003). The 'magic tail' of G protein-coupled receptors: an anchorage for functional protein networks. *FEBS Lett.,* **546,** 65–72.

BRADY, J.D., RICH, T.C., LE, X., STAFFORD, K., FOWLER, C.J., LYNCH, L., KARPEN, J.W., BROWN, R.L. & MARTENS, J.R. (2004). Functional role of lipid raft microdomains in cyclic nucleotide-gated channel activation. *Mol. Pharmacol.,* **65,** 503–511.

CARMAN, C.V., LISANTI, M.P. & BENOVIC, J.L. (1999). Regulation of G protein-coupled receptor kinases by caveolin. *J. Biol. Chem.,* **274,** 8858–8864.

CHUN, M., LIYANAGE, U.K., LISANTI, M.P. & LODISH, H.F. (1994). Signal transduction of a G protein-coupled receptor in caveolae: colocalization of endothelin and its receptor with caveolin. *Proc. Natl. Acad. Sci. U.S.A.,* **91,** 11728–11732.

CLAPHAM, D.E. & NEER, E.J. (1997). G protein beta gamma subunits. *Annu. Rev. Pharmacol. Toxicol.,* **37,** 167–203.

CLAPHAM, D.E., RUNNELS, L.W. & STRUBING, C. (2001). The TRP ion channel family. *Nat. Rev. Neurosci.,* **2,** 387–396.

COOPER, D.M. (2003). Molecular and cellular requirements for the regulation of adenylate cyclases by calcium. *Biochem. Soc. Trans.,* **31,** 912–915.

COUET, J., SARGIACOMO, M. & LISANTI, M.P. (1997). Interaction of a receptor tyrosine kinase, EGF-R, with caveolins. Caveolin binding negatively regulates tyrosine and serine/threonine kinase activities. *J. Biol. Chem.,* **272,** 30429–30438.

DARBY, P.J., KWAN, C.Y. & DANIEL, E.E. (2000). Caveolae from canine airway smooth muscle contain the necessary components for a role in Ca(2+) handling. *Am. J. Physiol. Lung Cell. Mol. Physiol.,* **279,** L1226–L1235.

DE WEERD, W.F. & LEEB-LUNDBERG, L.M. (1997). Bradykinin sequesters B2 bradykinin receptors and the receptor-coupled Galpha subunits Galphaq and Galphai in caveolae in DDT1 MF-2 smooth muscle cells. *J. Biol. Chem.,* **272,** 17858–17866.

DOHLMAN, H.G. & THORNER, J. (1997). RGS proteins and signaling by heterotrimeric G proteins. *J. Biol. Chem.,* **272,** 3871–3874.

ENGELMAN, J.A., CHU, C., LIN, A., JO, H., IKEZU, T., OKAMOTO, T., KOHTZ, D.S. & LISANTI, M.P. (1998). Caveolin-mediated regulation of signaling along the p42/44 MAP kinase cascade *in vivo*. A role for the caveolin-scaffolding domain. *FEBS Lett.,* **428,** 205–211.

FAGAN, K.A., SMITH, K.E. & COOPER, D.M. (2000). Regulation of the Ca2+-inhibitable adenylyl cyclase type VI by capacitative Ca2+ entry requires localization in cholesterol-rich domains. *J. Biol. Chem.,* **275,** 26530–26537.

FERGUSON, S.S. (2001). Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling. *Pharmacol. Rev.,* **53,** 1–24.

FERON, O., SALDANA, F., MICHEL, J.B. & MICHEL, T. (1998). The endothelial nitric-oxide synthase-caveolin regulatory cycle. *J. Biol. Chem.,* **273,** 3125–3128.

FERON, O., SMITH, T.W., MICHEL, T. & KELLY, R.A. (1997). Dynamic targeting of the agonist-stimulated m2 muscarinic acetylcholine receptor to caveolae in cardiac myocytes. *J. Biol. Chem.,* **272,** 17744–17748.

FREDRIKSSON, R., LAGERSTROM, M.C., LUNDIN, L.G. & SCHIOTH, H.B. (2003). The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. *Mol. Pharmacol.,* **63,** 1256–1272.

GAGNON, A.W., KALLAL, L. & BENOVIC, J.L. (1998). Role of clathrin-mediated endocytosis in agonist-induced down-regulation of the beta2-adrenergic receptor. *J. Biol. Chem.,* **273,** 6976–6981.

GALBIATI, F., RAZANI, B. & LISANTI, M.P. (2001). Emerging themes in lipid rafts and caveolae. *Cell,* **106,** 403–411.

GARCÍA-CARDEÑA, G., MARTASEK, P., MASTERS, B.S., SKIDD, P.M., COUET, J., LI, S., LISANTI, M.P. & SESSA, W.C. (1997). Dissecting the interaction between nitric oxide synthase (NOS) and caveolin. Functional significance of the nos caveolin binding domain *in vivo*. *J. Biol. Chem.,* **272,** 25437–25440.

GOODMAN JR, O.B., KRUPNICK, J.G., SANTINI, F., GUREVICH, V.V., PENN, R.B., GAGNON, A.W., KEEN, J.H. & BENOVIC, J.L. (1996). Beta-arrestin acts as a clathrin adaptor in endocytosis of the beta2-adrenergic receptor. *Nature,* **383,** 447–450.

GUZZI, F., ZANCHETTA, D., CASSONI, P., GUZZI, V., FRANCOLINI, M., PARENTI, M. & CHINI, B. (2002). Localization of the human oxytocin receptor in caveolin-1 enriched domains turns the receptor-mediated inhibition of cell growth into a proliferative response. *Oncogene,* **21,** 1658–1667.

HAKAK, Y., SHRESTHA, D., GOEGEL, M.C., BEHAN, D.P. & CHALMERS, D.T. (2003). Global analysis of G-protein-coupled receptor signaling in human tissues. *FEBS Lett.,* **550,** 11–17.

HALL, R.A., PREMONT, R.T. & LEFKOWITZ, R.J. (1999). Heptahelical receptor signaling: beyond the G protein paradigm. *J. Cell Biol.,* **145,** 927–932.

HANOUNE, J. & DEFER, N. (2001). Regulation and role of adenylyl cyclase isoforms. *Annu. Rev. Pharmacol. Toxicol.,* **41,** 145–174.

HERMANS, E. (2003). Biochemical and pharmacological control of the multiplicity of coupling at G-protein-coupled receptors. *Pharmacol. Ther.,* **99,** 25–44.

HILL, J., HOWLETT, A. & KLEIN, C. (2000). Nitric oxide selectively inhibits adenylyl cyclase isoforms 5 and 6. *Cell Signal,* **12,** 233–237.

HNASKO, R. & LISANTI, M.P. (2003). The biology of caveolae: lessons from caveolin knockout mice and implications for human disease. *Mol. Interventions,* **3,** 445–464.

HOOPER, N.M. (1999). Detergent-insoluble glycosphingolipid/cholesterol-rich membrane domains, lipid rafts and caveolae (review). *Mol. Membr. Biol.,* **16,** 145–156.

INSEL, P.A. (2003). Location, location, location. *Trends Endocrinol. Metab.,* **14,** 100–102.

KAWASAKI, H., SPRINGETT, G.M., MOCHIZUKI, N., TOKI, S., NAKAYA, M., MATSUDA, M., HOUSMAN, D.E. & GRAYBIEL, A.M. (1998). A family of cAMP-binding proteins that directly activate Rap1. *Science,* **282,** 2275–2279.

KENAKIN, T. (2001). Inverse, protean, and ligand-selective agonism: matters of receptor conformation. *FASEB J.,* **15,** 598–611.

KENAKIN, T. (2003). Ligand-selective receptor conformations revisited: the promise and the problem. *Trends Pharmacol. Sci.,* **24,** 346–354.

KOHOUT, T.A. & LEFKOWITZ, R.J. (2003). Regulation of G protein-coupled receptor kinases and arrestins during receptor desensitization. *Mol. Pharmacol.,* **63,** 9–18.

KOPPERUD, R., KRAKSTAD, C., SELHEIM, F. & DOSKELAND, S.O. (2003). cAMP effector mechanisms. Novel twists for an 'old' signaling system. *FEBS Lett.,* **546,** 121–126.

KRUPNICK, J.G. & BENOVIC, J.L. (1998). The role of receptor kinases and arrestins in G protein-coupled receptor regulation. *Annu. Rev. Pharmacol. Toxicol.,* **38,** 289–319.

LAHTINEN, U., HONSHO, M., PARTON, R.G., SIMONS, K. & VERKADE, P. (2003). Involvement of caveolin-2 in caveolar biogenesis in MDCK cells. *FEBS Lett.,* **538,** 85–88.

LEFKOWITZ, R.J. (1998). G protein-coupled receptors. III. New roles for receptor kinases and beta-arrestins in receptor signaling and desensitization. *J. Biol. Chem.,* **273,** 18677–18680.

LEVITZKI, A. & BAR-SINAI, A. (1991). The regulation of adenylyl cyclase by receptor-operated G proteins. *Pharmacol. Ther.,* **50,** 271–283.

LLOYD, P.G. & HARDIN, C.D. (2001). Caveolae and the organization of carbohydrate metabolism in vascular smooth muscle. *J. Cell. Biochem.,* **82,** 399–408.

LOCKWICH, T.P., LIU, X., SINGH, B.B., JADLOWIEC, J., WEILAND, S. & AMBUDKAR, I.S. (2000). Assembly of Trp1 in a signaling complex associated with caveolin-scaffolding lipid raft domains. *J. Biol. Chem.,* **275,** 11934–11942.

LOHN, M., FURSTENAU, M., SAGACH, V., ELGER, M., SCHULZE, W., LUFT, F.C., HALLER, H. & GOLLASCH, M. (2000). Ignition of calcium sparks in arterial and cardiac muscle through caveolae. *Circ. Res.,* **87,** 1034–1039.

LUDWIG, M.G. & SEUWEN, K. (2002). Characterization of the human adenylyl cyclase gene family: cDNA, gene structure, and tissue distribution of the nine isoforms. *J. Recept. Signal Transduct. Res.,* **22,** 79–110.

LUTTRELL, L.M. & LEFKOWITZ, R.J. (2002). The role of beta-arrestins in the termination and transduction of G-protein-coupled receptor signals. *J. Cell. Sci.,* **115,** 455–465.

MARTENS, J.R., O'CONNELL, K. & TAMKUN, M. (2004). Targeting of ion channels to membrane microdomains: localization of KV channels to lipid rafts. *Trends Pharmacol. Sci.,* **25,** 16–21.

MARTENS, J.R., SAKAMOTO, N., SULLIVAN, S.A., GROBASKI, T.D. & TAMKUN, M.M. (2000). Isoform-specific localization of voltage-gated K+ channels to distinct lipid raft populations: targeting of Kv1.5 to caveolae. *J. Biol. Chem.,* **13,** 13.

MCVEY, M., HILL, J., HOWLETT, A. & KLEIN, C. (1999). Adenylyl cyclase, a coincidence detector for nitric oxide. *J. Biol. Chem.,* **274,** 18887–18892.

MICHEL, J.J. & SCOTT, J.D. (2002). AKAP mediated signal transduction. *Annu. Rev. Pharmacol. Toxicol.,* **42,** 235–257.

MILLIGAN, G. (1996). The stoichiometry of expression of protein components of the stimulatory adenylyl cyclase cascade and the regulation of information transfer. *Cell Signal,* **8,** 87–95.

MILLIGAN, G., GRASSIE, M.A., WISE, A., MACEWAN, D.J., MAGEE, A.I. & PARENTI, M. (1995). G-protein palmitoylation: regulation and functional significance. *Biochem. Soc. Trans.,* **23,** 583–587.

MUMBY, S.M. (1997). Reversible palmitoylation of signaling proteins. *Curr. Opin. Cell Biol.,* **9,** 148–154.

NAIR, K.S., BALASUBRAMANIAN, N. & SLEPAK, V.Z. (2002). Signal-dependent translocation of transducin, RGS9-1-Gbeta5L complex, and arrestin to detergent-resistant membrane rafts in photoreceptors. *Curr. Biol.,* **12,** 421–425.

OH, P. & SCHNITZER, J.E. (1999). Immunoisolation of caveolae with high affinity antibody binding to the oligomeric caveolin cage. Toward understanding the basis of purification. *J. Biol. Chem.,* **274,** 23144–23154.

OH, P. & SCHNITZER, J.E. (2001). Segregation of heterotrimeric G proteins in cell surface microdomains. G(q) binds caveolin to concentrate in caveolae, whereas g(i) and g(s) target lipid rafts by default. *Mol. Biol. Cell,* **12,** 685–698.

OKA, N., YAMAMOTO, M., SCHWENCKE, C., KAWABE, J., EBINA, T., OHNO, S., COUET, J., LISANTI, M.P. & ISHIKAWA, Y. (1997). Caveolin interaction with protein kinase C. Isoenzyme-dependent regulation of kinase activity by the caveolin scaffolding domain peptide. *J. Biol. Chem.,* **272,** 33416–33421.

OKAMOTO, T., SCHLEGEL, A., SCHERER, P.E. & LISANTI, M.P. (1998). Caveolins, a family of scaffolding proteins for organizing 'preassembled signaling complexes' at the plasma membrane. *J. Biol. Chem.,* **273,** 5419–5422.

ORLANDI, P.A. & FISHMAN, P.H. (1998). Filipin-dependent inhibition of cholera toxin: evidence for toxin internalization and activation through caveolae-like domains. *J. Cell Biol.,* **141,** 905–915.

OSTROM, R.S., BUNDEY, R.A. & INSEL, P.A. (2004). Nitric oxide inhibition of adenylyl cyclase type 6 activity is dependent upon lipid rafts and caveolin signaling complexes. *J. Biol. Chem,* **279,** 19846–19853.

OSTROM, R.S., GREGORIAN, C., DRENAN, R.M., XIANG, Y., REGAN, J.W. & INSEL, P.A. (2001). Receptor number and caveolar co-localization determine receptor coupling efficiency to adenylyl cyclase. *J. Biol. Chem.,* **276,** 42063–42069.

OSTROM, R.S., LIU, X., HEAD, B.P., GREGORIAN, C., SEASHOLTZ, T.M. & INSEL, P.A. (2002). Localization of adenylyl cyclase isoforms and G protein-coupled receptors in vascular smooth muscle cells: expression in caveolin-rich and noncaveolin domains. *Mol. Pharmacol.,* **62,** 983–992.

OSTROM, R.S., NAUGLE, J.E., HASE, M., GREGORIAN, C., SWANEY, J.S., INSEL, P.A., BRUNTON, L.L. & MESZAROS, J.G. (2003). Angiotensin II enhances adenylyl cyclase signaling *via* Ca2+/calmodulin. Gq-Gs cross-talk regulates collagen production in cardiac fibroblasts. *J. Biol. Chem.,* **278,** 24461–24468.

OSTROM, R.S., POST, S.R. & INSEL, P.A. (2000a). Stoichiometry and compartmentation in G protein-coupled receptor signaling: implications for therapeutic interventions involving Gs. *J. Pharmacol. Exp. Ther.,* **294,** 407–412.

OSTROM, R.S., VIOLIN, J.D., COLEMAN, S. & INSEL, P.A. (2000b). Selective enhancement of beta-adrenergic receptor signaling by overexpression of adenylyl cyclase type 6: colocalization of receptor and adenylyl cyclase in caveolae of cardiac myocytes. *Mol. Pharmacol.,* **57,** 1075–1079.

PAGE, E., WINTERFIELD, J., GOINGS, G., BASTAWROUS, A. & UPSHAW-EARLEY, J. (1998). Water channel proteins in rat cardiac myocyte caveolae: osmolarity-dependent reversible internalization. *Am. J. Physiol.,* **274,** H1988–H2000.

PAPOUCHEVA, E., DUMUIS, A., SEBBEN, M., RICHTER, D.W. & PONIMASKIN, E.G. (2004). The 5-hydroxytryptamine(1A) receptor is stably palmitoylated, and acylation is critical for communication of receptor with Gi-protein. *J. Biol. Chem.,* **279,** 3280–3291.

PERRY, S.J., BAILLIE, G.S., KOHOUT, T.A., MCPHEE, I., MAGIERA, M.M., ANG, K.L., MILLER, W.E., MCLEAN, A.J., CONTI, M., HOUSLAY, M.D. & LEFKOWITZ, R.J. (2002). Targeting of cyclic AMP degradation to beta 2-adrenergic receptors by beta-arrestins. *Science,* **298,** 834–836.

PIERCE, K.L., LUTTRELL, L.M. & LEFKOWITZ, R.J. (2001). New mechanisms in heptahelical receptor signaling to mitogen activated protein kinase cascades. *Oncogene,* **20,** 1532–1539.

PIKE, L.J. (2003). Lipid rafts: bringing order to chaos. *J. Lipid Res.,* **44,** 655–667.

POST, S.R., HILAL-DANDAN, R., URASAWA, K., BRUNTON, L.L. & INSEL, P.A. (1995). Quantification of signalling components and amplification in the beta-adrenergic-receptor-adenylate cyclase pathway in isolated adult rat ventricular myocytes. *Biochem. J.,* **311,** 75–80.

RAPACCIUOLO, A., SUVARNA, S., BARKI-HARRINGTON, L., LUTTRELL, L.M., CONG, M., LEFKOWITZ, R.J. & ROCKMAN, H.A. (2003). Protein kinase A and G protein-coupled receptor kinase phosphorylation mediates beta-1 adrenergic receptor endocytosis through different pathways. *J. Biol. Chem.,* **278,** 35403–35411.

RAZANI, B., COMBS, T.P., WANG, X.B., FRANK, P.G., PARK, D.S., RUSSELL, R.G., LI, M., TANG, B., JELICKS, L.A., SCHERER, P.E. & LISANTI, M.P. (2002a). Caveolin-1-deficient mice are lean, resistant to diet-induced obesity, and show hypertriglyceridemia with adipocyte abnormalities. *J. Biol. Chem.,* **277,** 8635–8647.

RAZANI, B. & LISANTI, M.P. (2001). Caveolin-deficient mice: insights into caveolar function human disease. *J. Clin. Invest.,* **108,** 1553–1561.

RAZANI, B., RUBIN, C.S. & LISANTI, M.P. (1999). Regulation of cAMP-mediated signal transduction *via* interaction of caveolins with the catalytic subunit of protein kinase A. *J. Biol. Chem.,* **274,** 26353–26360.

RAZANI, B., WANG, X.B., ENGELMAN, J.A., BATTISTA, M., LAGAUD, G., ZHANG, X.L., KNEITZ, B., HOU JR, H., CHRIST, G.J., EDELMANN, W. & LISANTI, M.P. (2002b). Caveolin-2-deficient mice show evidence of severe pulmonary dysfunction without disruption of caveolae. *Mol. Cell. Biol.,* **22,** 2329–2344.

RAZANI, B., WOODMAN, S.E. & LISANTI, M.P. (2002c). Caveolae: from cell biology to animal physiology. *Pharmacol. Rev.,* **54,** 431–467.

ROSS, E.M. & WILKIE, T.M. (2000). GTPase-activating proteins for heterotrimeric G proteins: regulators of G protein signaling (RGS) and RGS-like proteins. *Annu. Rev. Biochem.,* **69,** 795–827.

RYBIN, V.O., GRABHAM, P.W., ELOUARDIGHI, H. & STEINBERG, S.F. (2003). Caveolae-associated proteins in cardiomyocytes: caveolin-2 expression and interactions with caveolin-3. *Am. J. Physiol. Heart Circ. Physiol.,* **285,** H325–H332.

RYBIN, V.O., XU, X., LISANTI, M.P. & STEINBERG, S.F. (2000). Differential targeting of beta-adrenergic receptor subtypes and adenylyl cyclase to cardiomyocyte caveolae. A mechanism to functionally regulate the cAMP signaling pathway. *J. Biol. Chem.,* **275,** 41447–41457.

RYBIN, V.O., XU, X. & STEINBERG, S.F. (1999). Activated protein kinase C isoforms target to cardiomyocyte caveolae: stimulation of local protein phosphorylation. *Circ. Res.,* **84,** 980–988.

SCHERER, P.E., LEWIS, R.Y., VOLONTÉ, D., ENGELMAN, J.A., GALBIATI, F., COUET, J., KOHTZ, D.S., VAN DONSELAAR, E., PETERS, P. & LISANTI, M.P. (1997). Cell-type and tissue-specific expression of caveolin-2. Caveolins 1 and 2 co-localize and form a stable hetero-oligomeric complex *in vivo*. *J. Biol. Chem.,* **272,** 29337–29346.

SCHERER, P.E., OKAMOTO, T., CHUN, M., NISHIMOTO, I., LODISH, H.F. & LISANTI, M.P. (1996). Identification, sequence, and expression of caveolin-2 defines a caveolin gene family. *Proc. Natl. Acad. Sci. U.S.A.,* **93,** 131–135.

SCHNITZER, J.E. & OH, P. (1996). Aquaporin-1 in plasma membrane and caveolae provides mercury-sensitive water channels across lung endothelium. *Am. J. Physiol.,* **270,** H416–H422.

SCHNITZER, J.E., OH, P., PINNEY, E. & ALLARD, J. (1994). Filipin-sensitive caveolae-mediated transport in endothelium: reduced transcytosis, scavenger endocytosis, and capillary permeability of select macromolecules. *J. Cell. Biol.,* **127,** 1217–1232.

SCHUBERT, W., FRANK, P.G., WOODMAN, S.E., HYOGO, H., COHEN, D.E., CHOW, C.W. & LISANTI, M.P. (2002). Microvascular hyper-permeability in caveolin-1 (−/−) knock-out mice: treatment with a specific NOS inhibitor, L-NAME, restores normal microvascular permeability in Cav-1 null mice. *J. Biol. Chem.,* **277,** 40091–40098.

SCHWENCKE, C., OKUMURA, S., YAMAMOTO, M., GENG, Y.J. & ISHIKAWA, Y. (1999a). Colocalization of beta-adrenergic receptors and caveolin within the plasma membrane. *J. Cell. Biochem.,* **75,** 64–72.

SCHWENCKE, C., YAMAMOTO, M., OKUMURA, S., TOYA, Y., KIM, S.J. & ISHIKAWA, Y. (1999b). Compartmentation of cyclic adenosine 3′,5′-monophosphate signaling in caveolae. *Mol. Endocrinol.,* **13,** 1061–1070.

SHAUL, P.W., SMART, E.J., ROBINSON, L.J., GERMAN, Z., YUHANNA, I.S., YING, Y., ANDERSON, R.G. & MICHEL, T. (1996). Acylation targets endothelial nitric-oxide synthase to plasmalemmal caveolae. *J. Biol. Chem.,* **271,** 6518–6522.

SMART, E.J., YING, Y.S., MINEO, C. & ANDERSON, R.G. (1995). A detergent-free method for purifying caveolae membrane from tissue culture cells. *Proc. Natl. Acad. Sci. U.S.A.,* **92,** 10104–10108.

SMITH, K.E., GU, C., FAGAN, K.A., HU, B. & COOPER, D.M. (2002). Residence of adenylyl cyclase type 8 in caveolae is necessary but not sufficient for regulation by capacitative Ca2+ entry. *J. Biol. Chem.,* **277,** 6025–6031.

SONG, K.S., SARGIACOMO, M., GALBIATI, F., PARENTI, M. & LISANTI, M.P. (1997). Targeting of a G alpha subunit (Gi1 alpha) and c-Src tyrosine kinase to caveolae membranes: clarifying the role of N-myristoylation. *Cell Mol. Biol. (Noisy-le-grand),* **43,** 293–303.

SONG, K.S., SCHERER, P.E., TANG, Z., OKAMOTO, T., LI, S., CHAFEL, M., CHU, C., KOHTZ, D.S. & LISANTI, M.P. (1996a). Expression of caveolin-3 in skeletal, cardiac, and smooth muscle cells. Caveolin-3 is a component of the sarcolemma and co-fractionates with dystrophin and dystrophin-associated glycoproteins. *J. Biol. Chem.,* **271,** 15160–15165.

SONG, S.K., LI, S., OKAMOTO, T., QUILLIAM, L.A., SARGIACOMO, M. & LISANTI, M.P. (1996b). Co-purification and direct interaction of Ras with caveolin, an integral membrane protein of caveolae microdomains. Detergent-free purification of caveolae microdomains. *J. Biol. Chem.,* **271,** 9690–9697.

SOWA, G., PYPAERT, M. & SESSA, W.C. (2001). Distinction between signaling mechanisms in lipid rafts *vs* caveolae. *Proc. Natl. Acad. Sci. U.S.A.,* **98,** 14072–14077.

STEINBERG, S.F. & BRUNTON, L.L. (2001). Compartmentation of g protein-coupled signaling pathways in cardiac myocytes. *Annu. Rev. Pharmacol. Toxicol.,* **41,** 751–773.

TANG, C.M. & INSEL, P.A. (2004). GPCR expression in the heart; 'new' receptors in myocytes and fibroblasts. *Trends Cardiovasc. Med.,* **14,** 94–99.

TANG, Z., SCHERER, P.E., OKAMOTO, T., SONG, K., CHU, C., KOHTZ, D.S., NISHIMOTO, I., LODISH, H.F. & LISANTI, M.P. (1996). Molecular cloning of caveolin-3, a novel member of the caveolin gene family expressed predominantly in muscle. *J. Biol. Chem.,* **271,** 2255–2261.

TASKEN, K. & AANDAHL, E.M. (2004). Localized effects of cAMP mediated by distinct routes of protein kinase A. *Physiol. Rev.,* **84,** 137–167.

TORIHASHI, S., FUJIMOTO, T., TROST, C. & NAKAYAMA, S. (2002). Calcium oscillation linked to pacemaking of interstitial cells of Cajal: requirement of calcium influx and localization of TRP4 in caveolae. *J. Biol. Chem.,* **277,** 19191–19197.

TOYA, Y., SCHWENCKE, C., COUET, J., LISANTI, M.P. & ISHIKAWA, Y. (1998). Inhibition of adenylyl cyclase by caveolin peptides. *Endocrine,* **139,** 2025–2031.

VALLEJO, J. & HARDIN, C.D. (2004). Metabolic organization in vascular smooth muscle: distribution and localization of caveolin-1 and phosphofructokinase. *Am. J. Physiol. Cell. Physiol.,* **286,** C43–C54.

VASSILATIS, D.K., HOHMANN, J.G., ZENG, H., LI, F., RANCHALIS, J.E., MORTRUD, M.T., BROWN, A., RODRIGUEZ, S.S., WELLER, J.R., WRIGHT, A.C., BERGMANN, J.E. & GAITANARIS, G.A. (2003). The G protein-coupled receptor repertoires of human and mouse. *Proc. Natl. Acad. Sci. U.S.A.,* **100,** 4903–4908.

VON ZASTROW, M. (2003). Mechanisms regulating membrane trafficking of G protein-coupled receptors in the endocytic pathway. *Life Sci.,* **74,** 217–224.

WILLIAMS, T.M. & LISANTI, M.P. (2004). The caveolin proteins. *Genome Biol.,* **5,** 214.

WONG, W. & SCHLICHTER, L.C. (2004). Differential recruitment of Kv1.4 and Kv4.2 to lipid rafts by PSD-95. *J. Biol. Chem.,* **279,** 444–452.

WOODMAN, S.E., PARK, D.S., COHEN, A.W., CHEUNG, M., CHANDRA, M., SHIRANI, J., TANG, B., JELICKS, L.A., KITSIS, R.N., CHRIST, G.J., FACTOR, S.M., TANOWITZ, H.B. & LISANTI, M.P. (2002). Caveolin-3 knock-out mice develop a progressive cardiomyopathy and show hyperactivation of the p42/44 MAP kinase cascade. *J. Biol. Chem,* **277,** 38988–38997.

XIANG, Y., DEVIC, E. & KOBILKA, B. (2002a). The PDZ binding motif of the beta1 adrenergic receptor modulates receptor trafficking and signaling in cardiac myocytes. *J. Biol. Chem.,* **277,** 33783–33790.

XIANG, Y. & KOBILKA, B. (2003). The PDZ-binding motif of the beta2-adrenoceptor is essential for physiologic signaling and trafficking in cardiac myocytes. *Proc. Natl. Acad. Sci. U.S.A.,* **100,** 10776–10781.

XIANG, Y., RYBIN, V.O., STEINBERG, S.S. & KOBILKA, B. (2002b). Caveolar localization dictates physiologic signaling of beta2 adrenoceptors in neonatal cardiac myocytes. *J. Biol. Chem.,* **277,** 34280–34286.

XIAO, R.P., AVDONIN, P., ZHOU, Y.Y., CHENG, H., AKHTER, S.A., ESCHENHAGEN, T., LEFKOWITZ, R.J., KOCH, W.J. & LAKATTA, E.G. (1999). Coupling of beta2-adrenoceptor to Gi proteins and its physiological relevance in murine cardiac myocytes. *Circ. Res.,* **84,** 43–52.

XIAO, R.P., HOHL, C., ALTSCHULD, R., JONES, L., LIVINGSTON, B., ZIMAN, B., TANTINI, B. & LAKATTA, E.G. (1994). Beta 2-adrenergic receptor-stimulated increase in cAMP in rat heart cells is not coupled to changes in Ca2+ dynamics, contractility, or phospholamban phosphorylation. *J. Biol. Chem.,* **269,** 19151–19156.

YAMABHAI, M. & ANDERSON, R.G. (2002). Second cysteine-rich region of epidermal growth factor receptor contains targeting information for caveolae/rafts. *J. Biol. Chem.,* **277,** 24843–24846.

YARBROUGH, T.L., LU, T., LEE, H.C. & SHIBATA, E.F. (2002). Localization of cardiac sodium channels in caveolin-rich membrane domains: regulation of sodium current amplitude. *Circ. Res.,* **90,** 443–449.

YATANI, A., TAJIMA, Y. & GREEN, S.A. (1999). Coupling of beta-adrenergic receptors to cardiac L-type Ca2+ channels: preferential coupling of the beta1 *versus* beta2 receptor subtype and evidence for PKA-independent activation of the channel. *Cell Signal,* **11,** 337–342.

ZACCOLO, M. & POZZAN, T. (2002). Discrete microdomains with high concentration of cAMP in stimulated rat neonatal cardiac myocytes. *Science,* **295,** 1711–1715.

ZACHARIAS, D.A., VIOLIN, J.D., NEWTON, A.C. & TSIEN, R.Y. (2002). Partitioning of lipid-modified monomeric GFPs into membrane microdomains of live cells. *Science,* **296,** 913–916.

ZHENG, B., DE VRIES, L. & GIST FARQUHAR, M. (1999). Divergence of RGS proteins: evidence for the existence of six mammalian RGS subfamilies. *Trends Biochem. Sci.,* **24,** 411–414.

ZHENG, B., MA, Y.C., OSTROM, R.S., LAVOIE, C., GILL, G.N., INSEL, P.A., HUANG, X.Y. & FARQUHAR, M.G. (2001). RGS-PX1, a GAP for Galpha s and sorting Nexin in vesicular trafficking. *Science,* **294,** 1939–1942.

ZHONG, H., WADE, S.M., WOOLF, P.J., LINDERMAN, J.J., TRAYNOR, J.R. & NEUBIG, R.R. (2003). A spatial focusing model for G protein signals. Regulator of G protein signaling (RGS) protein-mediated kinetic scaffolding. *J. Biol. Chem.,* **278,** 7278–7284.

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