Caveolae as Organizers of Pharmacologically Relevant Signal Transduction Molecules

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Abstract

Caveolae, a subset of membrane (lipid) rafts, are flask-like invaginations of the plasma membrane that contain caveolin proteins, which serve as organizing centers for cellular signal transduction. Caveolins (-1, -2, and -3) have cytoplasmic N and C termini, palmitolylation sites, and a scaffolding domain that facilitates interaction and organization of signaling molecules so as to help provide coordinated and efficient signal transduction. Such signaling components include upstream entities (e.g., G protein–coupled receptors (GPCRs), receptor tyrosine kinases, and steroid hormone receptors) and downstream components (e.g., heterotrimeric and low-molecular-weight G proteins, effector enzymes, and ion channels). Diseases associated with aberrant signaling may result in altered localization or expression of signaling proteins in caveolae. Caveolin-knockout mice have numerous abnormalities, some of which may reflect the impact of total body knockout throughout the life span. This review provides a general overview of caveolins and caveolae, signaling molecules that localize to caveolae, the role of caveolae/caveolin in cardiac and pulmonary pathophysiology, pharmacologic implications of caveolar localization of signaling molecules, and the possibility that caveolae might serve as a therapeutic target.

INTRODUCTION

The cell surface organization of receptors and their signaling partners has been a topic of long-standing interest in several biological disciplines, including biochemistry, cell biology, physiology, and pharmacology. Recent data have emphasized the importance of colocalization of receptors, including G protein–coupled receptors (GPCRs), with their signaling partners in discrete microdomains so as to facilitate the activation of cellular events. The existence of such domains was initially inferred from the compartmental organization of various cell types. For example, myocytes and neurons have anatomically and functionally discrete cellular regions (e.g., T-tubules and intercalated discs in skeletal and cardiac muscle; synaptic densities in neurons), whereas endothelial and epithelial cells have luminal and antiluminal membranes that can be distinguished by microscopic appearance and functional activities. Much of the initial information regarding subcellular compartments was thus obtained by microscopy.

One such subcellular compartment, detected more than 50 years ago (1, 2), was termed a caveola, "little cave", due to its flask-like (<100 nm diameter), invaginated appearance in the plasma membrane (**Figure 1**). Caveolae are found in numerous cell types, especially pulmonary vascular endothelial cells (3). A subset of lipid rafts, caveolae are membrane regions enriched in particular lipids (e.g., cholesterol, glycosphingolipids) and possess scaffolding proteins (e.g., caveolins) that interact with a wide variety of proteins. Unlike caveolae, lipid rafts cannot be identified at the electron microscopic level and must be studied with alternative techniques (4–6).

Debate has existed regarding the precise nature of lipid rafts. A recent, consensus definition replaced the name lipid rafts with membrane rafts and defined them

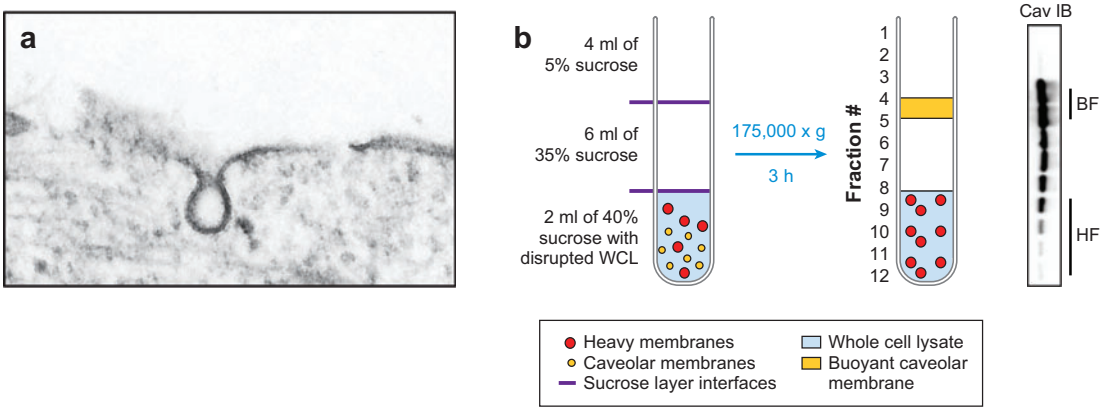

Figure 1

Microscopic and biochemical evaluation of caveolae. (*a*) Electron microscopic image of a caveola in pulmonary artery smooth muscle cells (8900x). (*b*) Sucrose density gradient separation of caveolae from disrupted cellular membranes. Resulting fractions were probed on the caveolin immunoblot (Cav IB) for the caveolar marker caveolin (*right*) and show enrichment in buoyant fractions (BF), which are thus representative of caveolae, but were not present in heavy fractions (HF), which localize other cellular membranes.

as "small (10–200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes. Small rafts can sometimes be stabilized to form larger platforms through protein-protein and protein-lipid interactions" (5). Key elements of this definition are that (*a*) both proteins and lipids contribute to the structure of rafts, (*b*) such domains may exist in intracellular membranes as well as in the plasma membrane, and (*c*) caveolae are members of the membrane (lipid) raft family. In spite of this consensus definition, precise information about certain aspects of rafts remains limited, in part because of the techniques available for their study (4).

This review focuses on caveolae as morphologically distinct entities that organize lipid and protein components. Caveolae contain caveolins (**Figure 2**), ~20 kDa caveolae-resident proteins with a unique hairpin structure and cytoplasmic amino and carboxy termini; the three caveolins (caveolins-1, -2, and -3) differ in their patterns of expression in different cell types (7). Although caveolins were named based on their identification in caveolae, they are also expressed in other cellular locations (8).

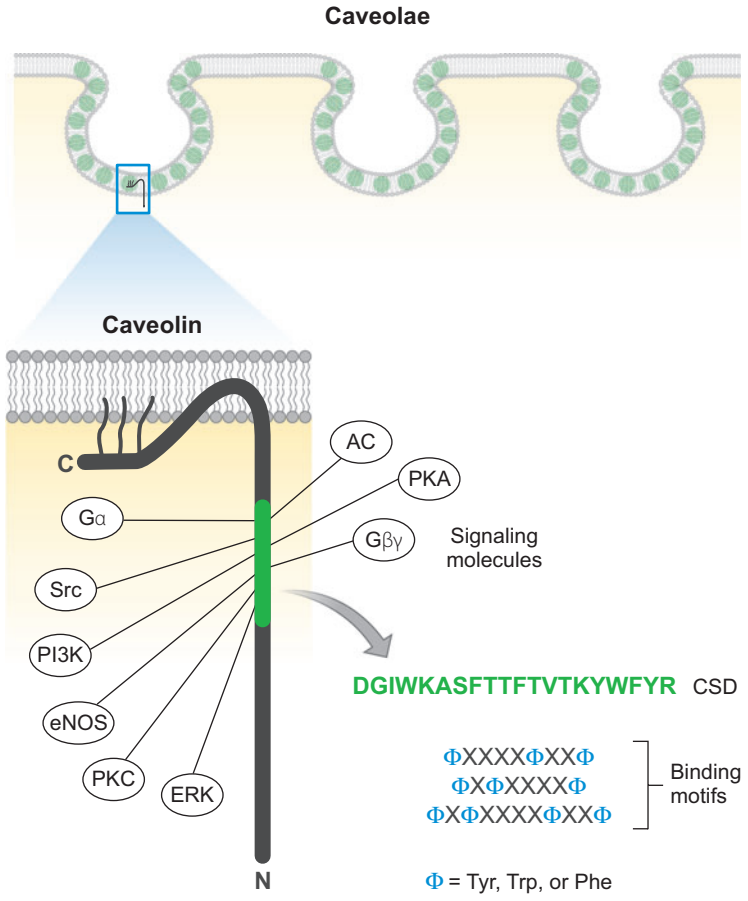

Figure 2

Caveolin scaffolding domain. Schematic depicting caveolae, resident structural proteins, caveolin (with its topology in the plasma membrane), and certain binding partners that interact with the caveolin scaffolding domain (CSD, *green*). The CSD is a peptide sequence (with the single letters reflecting its amino acids) that contains binding motifs [Φ representing aromatic amino acids, e.g., tyrosine (Tyr), tryptophan (Trp), or phenylalanine (Phe), and X representing other amino acids] that scaffold signaling molecules: adenylyl cyclase (AC), heterotrimeric Gα and Gβγ, Src, PI3 kinase (PI3K), endothelial nitric oxide synthase (eNOS, NOS 3), protein kinase A (PKA), protein kinase C (PKC), and mitogen activated protein kinase (MAPK, ERK).

Caveolins possess a domain (the caveolin scaffolding domain, CSD) to which signaling molecules bind in an inactive state; activation leads to conformational changes that release and activate the signaling proteins (9, 10) (**Figure 2**). In this review, we summarize methods used to study caveolae and provide an update on signaling components that interact with caveolins, as well as on roles of caveolae and caveolins in two organ systems, heart and lung, with an emphasis on pathophysiologic and pharmacologic implications of such findings. Other recent reviews describe additional aspects of the biology of caveolae and membrane rafts (4–8, 11–13).

METHODS TO STUDY CAVEOLAE

Many experimental approaches, including biochemical, pharmacological, molecular biological, and microscopic methods, have been used to study caveolae, in particular the caveolar localization of signaling molecules. Descriptions of such methods are available elsewhere (e.g., 14). The most widely used biochemical technique is subcellular fractionation, which is typically performed following cellular disruption by detergents (usually nonionic detergents) or high pH. Fractionation, with continuous or discontinuous gradients, is accomplished using sucrose or other entities and immunoprecipitation, in particular, with caveolin antibodies.

A pharmacologic approach involves the treatment of cells with agents that deplete membranes of cholesterol, a key component of membrane rafts and caveolae; methyl-β-cyclodextrin (MβCD) has been the most widely used agent of this type. When using MβCD, one should include a control in which cells are treated with cholesterol and MβCD to assess for effects of MβCD other than removal of cholesterol. Statin drugs have the potential to deplete membrane cholesterol and thereby alter expression of caveolae (and perhaps membrane rafts), effects that may contribute to the pleiotropic actions of statins (15).

Treatment with MβCD alters the cholesterol content and thereby the structure of caveolae. Other methods can be used to modify the expression of protein constituents in caveolae. The use of siRNAs targeted to particular caveolins or DNA constructs that increase caveolin expression provide one such approach (e.g., 16). Because caveolae are defined by their microscopic appearance, we believe that studies designed to alter the expression of caveolins, cholesterol, or other lipids in caveolae should assess the impact of such alterations by electron microscopy—an approach not commonly reported by investigators conducting such studies.

An alternative approach for the study of caveolae is the use of knockout (KO) mice (as reviewed in References 17, 18). Caveolin-KO mice (caveolin-1, -2, -3 and caveolin-1/-3 double KO mice have been generated), although viable and fertile, display numerous phenotypes (**Table 1**). Caveolin-2 KO mice retain normal expression of caveolae. Caveolin-1 KO mice also lack caveolin-2 and caveolae (in certain cell types) and develop cardiomyopathy, right ventricular hypertrophy, pulmonary arterial hypertension, and remodeling in the lung. It is unclear whether the phenotypes of caveolin KO mice result directly or indirectly from the loss of expression of caveolins and caveolae. Thus, it will be of interest to determine the phenotypes in animals that have temporally and spatially specific loss of expression of individual caveolins.

Table 1 Examples of phenotypes in caveolin knockout mice

Phenotype	Cav-1 KO	Cav-2 KO	Cav-3 KO
Presence of caveolae	Present in heart and skeletal muscle	Present in all organs	Present in all organs except heart and skeletal muscle
Cardiac			
Cardiac hypertrophy	p42/44 MAPK hyperactivation (124)		p42/44 MAPK hyperactivation (125)
Ischemia/reperfusion injury	Src activation/inactivation abnormalities (52)		Loss of caveolae-associated protective molecules (personal observations)
Extracellular matrix	Enhanced matrix metalloproteinase-2 activity (190)		
Vascular			
Reduced aortic contractile tone	Increased eNOS activity (79, 161)		
Impaired angiogenic response	Altered VEGF interactions (191)		
Neointimal hyperplasia	Increased cyclin D1 and ERK 1/2 levels (192)		
Microvascular permeability	Altered tight junctions (167)		
Pulmonary			
Lung remodeling	Hyper phosphorylation of STAT3; cyclin D1 and D3 activation (193)	Hyper phosphorylation of STAT3; cyclin D1 and D3 activation (193)	
Constricted alveolar spaces	Thickened alveolar wall (79, 161)	Thickened alveolar wall (163)	
Pulmonary hypertension	Multiple causes (160, 162, 193)		
Urogenital			
Impaired renal calcium absorption	Abnormal function of plasma membrane calcium ATPase (194)		
Enlarged seminal vesicles	Engorgement of seminal fluid (195)		
Bladder hypertrophy	Smooth muscle abnormalities (195–197)		
Cancer			
High sensitivity to carcinogens	Increased cyclin D1 and ERK 1/2 levels (198)		
Increased tumor permeability and growth	Unsuppressed VEGF signaling (191)		
Endocrine-metabolic			
Lipid abnormalities	Altered lipid homeostasis (199, 200)		Increased serum lipids (113)

(*Continued*)

Table 1 (*Continued*)

Phenotype	Cav-1 KO	Cav-2 KO	Cav-3 KO
Impaired lipolytic activity; altered lipid droplet architecture	Altered perilipin phosphorylation (199)		
Accelerated mammary gland development	Hyperactivation of prolactin signaling (201)		
Exercise tolerance	Restrictive lung disease (161)	Restrictive lung disease (163)	
Decreased glucose uptake	Insulin resistance and altered glucose transporter localization (112)		Insulin resistance and altered glucose transporter localization (113, 202)
Skeletal			
Muscle abnormalities	Tubular aggregation (203)	Tubular aggregation (203)	Loss of dystrophin complex formation (204)
Neuronal			
Neuronal injury	Increased cerebral infarct volume and apoptosis (205)		
Neurologic abnormalities	Motor and behavioral defects (206)		

For phenotype of caveolin-1/3 double knockout mice see Park et al. (129).

Because no single method to assess caveolae (and the roles of caveolae expression in drug action) is ideal, it is important to use a variety of techniques for such assessment. We believe that complementary data from studies that employ cell fractionation, immunoprecipitation, electron microscopy, and methods that alter caveolin expression (including KO animals) should be used to infer functional roles for caveolae.

Proteomic approaches have revealed that a large number of proteins localize to caveolae (19–22), either via interaction of proteins with the CSD or in the lipid microenvironment of caveolae. Such approaches have the potential to reveal the full range of protein partners in caveolae, as well as physiological or disease-related changes in the amount and nature of these partners. A substantial variety and number of signal transduction proteins localize in caveolae (**Table 2**); in the following sections we discuss signal transduction proteins and changes in their localization that may contribute to pathophysiology.

GPCR/G PROTEINS

Caveolae can localize complexes that include GPCRs, heterotrimeric G proteins, and G–protein–regulated effectors. A large number of GPCRs have been localized to membrane rafts/caveolae. In some cases, preassembled signaling complexes localize in those domains, but in others interaction with agonists facilitates migration of GPCR to rafts/caveolae as part of the events involved in desensitization and internalization (23, 24). Results for the angiotensin-1 receptor have implicated a role for

Table 2 Examples of signal transduction proteins that localize in caveolae and/or interact with caveolin

Signal transduction protein	References
Receptors	
GPCRs (e.g., angiotensin-1, serotonin, endothelin, opioid, adenosine, α_1-, adrenergic)	(23–25, 35, 150, 151, 155, 166)
Steroid hormone receptors (e.g., estrogen receptor α)	(44–46)
Bone morphogenetic receptor-II	(169)
Tyrosine kinase (e.g., insulin, EGF, NGF, IGF, PDGF)	(116–118)
Ion channels, transporters, and exchangers	
Ca^{2+}-ATPase	(60)
IP_3R	(59, 60)
Ca^{2+} pumps	(61)
L-type Ca^{2+}	(61, 62)
Large-conductance Ca^{2+}-activated K^+	(63)
TRPC	(64–68)
Na/K-ATPase	(71, 72)
Voltage-gated K^+	(73, 74)
K_{ATP}	(75)
Kinases	
Src-family	(27, 29, 32, 48, 49, 54, 50–53)
PKA	(96–99)
PKC	(101–103)
p42/p44 MAPK	(43, 123, 126)
p38 MAPK	(126)
PI3K	(108, 109)
Akt	(111, 114, 115, 104–109)
Other postreceptor components	
Gα subunits	(17, 26, 30–32, 34–38)
Gβγ	(36)
Ras	(39–41)
AC (AC3, 5, and 6)	(69, 70, 84, 86)
PDE (PDE3B)	(93)
eNOS	(76–81, 98, 99)

GPCR–caveolin interaction as more important for receptor sorting and delivery to the plasma membrane than for localization in membrane caveolae (25). Other data indicate poorly understood receptor- and cell-specific differences in the contribution of membrane rafts/caveolae to GPCR desensitization and internalization (24).

As implied by the caveolin signaling hypothesis, caveolae bring downstream effectors in proximity to receptors (e.g., GPCRs) so as to help initiate receptor-, tissue-, and cell-specific signal transduction (17, 18, 26). These effectors are thought to reside in caveolae due to direct interaction with caveolins (via the CSD) or by other

caveolae-associated proteins. Palmitoylation may aid or enhance caveolar localization of proteins; the reversibility of palmitoylation may help regulate the movement of molecules into and out of caveolae in response to stimulation by agonists (27–29).

G proteins are enriched in caveolae and directly interact with caveolin (26, 30). This interaction retains Gα proteins in an inactive GDP-bound state (31–34). Agonist stimulation of Gαq/11 or Gαi3 causes exchange of GTP for GDP and Gα redistribution to the cytosol, an effect blocked by the CSD (34), suggesting that binding to the CSD regulates G protein function. Caveolar localization of Gα subunits may favor coupling to specific signaling pathways and thus determine cellular regulation by GPCRs (31, 35–38). By contrast, downregulation of caveolin-1 by siRNA facilitates interactions of Gαq with selected GPCRs (37). Certain heterotrimeric G protein subunits, such as Gαs, show a diffuse distribution pattern on sucrose gradients and are detected in both buoyant (i.e., caveolin-enriched) and nonbouyant fractions in a cell-specific manner, including in endothelial cells and cardiac myocytes (14, 16, 17). In rat lung and endothelial cells, different G protein subunits differentially localize to caveolae compared with other cholesterol-rich domains (31, 35, 38). Palmitoylation enhances targeting of Gα subunits to caveolae (29); Gβγ increases palmitoylation of Gα, suggesting enhanced interaction of caveolae/Gα (36). Segregation of Gα subunits in caveolae and interaction with the CSD may thus determine cell-specific patterns of initiation of G protein pathways (38).

Small GTP-binding proteins of the Ras superfamily also reside in caveolae (39, 40). Palmitoylation of the C-terminal hypervariable region (CAAX motif) of Ras isoforms (e.g., H-Ras) may aid in their targeting to caveolae (39, 41). In addition, the CSD can hold H-Ras in an inactive state (31, 40). Activating mutations in H-Ras (such as G12V) prevent its interaction with caveolin and keep the protein in an active conformation; because such mutations are found in a number of human cancers, agents able to mimic this inhibitory action of caveolin may have therapeutic potential (42, 43).

STEROID HORMONE RECEPTORS

Several steroid hormone receptors associate with caveolae, an interaction that may be important for nongenomic effects of steroids (44). Targeting of estrogen receptor α (ERα) to caveolae depends on both caveolin-1 and palmitoylation (Cys^{447}) (44–46). A conserved nine–amino acid motif in the binding domain of ERα, progesterone, and androgen receptors appears to mediate palmitoylation and association with caveolin-1 (45). The compartmentalization of a population of ERα (46) in caveolae facilitates their interaction with what has been termed the steroid receptor fast-action complex, which is essential for estrogen-induced endothelial nitric oxide synthase (eNOS) activation and NO formation. Such activation likely also derives from the localization of eNOS in caveolae (see below); thus, caveolar localization of ERα may be responsible for this and other rapid effects of estrogen (46). Mutational loss of caveolin-1 expression is found in human ERα-positive breast tumors and may contribute to estrogen-dependent mammary epithelial cell growth (47).

SRC FAMILY KINASES (FYN, YES, LCK, AND LYN)

Certain nonreceptor tyrosine kinases, such as members of the Src family (c-Src, Fyn, Lyn), are enriched in caveolae due to N-terminal myristoylation and formation of complexes with caveolin; palmitoylation of caveolin-1 at Cys^{156} is essential for caveolae/c-Src interaction (27, 29). Caveolin-1, due to interaction via the CSD, suppresses the activity of c-Src and Fyn (32). Tyrosine phosphorylation of caveolin-1 (Tyr^{14}) and caveolin-2 (Tyr^{19}) facilitates recruitment of SH2 domain-containing proteins, such as Grb7 or matrix metalloproteinases (32, 48, 49). Phosphocaveolin-1 localizes to focal adhesions (protein complexes in cells that integrate cell adhesion and signaling), a key site of tyrosine kinase signaling and one that mediates cytoskeleton rearrangement (49–51). Phosphorylation of caveolin by Src can influence shape change, muscle degeneration, and inflammatory gene expression, and has been implicated in the development of cancer and protection from ischemic injury (52–54). Caveolin-1 mediates the association of integrins with Src kinases, which can activate ERK and promote cell cycle progression, interactions that may contribute to clinical disorders (51, 55).

ION CHANNELS, TRANSPORTERS, AND EXCHANGERS

The subcellular localization of ion channels and pumps is critical for their regulation and impact on cell function; particular Ca^{2+}, K^+, Na^+, and Cl^- channels are targeted to caveolae and associate with caveolins (56–58). In several cell types, including smooth muscle and endothelial cells, mediators of calcium signaling, such as Ca^{2+}-ATPase; inositol 1, 4, 5-trisphosphate receptors (IP_3R); Ca^{2+} pumps; L-type Ca^{2+} channels; large-conductance Ca^{2+}-activated K^+ channels; calmodulin; and transient receptor potential (TRP) channels, localize in cholesterol-rich membrane domains; such localization suggests that membrane rafts and/or caveolae have a role in Ca^{2+} handling, Ca^{2+} entry, and Ca^{2+} sparks (small, intense bursts of calcium) that control excitation-contraction or excitation-secretion (59–63). In smooth muscle cells, caveolae are in close (i.e., within 10–40 nm) association with the peripheral sarcoplasmic reticulum, a major site for Ca^{2+} release and postulated to be the preferred site of Ca^{2+} entry in response to depletion of calcium [i.e., calcium-induced Ca^{2+} entry (CCE)] (64). TRP channels, in particular TRPC1, -3, and -4, are enriched in caveolae (65, 66); caveolin-1 regulates the plasma membrane localization and function of TRPC1 (66). Such results suggest that caveolae regulate CCE, an idea consistent with evidence that depletion of cholesterol by MβCD reduces colocalization of caveolin-1 and TRPC1 and redistributes TRPC1, thus preventing Ca^{2+} influx (65, 67). Furthermore, caveolin-1 deficiency in endothelial cells impairs plasma membrane Ca^{2+} entry and release from internal stores, in part, due to membrane redistribution of TRPC4 and reduced TRPC4-IP_3R complex formation (68).

Caveolae may serve as scaffolds to bring Ca^{2+} in close contact with downstream effectors or Ca^{2+} channels closer to activators: TRPC1, IP_3R, Gαq/11, and caveolin-1 appear to form a preassembled signaling moiety (65). Ca^{2+}-dependent NO

production requires that AC6, eNOS, and calmodulin are together in caveolin-rich membranes and caveolae (69, 70). Moreover, the Na^+ pump, Na/K-ATPase, contains two caveolin-binding motifs and resides in caveolae in a number of cells, including smooth muscle and cardiac myocytes, thereby helping to maintain the Na^+ gradient and facilitating the formation of a Src/EGFR/ERK complex (71, 72).

Targeting of voltage-gated K^+ channels to caveolae occurs in a cell- and isoform-dependent manner and appears to be important for cellular excitability: In fibroblasts the Kv1.5 subunit targets to caveolae and colocalizes with caveolin-1; Kv2.1 localizes in membrane rafts, whereas Kv4.2 is not associated with buoyant fractions; and depletion of cholesterol with MβCD redistributes and alters the function of K^+ channels (73). MβCD has similar functional effects in cardiac myocytes, but in these cells Kv1.5 is found in cholesterol-rich fractions that are not caveolae, implying cell-specific localization of such channels (74). In smooth muscle cells, a subunit of ATP-sensitive potassium channels (K_{ATP}), which are important for the regulation of vascular tone, forms a complex with adenylyl cyclase (AC) in caveolae, thereby allowing for sustained activation by protein kinase A (75). Although some channels (e.g., TRPC1) directly interact with caveolins, others, such as Kv1.5, lack caveolin-binding motifs, which suggests that other proteins mediate their localization to caveolae (33). The findings related to ion channels imply that alterations in caveolin/caveolae expression, such as by disease or drugs, may shift the localization of channels, thereby altering cellular excitability and functional activity.

EFFECTOR ENZYMES

Numerous enzymes [e.g., eNOS, AC, cyclic nucleotide phosphodiesterase (PDE), and protein kinases] that regulate the levels and action of second messengers and cellular function by the regulation of proteins and lipids interact with the CSD and localize to caveolae.

ENDOTHELIAL NITRIC OXIDE SYNTHASE

Among the binding partners of caveolin, its interaction with eNOS has been the most extensively studied (76). Binding of eNOS to the CSD inhibits enzyme activity (77). Regions on caveolin-3 corresponding to the CSD on caveolin-1 have a similar suppression of eNOS activity, implying that this is a conserved binding and regulatory sequence motif in caveolins (78). Loss of caveolin expression upregulates eNOS activity (79), implying that caveolin regulates this activity under basal conditions. The interaction of eNOS and caveolin, with enrichment in the microdomain associated with reduced enzyme activity, led to the "caveolar paradox," whereby enrichment does not lead to enhanced activity (80). This paradox was recently resolved with the proposition that eNOS is dually regulated: direct interaction with caveolin under basal conditions to maintain an inactive enzyme and enrichment of eNOS in caveolae to provide for a rapid, high-fidelity response upon stimulation (81).

AC/cAMP

The regulation of AC in specific microdomains has been recently reviewed in detail by Willoughby & Cooper (82). As with eNOS, caveolin is thought to negatively regulate isoforms of AC. The CSD peptides of caveolin-1 and -3, but not caveolin-2, inhibit AC activity, and this inhibition is AC isoform-specific, for example, with greater inhibition of AC types 5 and 3 (83). Caveolae have enriched expression of certain isoforms of AC and regulators of AC (e.g., GPCR, G proteins, eNOS, etc.), thus providing a second level of regulation (69). ACs in caveolae couple to particular GPCRs with different efficiencies (84). Recent data suggest that superactivation of AC is dependent on the long-term stimulation of mu opioid receptors localized to membrane rafts/caveolae (85). Disruption of membrane rafts/caveolae using MβCD, colchicine (to disrupt microtubules), or cytochalasin D (to disrupt microfilaments) relocates AC from rafts/caveolae to nonbuoyant membrane regions in parallel with an enhancement in isoproterenol-stimulated cAMP generation, implying that interaction of cytoskeletal components with rafts/caveolae regulates activation of AC by GPCR (86). Because stimulation of cAMP synthesis can reduce the expression of caveolin mRNA and protein expression (87), a feedback loop may exist between cAMP levels and caveolin expression. The findings with the cytoskeletal inhibitors, several of which are used clinically (e.g., colchicine for gout and vinblastine for cancer chemotherapy), suggest that regulation of rafts/caveolae may contribute to the therapeutic utility of such agents. Additionally, it has been shown that the cytosolic domains of the Ca^{2+} sensitive ACs (AC5 and AC8) target them to membrane rafts (88). These findings reiterate and generalize the idea (81) that rafts/caveolins regulate responses under both basal and stimulated conditions.

CYCLIC NUCLEOTIDE PHOSPHODIESTERASE

Cyclic nucleotide PDEs, which hydrolyze cAMP and cGMP, compartmentalize cyclic nucleotide signals by acting as cellular barriers that establish cAMP/cGMP gradients and influence downstream responses (89, 90). Specific PDEs can link to scaffolding complexes in subcellular compartments, in part, due to PDE isoform-unique N-terminal regions (91). PDEs can associate with membrane rafts (and perhaps caveolae) before or after agonist stimulation (92). For example, in T-cells PDE4 isoforms are recruited to membrane rafts upon T-cell receptor and CD28 costimulation, thereby decreasing cAMP at the membrane and influencing immune response (92)

The targeting of PDEs to caveolae is somewhat counterintuitive because such targeting might promote the decay of cAMP/cGMP signals. PDE3B has been localized to caveolae and coimmunoprecipitated with caveolin-1 (93); PDE3B expression is reduced in adipocytes from caveolin -/- mice or when caveolin-1 is lowered by MβCD. These data suggest that caveolin-1 may have a stabilizing/activating effect on PDE3B. We have found an association of caveolin-1 with PDE5 (cGMP-specific PDE) in pulmonary artery smooth muscle cells; moreover, overexpression of caveolin-1 decreases PDE5 expression and siRNA-promoted decrease in caveolin-1 expression

increases PDE5 expression (94). This inverse relationship apparently does not result from a direct interaction of caveolin-1 with PDE5 but rather via an indirect mechanism. Perhaps PDEs translocate to caveolae after the initiation of specific signaling pathways through adapter proteins; for example, PDE4A4 interacts with the SH3-domain of Src protein tyrosine kinases (95). Future work with techniques that visualize protein movement should aid in defining the interactions of PDEs in caveolae.

PROTEIN KINASES (PKA/PKC)

Little is known regarding PKA localization to caveolae, but both the CSD and C terminus of caveolin-1 can interact with PKA and inhibit its activity (96, 97). Localization of PKA in caveolae is linked to its regulation of proteins, such as ATP-dependent K^+ channels in smooth muscle cells (75) and eNOS in endothelial cells (98). The interaction of caveolin-1, PKA and eNOS occurs via the CSD, such that the multiprotein complex may depend on oligomerized caveolin to organize these entities in a manner to facilitate the inhibition of enzyme activity (99). Enzyme activation would then occur by signaling events that de-oligomerize caveolins and release the "caged" enzymes.

The PKC family of enzymes translocate to cellular compartments in response to stimuli (100) and isoforms of PKC can target to caveolae to enhance the regulation of caveolar-localized proteins (101). Ceramide is one such molecule that can recruit and activate PKC in caveolae (102). Caveolae can also contribute to the inactivation of PKCα signaling via facilitation of endosomal delivery (103). The localization of PKC, PKA, and other protein kinases in caveolae and interaction with caveolin thus appear to regulate caveolar resident proteins and, by phosphorylation, various cellular processes.

PI3K/PKB (Akt)

The phosphatidylinositol-3-kinase/protein kinase B (PI3K/PKB, Akt) pathway is another protein kinase system that interacts with caveolin, an interaction that may regulate cell survival. For example, caveolin maintains Akt in a phosphorylated (activated) form in prostate cancer (104), presumably via interaction of the CSD with, and inhibition of, protein phosphatase 1 and 2A (105). The increased expression of caveolin-1 and Akt activity have also been correlated in colorectal cancer (106). Conversely, caveolin expression can enhance cell death via activation of Akt, for example, sensitizing HepG2 cells to killing by TNF-α (107) or arsenite (108). In muscle, a balance appears to exist between the expression of caveolin-3 and activation of the PI3K/Akt pathway in the regulation of cell survival (109). Interaction of Akt may be recruited by PI3K, which binds to caveolin (110). Caveolin and Akt have also been linked to insulin signaling in muscle cells (111), a linkage that may be altered by insulin resistance (111–113). In addition, the phosphorylated form of caveolin is involved in EGF receptor transactivation, which is dependent on Src and Akt phosphorylation and for which caveolin helps integrate this signaling cascade (114). Such integration

is also important for a physiological stress, cyclical stretch of smooth muscle, in which PI3K/Akt activation depends on caveolin expression (115).

MITOGEN ACTIVATED PROTEIN KINASES

Receptor tyrosine kinases (RTK) have been localized to caveolae [e.g., EGF, NGF, IGF, PGDF, and insulin (116–118)]. Regulation of MAPK, the downstream effectors of RTK, by caveolin is important for numerous cellular processes; these include shear, mechanical, and osmotic stress (53, 119); lung fibrosis (120); cellular proliferation (121); and angiogenesis (122). p42/44 MAPK localize to caveolae and are negatively regulated by interaction with caveolin-1 (43, 123). Overexpression of caveolin-1 inhibits the MEK/ERK signaling pathways, an inhibition that is dependent on the CSD (43). Consistent with this action, caveolin-1 and -3 KO mice show increased activation of p42/44 ERK (124, 125). An ischemia/reperfusion model shows differential activation of p42/44 ERK and p38 MAPK in caveolar fractions, implying differences in the regulation of these kinases by caveolins (126). This idea is supported by studies that have assessed the role of carbon monoxide in cellular signaling and found that upregulation of caveolin-1, secondary to the activation of p38 MAPK, contributes to the antiproliferative properties of carbon monoxide (127).

CAVEOLINS, CAVEOLAE, AND PATHOPHYSIOLOGY

Because the efficiency of intracellular signaling can be influenced by the localization of signaling components in caveolae and interaction of such components with caveolins, it is not surprising that altered caveolae expression can contribute to disease phenotypes. In the next section, we focus on diseases of two organ systems, heart and lung, for which recent data implicate important roles for caveolin expression and caveolae.

Caveolins in the Heart: General Principles and Role in Hypertrophy and Reperfusion Injury

The potential role of caveolin in cardiovascular pathophysiology has been highlighted by the discovery that caveolin-1 and -3 single and double KO and caveolin-3 transgenic mice have cardiomyopathic phenotypes (124, 125, 128, 129). The regulation of cardiac physiology by caveolins may depend on cell-specific expression and compartmentation of signaling complexes. The heart is comprised of terminally differentiated cardiac myocytes surrounded by a network of cardiac fibroblasts and with less abundant vascular smooth muscle and endothelial cells. Below we discuss caveolin and caveolae in cardiac tissue with a particular focus on mechanisms of their impact on cardiac disease.

There has been controversy regarding expression of caveolin isoforms in the heart. What was known with certainty is that cardiac myocytes express the muscle-specific isoform caveolin-3 (130, 131) and that other cell types in the heart express caveolin-1 and -2. Recent studies have provided evidence for the existence of and a signaling

role for caveolin-1 in cardiac myocytes (52). It was previously thought that caveolin-1 and caveolin-2 form hetero-oligomers and caveolin-3 forms homo-oligomers (131, 132); however, recent data indicate coexpression and interaction of caveolin-2 and -3 in neonatal cardiac myocytes (133) and interaction of caveolin-1, -2, and -3 in adult cardiac myocytes (86, 134). Other findings have shown that cell type–specific environments may regulate the interaction of caveolins: In fibroblasts caveolins-1 and -2, but not caveolin-3, interact, but in myoblasts all three caveolin isoforms coimmunoprecipitate (135). Although the heart expresses all three forms of caveolin, most studies have emphasized caveolin-3; we thus focus our discussion of cardiac disease on this isoform.

Caveolins are developmentally regulated in cardiac tissue. Immunohistochemistry reveals that caveolin-3 expression is punctuated with linear patterns from postnatal days 1–5 but is more continuously linear after day 14; such patterns parallel an increase in expression at birth and more stabilized expression postnatally (136). This change in caveolin-3 expression may contribute to its involvement in transverse tubule development (137). Caveolin-3 is the only isoform that shows cardiac and skeletal muscle–specific expression, presumably because its promoter region contains an E box element that serves as a binding site for myogenin, a transcription factor involved in differentiation of cardiac myocytes (138). By contrast with the change in expression of caveolin during development, aging is associated with dissociation of caveolin from caveolae (139).

Cardiac Hypertrophy

Hypertrophy is a growth response of cardiac myocytes to increase their size and thereby change the size and geometry of the heart. The growth response is due to stimulants (stresses) that increase demand for energy. In certain settings (e.g., exercise) this stimulation is physiologic and increases efficiency of the heart; however, in other situations (e.g., myocardial ischemia, pulmonary or systemic arterial hypertension, or valvular heart disease) an initially compensatory hypertrophy becomes detrimental, such that the heart is unable to function normally.

Overexpression of caveolin-3 in neonatal cardiac myocytes decreases the ability of the adrenergic agonist phenylephrine or endothelin-1 to increase cell size (140). A similar effect is seen in cardiac myoblasts (H9C2) in which caveolin-3 reduces angiotensin II–promoted hypertrophy (141). Other studies indicate that cardiac hypertrophy results in decreased expression of caveolin-3 (142, 143) and that right heart [and perhaps left heart (144)] hypertrophy is enhanced in caveolin-1 KO and caveolin-1/3 double KO mice (142). Interestingly, exercise training of spontaneously hypertensive rats, which undergo pathologic hypertrophy as a consequence of hypertension, produces changes that show a return to a more normal phenotype with increased expression of caveolin-3 mRNA, suggesting that increased expression of caveolin-3 is associated with regression of the hypertrophic phenotype (145). In another setting of altered cardiac function, ischemia-induced heart failure, caveolins-1 and -3 dissociate from caveolae (139). Collectively, these studies suggest that increased expression of caveolin-3, and possibly other caveolins, results in a decreased growth

response and that conversely, decreased caveolin-3 expression can result in cardiac hypertrophy. Other studies indicate that increased expression of caveolin-3 accompanies hypertrophy (146, 147) and pacing-induced heart failure (148). The discrepancy in these studies might be attributed to the nature of the models used to study hypertrophy. Perhaps signaling molecules localized to caveolae respond differently to different physiological or pathological stimuli.

The mechanism by which increased expression of caveolin suppresses pathologic myocyte growth appears to involve the down-regulation of growth signals. Caveolin-1 and -3 KO mice show hyperactivation of p42/44 MAPK (124, 125) and upregulation of eNOS activity and nitrosative stress (143, 144). By contrast, increased caveolin expression downregulates activity of those entities (140, 149). Chronic myocardial hypoxia increases eNOS expression while decreasing the expression of caveolin-3, consistent with the idea that the expression and activity of eNOS is dependent on caveolin (69, 78). Caveolae also show enrichment of α_1-adrenergic receptor signaling components; downregulation of caveolin-3 during hypertrophy may lead to dysregulation of α_1 adrenergic receptor signaling and progression of cardiac hypertrophy (150, 151). β-adrenergic receptor stimulation (via continuous isoproterenol infusion) downregulates caveolin-1 and -3 expression in the heart (152). Such studies reveal a potential role for caveolin expression in cardiac hypertrophy, but it is unclear if changes in expression of caveolin isoforms are causative or a secondary consequence of hypertrophy. Alterations in caveolin expression almost certainly change the ability of the hypertrophied heart to respond to a variety of physiologic and pharmacologic agonists.

Myocardial Ischemia/Reperfusion Injury

Myocardial ischemia continues to be a major cause of morbidity and mortality worldwide. One intervention for protection from such injury is ischemic preconditioning, whereby an initial, nonlethal injury protects the heart from subsequent lethal injury. Although preconditioning was discovered more than two decades ago (153), it has not yet been translated into a useful therapeutic approach, in part, because of the absence of an optimal therapeutic target after the onset of ischemia, i.e., when patients have symptoms. Ischemic preconditioning involves the activation of multiple pathways (154) whose spatial and temporal relationships remain to be fully defined. Recent studies implicate the possible involvement of caveolins and caveolae in protecting the heart from ischemia/reperfusion injury. Certain GPCRs, e.g., opioids (35) and adenosine (155), that can promote cardiac protection as well as postreceptor components that can enhance protection, localize to caveolae and coimmunoprecipitate with caveolins. The latter include Gα subunits of heterotrimeric G proteins, Src kinases, PI3K, eNOS, PKC isoforms, and ERK, all of which bind to the CSD and are regulated by caveolin (110, 126).

Evidence that caveolins may be involved in protecting the myocardium includes the finding that infusion of the CSD peptide of caveolin-1 into ischemic/reperfused hearts results in recovery of cardiac function (156). Ischemia/reperfusion injury activates p42/44 and p38 MAPK, redistributes caveolin-3 and downregulates

expression of caveolin-1 (126), thereby limiting the negative impact of caveolin-1 on eNOS. The latter effect suggests that one mechanism by which preconditioning produces cardiac protection is via increased generation of NO during lethal ischemia (157). Disruption of caveolae using MβCD eliminates the ability of ischemia and pharmacological preconditioning to protect cardiac myocytes from injury (158), implying that caveolae are essential for cardiac protection from such injury, an idea supported by the decreased ability of caveolin-1 KO mice to undergo pharmacological preconditioning (52).

The molecular mechanism of caveolin-associated protection from ischemic injury is unclear but may involve the regulation of Src activity (52). Ischemic preconditioning may modulate the microenvironment of caveolae and caveolin-associated protein interactions so as to enrich for proteins that promote cardiac protection. This idea is consistent with findings indicating that eNOS and the glucose transporter GLUT-4 translocate to caveolae after preconditioning (159). Future studies are needed to define the proteome of preconditioned caveolae to identify the proteins and signaling networks that may mediate and modulate cardiac protection.

Caveolins in the Lung: General Principles and a Role in Pulmonary Hypertension

The lungs, in particular the pulmonary vasculature, have a very high expression of caveolae (160). Moreover, caveolin-1 KO mice have a pulmonary phenotype (**Table 1**). Recent data have helped to identify why the absence of caveolin-1 has such adverse pulmonary effects and whether this is a cell- or species-specific response. From a therapeutic point of view, one could ask: Do caveolin-1 KO mice help identify novel approaches for the treatment of pulmonary disorders or are findings in such mice not readily extrapolated to human disease phenotypes (18)?

Caveolin-1 and -2 KO mice have multiple pulmonary defects [e.g., alveolar constriction, septal thickening, hypercellularity due to increased pulmonary artery endothelial cells (PAEC), and increased basement membrane deposition], as well as exercise intolerance (161, 162) (**Table 1**). The explanation for such complex phenotypes is that caveolin-2 expression is reduced in caveolin-1 KO mice because caveolin-1 is necessary to form caveolin-1 and -2 hetero-oligomers; caveolin-1 KO mice thus have no caveolin-2 expression. Aspects of the pulmonary phenotype, such as constricted alveolar spaces, associated with a hypercellular thickening of the alveolar walls, observed in both the caveolin-1 KO and caveolin-2 KO mice, may thus be attributable to the loss of caveolin-2 and not necessarily to reduced caveolin-1 and caveolae expression (161, 163). Because the development of pulmonary arterial hypertension (PAH) occurs in caveolin-1 KO, but not in the caveolin-2 KO, PAH appears to be caveolae- and caveolin-1-dependent.

PAH in humans occurs as both a primary (idiopathic PAH, IPAH) and secondary disorder, the precise molecular mechanisms of which are not known (164, 165). PAH is characterized by smooth muscle proliferation and impaired endothelial-dependent vasodilatation. A number of signaling entities that play a role in PAH target to caveolae and/or interact with caveolin-1. These include bone morphogenetic

receptor-II [BMPR-II, mutations of which are found in a number of IPAH-patients (164, 165)], eNOS, TRPC, reactive oxygen species (ROS), heme-oxygenase-1 (HO-1), serotonin (5HT), and endothelin receptors; it is thus not surprising that altered expression of caveolae/caveolins might increase susceptibility to this disease (37, 67, 166–169). Consistent with data obtained from caveolin-1 KO mice, caveolin-1 expression is reduced in the lungs of patients with IPAH and in animal models of PAH, such as hypoxic- and monocrotaline-treated rats (168, 170–172, F. Murray, R.Y. Suda, X. Li, P.A. Thistlethwaite, and P.A. Insel, unpublished data). A reduction in caveolin-1 expression is observed in the plexiform lesions (comprised of proliferating endothelial cells) that accompany IPAH and in the endothelial layer of the PA, which likely accounts for the overall reduction in caveolin expression in the lung (168). In caveolin-1 KO mice, pulmonary vascular endothelial cells, the major source of caveolae in the lungs, demonstrate uncontrolled proliferation (162, 161).

Caveolin-1 can regulate proliferation and cell cycle progression and can, in turn, be regulated by these processes (79, 125, 161, 167, 173). Development of PAH in caveolin-1 KO mice thus may derive from removal of caveolin-mediated inhibition of signal transduction pathways that contribute to cellular proliferation as a result of enhanced activity of enzymes such as p42/p44 MAPK, PI3K, and AKT; increased NO production; and altered Ca^{2+} signaling (79, 125, 161, 167, 173). Injection of caveolin-1 KO mice with an eNOS inhibitor reverses aspects of the PAH phenotype and, thus, loss of the inhibition of eNOS in PAEC may be particularly important for the development of PAH (167). Immunohistochemistry and gradient fractionation of PAEC reveal that BMPR-II and BMP-II-mediated Smad (regulators of transcription that transduce signals from TGFβ receptors) signaling, which may underlie aspects of PAH, localize in caveolae and can interact with caveolin-1 (164, 165, 169, 174). Reduction in caveolin-1 in PAEC after monocrotaline treatment leads to phosphorylation and activation of the transcription factor STAT3, which upregulates proliferative and antiapoptotic genes (171). Downregulation of caveolin-1 in PAEC may thus alter the integrity of the endothelium, including the localization and efficiency of signaling components that are important for the development of PAH.

Caveolae have an inhibitory role on cell growth, a conclusion inferred from multiple types of data, including reduced caveolae formation and caveolin-1 expression in human cancers (e.g., breast, ovarian, and lung) and several cancer cell lines (175, 176). Serum and growth factors (e.g., TGF, EGF, and PDGF) can downregulate caveolin-1 expression (177, 178). In addition, lung tissue and pulmonary fibroblasts isolated from patients with idiopathic pulmonary fibrosis or scleroderma show decreased caveolin-1 expression, which may contribute to increased kinase signaling, Smad phosphorylation and translocation, and collagen accumulation (178). Negative regulation of proliferation by caveolin-1, especially in pulmonary endothelial cells and fibroblasts, may thus contribute to the development of PAH. Moreover, the number of caveolae decreases as smooth muscle cells change from a contractile to a synthetic, proliferative phenotype (179).

In contrast with such observations, we have found that pulmonary artery smooth muscle cells (PASMC) from IPAH patients show a prominent increase in caveolin-1 expression and number of caveolae, increases that are associated with

hyperproliferation and increased CCE (172). In spite of the increased caveolin-1 expression in IPAH from PASMC patients, there is an overall decrease in caveolin-1 in their lungs and in PAEC, implying cell-specific changes in caveolin-1 expression in this disease. The latter results highlight problems associated with inferring the expression of proteins on PASMC from the use of whole lungs and of (incorrectly) extrapolating conclusions to humans from results obtained with KO mice that have whole-body, entire-lifetime loss of caveolins. The development of PAH in caveolin-1 KO mice may also be secondary to other pathologies (e.g., cardiac dysfunction, pulmonary fibrosis). How increased caveolae and caveolin-1 contribute to the enhanced PASMC proliferation that occurs with IPAH is an unanswered question (179, 180).

The proliferative effect of caveolin-1 in PASMC is consistent with data that indicate its expression is elevated in certain carcinomas, such as prostate and bladder (181, 182). Williams & Lisanti reviewed mechanisms that may contribute to the opposing role of caveolin-1 in relation to tumor progression and speculated that Tyr and Ser phosphorylation of caveolin-1 and mutations of the gene may help explain its dual function (183). Tyr^{14} phosphorylation of caveolin-1 recruits proteins with SH2-domains, such as the c-Src/Grb7 complex, and stimulates growth by promoting migration and anchorage-independent proliferation, whereas Ser^{80} phosphorylation of caveolin-1, a site close to the CSD, increases proliferation by converting it from being a membrane-bound protein to a secreted form (27, 184). In addition, dominant-negative mutations, e.g., caveolin-1 (P132L), occur in breast cancer patients and may explain its poor tumor suppressor function (175). Whether such mechanisms contribute to the mitogenic effect of caveolin-1 in IPAH-PASMC requires further investigation.

Increased expression of caveolin-1 and caveolae in PASMC may increase contraction because caveolar localization of specific channels may contribute to increased tone. Kv1.5 associates with $5\text{-}HT_{2A}$ receptors and caveolin-1 and internalizes upon 5-HT stimulation, thereby inhibiting Kv current (166). Furthermore, the increase in CCE and $[Ca^{2+}]_i$ in IPAH-PASMC that is associated with an increase in caveolae and caveolin-1 may be due to increased TRPC expression and plasma membrane localization (172); these interactions may be important in IPAH because 5-HT, Kv1.5, and TRPC all likely contribute to its pathophysiology (164, 165). In resistance vessels, which are extensively remodeled in PAH, caveolae play important roles in Ca^{2+} sensitization of the contractile apparatus and in the translocation of RhoA, which contributes to pressure-induced myogenic tone (185). The phenotype of PASMC differs throughout the pulmonary arterial tree (164, 165); therefore, the contribution of caveolae and caveolin-1 in controlling tone in each branch may differ.

From a therapeutic perspective, agents such as statins, which deplete cholesterol and reduce caveolae expression, may be beneficial in the treatment of PAH by virtue of their ability to restore eNOS activity, reduce CCE, attenuate Ca^{2+} sensitization and alter TRPC localization (65, 67, 172). Moreover, statins can attenuate PAH in animal models of the disease (186, 187). We believe that further investigation is required to determine the contribution of caveolar disruption to effects of statins in cardiovascular disease.

Caveolae-directed therapy for PAH will likely need to be cell-specific because downregulation of caveolin-1 is detrimental in PAEC and reconstituting caveolin-1 into the endothelium can reverse the aspects of the phenotype in the caveolin-KO mice (188). Such findings suggest that it may be therapeutically useful in IPAH to increase caveolae and caveolin-1, so as to mimic the activity of the CSD, in PAEC while in parallel decreasing caveolin expression or activity in PASMC.

FUTURE DIRECTIONS AND CONCLUDING REMARKS

Some questions to consider for future studies include the following: (*a*) What are the mechanisms and determinants by which signaling components localize in caveolae (only by binding to the CSD of caveolins?) and what determines association and dissociation from the microdomain? (*b*) Could caveolae be used to facilitate cellular drug therapy (189)? (*c*) How do pharmacologic agonists and other activators release/stimulate caveolae-localized signaling molecules? Do caveolae and caveolins only serve as brakes on signaling or are they accelerators as well? (*d*) What is the full range of proteins, in particular, the signaling proteins, that bind to caveolins; are there cell-specific patterns of such proteins; and does interaction with agonists change this proteome? (*e*) What mechanisms mediate the assembly and disassembly of caveolar protein complexes? (*f*) Are there protein-protein networks that assemble and then disassemble, and if so, what are the rules for the formation and organization of specific pathways? (*g*) Are there unique patterns of lipids expressed in membrane rafts and caveolae in different tissues, and if so, how are these patterns regulated? (*h*) Do individual cells have multiple "species" of caveolar microdomains, and if so, how do these change during development, in states of altered physiology and with disease and drug treatment? (*i*) Does disruption of caveolae contribute to the efficacy and actions of cholesterol-lowering drugs, such as statins?

Information regarding caveolae initially accrued slowly after their discovery over a half-century ago, but in the past decade there has been a major advance in information regarding these unique cellular regions. Original morphological descriptions have given way to more sophisticated biochemical and molecular characterization. Use of caveolin KO mice has yielded insights into potential physiologic and pathologic roles and the contribution of caveolins and caveolae to signal transduction pathways. We feel confident in predicting that the next half-century will provide even greater insights than the first 50 years of research into caveolae.

DISCLOSURE STATEMENT

The authors are not aware of any biases that might be perceived as affecting the objectivity of this review.

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