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COMPARTMENTATION OF G PROTEIN-COUPLED SIGNALING PATHWAYS IN CARDIAC MYOCYTES

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■ **Abstract** There is a large body of functional data that supports the existence of subcellular compartmentation of the components of cyclic AMP action in the heart. Data from isolated perfused hearts and from purified ventricular myocytes imply a fixed and hormone-specific spatial relationship amongst components of cyclic AMP synthesis, response, and degradation. Available data demonstrate that within a cardiac myocyte, not all cyclic AMP gains access to all cyclic AMP-dependent protein kinase (PKA), that not all PKA interacts with all possible cellular substrates of PKA, and that only a subset of the myocyte's phosphodiesterases (PDEs) may degrade cyclic AMP after a given synthetic stimulus. Molecular mechanisms contributing to compartmentation are being discovered: localization of receptors, G proteins, and adenylyl cyclases in caveolar versus noncaveolar regions of the sarcolemma; localization of PKA by A-kinase anchoring proteins; localization of PKA substrates, PDE isoforms, and phosphoprotein phosphatases in discrete subcellular regions; and differential regulation of multiple isoforms of adenylyl cyclase, phosphoprotein phosphatase, and PDE in distinct subcellular compartments.

INTRODUCTION

The central postulate of cyclic AMP (cAMP)-mediated hormone action is that hormones regulate cAMP production, cAMP activates the cAMP-dependent protein kinase (PKA), and PKA phosphorylates and thereby regulates cellular substrates (1). As noted by Ted Rall, codiscoverer of cAMP, this scheme in its simplest form presents "the unsatisfying picture of the catalytic subunit of protein kinase swimming about, happily phosphorylating a variety of cellular constituents whether they need it or not" (2). In response to Dr. Rall's comment, subcellular

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compartmentation[1] of cAMP action was proposed as a necessary control 20 years ago (3). At that time, the molecular basis of such compartmentation was unknown. Regarding arguments that invoked compartments as acts of desperation by "scoundrel" scientists at a loss to explain their data, researchers in transmembrane signaling resisted the idea of nonrandom spatial organization of the components of signaling. Perhaps this reluctance reflected the popularity of the fluid mosaic model of membrane structure (4). This provocative model accounts for the thermodynamics of phospholipid associations into membranes containing intrinsic and extrinsic proteins. However, a model that describes proteins floating freely in a sea of lipid does not correspond to what is known today about nonrandomness in membranes. This model also has become increasingly untenable from the thermodynamic standpoint. For example, sympathetic neural regulation of cardiac contractile function must be accomplished on a millisecond timescale. Yet progress in signal transduction research has revealed a dauntingly large number of molecules that must undergo a series of productive interactions for this to occur (with elements such as the adenylyl cyclase enzyme expressed at limiting levels). This task seems best accomplished by the preassembly of components of signaling pathways in clusters or on scaffolds, a notion that has gained wide acceptance with the identification of molecular associations of signaling components within and across the membrane and within the cell. Familiar examples include hormone-receptor and G protein-effector complexes, protein stacking mediated by SH2 domains originating at phosphotyrosine residues on the intracellular tails of membrane receptors for growth factors, membrane binding proteins for intracellular and extracellular filaments, and clustering and internalization of ligand-occupied receptors. Even the older literature provides ample evidence that subcellular structural organization limits or channels intracellular messages, such as the organization of the glycogen particle with the enzymes necessary for storage and mobilization of glycogen, e.g. glycogen synthase, glycogen phosphorylase, phosphorylase kinase, phosphorylase phosphatase, and PKA (5–8). Recent data provide details of the molecular framework by which we can begin to understand how compartmentation of signaling occurs. In this review, we summarize evidence supporting compartmentation of hormone action mediated via G proteins, principally by the subcellular targeting of receptors, transducers, and effectors in the cAMP pathway in cardiac cells.

[1]We prefer the term compartmentation to compartmentalization, which is popular with many of our colleagues, one of whom has been heard to say compartmentalizationize. Why our preference? For economy of space and from a desire to avoid the redundancy of making an overly long noun (compartmentalization) from an already awkwardly long verb (compartmentalize), which itself comes from the perfectly useful and shorter noun (and verb) [to]compartment. The infinitive "to compart" may sound Dickensian to the modern ear. That notwithstanding, when we compart or compartment, we get compartments (spaces that are compartmental) and achieve compartmentation. Enough is enough. More is too much.

FUNCTIONAL COMPARTMENTS OF cAMP SIGNALING

The PKA holoenzyme is a heterotetramer consisting of two regulatory (R) and two catalytic (C) subunits; isoforms of PKA are classified as either type I or type II depending on the type of R subunit. By the late 1970s, there were examples of isoform-selective activation of PKA in isolated cell systems (e.g. 9). Early data on cardiac tissue were obtained in isolated, perfused hearts and relied on the pioneering work of Corbin and his colleagues (10), who described two isoforms of PKA, the differential distribution of these isoforms in hearts of various rodents, and the localization of a portion of the generally soluble PKA holoenzyme to sarcolemma. In the late 1970s, Keely reported that both prostaglandin E_1 (PGE_1) and β-adrenergic receptor (β-AR) activation with epinephrine increased cAMP and activated PKA in isolated perfused rat heart, but that only epinephrine caused activation of glycogen phosphorylase (11, 12). Hayes et al (13) found that this dichotomy between the effects of PGE_1 and those of the β-adrenergic agonist isoproterenol extended to a variety of events thought to be cAMP dependent. Perfusion of isolated rat hearts with isoproterenol enhanced left ventricular dP/dt, elevated tissue cAMP, activated PKA in soluble extracts, increased the activation states of phosphorylase kinase and glycogen phosphorylase, and enhanced the conversion of glycogen synthase to a less-active form. PGE_1 elevated cAMP content and activated PKA but caused no changes in contractile activity or in the activities of the PKA substrates that regulate glycogen metabolism. PGE_1 exerted no inhibitory influence on the β-adrenergic response, and the data seemed not to result from activation of a phosphoprotein phosphatase by PGE_1. Brunton et al (14) extended this comparison to the contractile protein troponin I, which was phosphorylated in response to isoproterenol but not in response to PGE_1. This hormone-specific activation of PKA could not be explained on the basis of PKA isoform profiles and the selective activation of an essential isoform by isoproterenol but not by PGE_1: similar results were obtained with guinea pig (>90% type II PKA) and rat (>80% type I PKA) hearts (15). Furthermore, in experiments with isolated rabbit hearts (with a more nearly equal distribution of the two isoforms), perfusion with isoproterenol resulted in the activation of both isoforms of PKA, as assessed by R subunit labeling with the photoaffinity analog [^{32}P]8-azido-cAMP (3). The key experiment applied the findings of Corbin et al (10) that up to 50% of PKA in rabbit heart homogenates is associated with a low-speed (3000 $\times$ g for 10 min) particulate fraction and that cAMP binding to the particulate R subunits causes the dissociation of C subunits, which are then recovered in the low-speed supernatant fraction. Perfusion of rabbit hearts with isoproterenol or PGE_1 increased the activity ratio of the soluble-fraction PKA, but only isoproterenol activated particulate PKA. Moreover, only isoproterenol caused the activation of phosphorylase and a positive inotropic effect (15). Thus, the β-adrenergic effects correlated with activation of the particulate or membrane-bound PKA; elevation of cAMP in the soluble fraction and the consequent activation of PKA in the soluble pool were

without functional consequences. On the basis of these data, Hayes & Brunton (16) postulated that differences in the heart's responses to isoproterenol and PGE_1 are the consequences of activation of cAMP signal transduction pathways that are confined to distinct intracellular compartments, and that activation of the particulate fraction of PKA is essential for the heart's metabolic and inotropic responses to elevated cAMP. Data from Aass et al (17) support this idea: comparing two β agonists, isoproterenol and the partial agonist prenalterol, these workers found that the inotropic effects of these agents correlated with their capacity to increase bound cAMP in the particulate fraction of rat heart.

Because these analyses (10–17) were conducted on freeze-clamped ventricles of isolated perfused hearts, it was possible that the apparent compartmentation involved selective responsiveness of the multiple cell types of the ventricle (myocytes, fibroblasts, endothelium, smooth muscle). Although additivity experiments argued against this, the most conclusive evidence came from experiments in isolated myocytes. Using cultured neonatal rat myocytes, Hayes et al (18) found that isoproterenol treatment stimulated PKA activity, activated phosphorylase, enhanced spontaneous contractile activity, and caused the phosphorylation of 16 proteins; PGE_1, by contrast, activated PKA but caused no other measurable response. Moreover, in purified rabbit ventricular myocytes, isoproterenol caused the selective activation of particulate PKA and the activation of phosphorylase, whereas PGE_1 elevated cAMP and activated PKA in a soluble fraction but did not activate phosphorylase (19).

Compartmentation has also been invoked to explain data from experiments that have compared the effects of forskolin and isoproterenol on cAMP accumulation and various sequelae. In general, achieving equal post-PKA responses requires a smaller elevation of cAMP by isoproterenol than that by forskolin. Conversely, if one uses concentrations of isoproterenol and forskolin that elevate tissue or cellular cAMP to similar levels, the downstream effects are larger with isoproterenol. England & Shahid (20) made this point in studies of protein phosphorylation and contractility in isolated perfused rat heart, concluding that forskolin increased cAMP in a compartment not accessible to PKA and not coupled to the measured responses. Hohl & Li (21) made similar observations in studies of the relationship of cAMP and the modulation of the properties of Ca^{2+} transients in isolated canine ventricular myocytes. Effects of increased cAMP on parameters of Ca^{2+} transients correlated best with accumulation of cAMP in the particulate fraction, which was proportionally greater with isoproterenol than with forskolin. A most elegant series of experiments are those of Jurevicius & Fischmeister (22), who performed whole-cell patch-clamp recording of Ca^{2+} transients on two physically isolated portions of single isolated frog ventricular myocytes to probe the role of subcellular compartmentation of cAMP in the β-adrenergic stimulation of sarcolemmal L-type Ca^{2+} channels. The premise was that with two separated recording sites, it would be possible to assess the regulation of I_{Ca} by local and distant pools of cAMP. The result was that local application of isoproterenol caused local activation of I_{Ca}, whereas local application of forskolin caused activation of I_{Ca} throughout the

cell, i.e. at local and distant sites. The results support the concept that β-AR–dependent cAMP accumulation and responsiveness are compartmented in at least some myocardial cells.

In these and many similar experiments with forskolin, dissociation of cAMP accumulation from response may result from excessive accumulation of cAMP, reflecting the common observation, especially in the presence of phosphodiesterase (PDE) inhibitors, that a cell's capacity to generate and accumulate cAMP exceeds what is needed for a maximal physiologic response. Nonetheless, at equivalent and submaximal activations of PKA, such comparisons seem valid. One could argue that β-adrenergic stimulation delivers some additional message besides an elevation of cAMP. Were this the case, forskolin would prove ineffective at causing a physiologic response, such as positive inotropism; however, forskolin is effective. We conclude that β-AR–stimulated accumulation of cAMP occurs in just the right place in the cell to be effective and may originate from a subset of cellular adenylyl cyclases, whereas forskolin generally stimulates most or all available adenylyl cyclases.

Several lines of evidence indicate that PDEs, the enzymes that degrade cellular cAMP, may also contribute to compartmentation. For instance, certain PDE inhibitors permit PGE_1 to be an effective stimulator of myocyte glycogen phosphorylase; in this case, inhibition of cAMP breakdown appears to cause "spill over" of cAMP from the soluble into the particulate compartment of rabbit ventricular myocytes (DC Bode, LL Brunton, unpublished observation). Some hormones seem to alter PDE activities in discrete subcellular regions. In rat myocytes, α_1-adrenergic stimulation activates cAMP degradation in a subcellular compartment that, as followed by [^{3}H]adenine labeling, may be selectively depleted of [^{3}H]cAMP (23). In studies with frog myocytes, Brechler et al (24) have found evidence that glucagon, acting through a pertussis toxin (PTX)-sensitive pathway, inhibits a membrane-bound PDE that has a high affinity for cAMP, is inhibited by cyclic GMP, and is selectively inhibited by milrinone; the soluble form of this PDE is not affected by treatment of myocytes with glucagon. The authors have noted a similar effect of glucagon in tissue from mouse and guinea pig hearts and postulate that it mediates a portion of the positive inotropic effect of the peptide.

Pharmacologic inhibition of phosphodiesterases has also been used to make a point similar to that made with forskolin (see above): Analysis of agonist actions plus or minus PDE inhibition indicates that the magnitudes of cAMP accumulation and functional responses can often be dissociated. Hohl & Li (21) and Jurevicius & Fischmeister (22) have used PDE inhibitors to exaggerate cAMP accumulation (or its effects) and have concluded that inhibition of PDEs reduces apparent compartmentation, as though PDE activities were localized to prevent the spread of β-adrenergic signals from particulate (membrane) locations to soluble fractions. Isoform-specific PDE inhibitors produce similar dissociations between cAMP accumulation and functional responses, often seeming to enhance cAMP accumulation in a specific subcellular compartment (e.g. see 25–27). In an extreme case, the PDE4 inhibitor rolipram elevated cAMP content and promoted the activation of

PKA without producing a positive inotropic response in isolated, perfused guinea pig heart (28). Such results, plus the positive inotropic effects of isoform-specific PDE inhibitors such as milrinone (25, 26), a PDE3 inhibitor, imply local regulation of cellular cAMP levels by identifiable, localized PDE isoforms. Interpretation of experiments using PDE inhibitors must be made with the awareness that specificity is relative and generally depends on using the proper concentration, that methylxanthines may interact with other systems such as adenosine receptors, and that the distribution of PDE isoforms in a given organ, such as heart, varies greatly with species (27) and cell type within the heart (29). The burgeoning literature on the PDE superfamily currently describes 19 genes and 10 PDE families. PDEs surely play an important role in compartmentation of cAMP signaling. Individual PDE isoforms are differentially regulated and may associate with subcellular structures such as membranes, SH3 groups, and scaffolding proteins (30–33). However, full treatment of this topic is beyond the scope of this review.

Collectively, these data indicate that there is compartmentation of the cAMP signaling pathway in cardiomyocytes and that the compartmentation occurs at multiple loci in the signal transduction pathway. More specifically, the data demonstrate three principles of compartmentation when ventricular myocytes generate cAMP in response to hormones:

1. Not all cAMP gains access to all PKA.
2. Not all PKA has access to all possible substrates.
3. Not all cAMP has access to all cellular PDEs.

What conditions of cellular organization can accommodate these conclusions? It is difficult to explain these findings without invoking a fixed spatial geometry of activated adenylyl cyclase, cAMP, PKAs, PDEs, and specific intracellular compartments containing (or lacking) substrates for PKA, i.e. physical compartmentation. It seems likely that phosphoprotein phosphatases are also differentially compartmented and regulated in cardiac myocytes (e.g. see 34).

The workers whose experiments are summarized above did not interpret their data in terms of known subcellular features of the cardiac myocyte; rather, they focused on functional compartmentation. However, functional compartmentation has physical requirements. The cardiac myocyte is a large ($\sim$120 μm $\times$ 25 μm in cross section), highly structured cell that invites subcellular investigation (35, 36). We now turn to recent data on the molecular basis by which nonrandom spatial organization of signaling components occurs in cardiac myocytes.

MOLECULAR BASIS OF COMPARTMENTATION OF SIGNALING COMPONENTS

β-Adrenergic Receptor Subtypes

The general consensus of results from several laboratories is that β_1- and β_2-ARs are coupled to distinct functional responses in adult rat ventricular myocytes.

β_1-AR activation leads to the classical catecholamine-dependent inotropic response, namely an increase in the amplitude and the relaxation kinetics of the Ca^{2+} transient and twitch. In contrast, β_2-ARs activation enhances the amplitude of the Ca^{2+} transient and twitch without any associated increase in their relaxation kinetics, i.e. without a positive lusitropic response (37, 38). Xiao et al (39) have espoused a functional compartmentation model to explain these distinct β_1- and β_2-AR actions. They argue that the cAMP signal generated by agonist-activated β_1-ARs is broadcast throughout the cell, whereas the cAMP signal generated by β_2-ARs is localized to the sarcolemma because of the actions of an intracellular G_i-activated phosphoprotein phosphatase 1, a phosphatase activated by the putative linkage of β_2-ARs, but not β_1-ARs, to G_i. Their model is based upon the following experimental results. (*a*) Activation of either β_1- or β_2-ARs elevates cAMP to similar levels, but in different locations in the myocyte: β_1-AR agonists elevate cAMP throughout the cell, whereas β_2-AR agonists preferentially elevate cAMP in a soluble compartment (39); this is difficult to reconcile with a model for β_2-AR action that puts regulatory control of Ca^{2+} cycling and contractile function at the sarcolemma and other particulate compartments. (*b*) Pretreatment of myocytes with PTX, to inhibit activation of the G_i pathway, potentiates the amplitude of the twitch in response to β_2-AR agonists without affecting the response to β_1-AR agonists. (*c*) PTX uncovers a de novo β_2-AR–dependent positive lusitropic response associated with the phosphorylation of phospholamban. (*d*) The capacity of PTX to broadcast the cAMP signal to sarcoplasmic reticular targets is reproduced by calyculin A (34), an inhibitor of phosphoprotein phosphatases 2A and 1. This constitutes the evidence that the target of the PTX-sensitive G proteins is an intracellular phosphatase that acts on phospholamban.

This model has gained widespread acceptance in the literature; however, key experimental results are from a single laboratory and one fundamental result that constitutes a basic tenet of this model has not been reproduced by two other laboratories. Specifically, two laboratories report that β_2-AR agonists do not detectably elevate cAMP levels in adult rat ventricular myocytes (40, 41). This disparity may result from methodologic differences between the studies of Xiao et al and those of others. First, Xiao et al use norepinephrine (NE) as a specific β_1-AR agonist, asserting that it acts almost exclusively on β_1-ARs; biochemical studies from other laboratories have employed β-AR agonists in the presence of subtype-specific β-AR antagonists to ensure selectivity for β_1-AR or β_2-AR activation. Although NE interacts more potently with β_1-ARs, it has the potential to be a satisfactory β_2-AR agonist at higher concentrations. In rats, the relative affinities (or K_{act} values for the stimulation of adenylyl cyclase) for NE at β_1-ARs (heart) and β_2-ARs (lung) reportedly differ by about ninefold (42); in human heart, the difference is about 20-fold (43). By these numbers, half saturation of β_1-ARs by NE could lead to 5%–9% occupancy of β_2-ARs and nearly full saturation of β_1-ARs ($[NE] = 10\ K_d$) would occupy 33%–50% of β_2-ARs. Thus, the specificity of NE is relative, not absolute, and depends on careful choice of concentration for a specific system in which affinities of NE at β_1- and β_2-adrenergic

receptors are known. The drawbacks of NE as a specific β_1-AR agonist become most pronounced under conditions where there is simultaneous stimulation of cAMP formation by β_1- and β_2-ARs, precisely the thesis espoused by Xiao et al (34, 39) and disputed by others (40, 41). The use of NE as an exclusive β_1-AR agonist also neglects the α_1-AR component of NE action: Activation of α_1-ARs on rat myocytes can reduce cAMP accumulation (in response to β_1-AR agonists) by activating cAMP degradation (23, 44). Data from Buxton & Brunton (23) and Laflamme & Becker (41) demonstrate that NE is not as efficacious as isoproterenol in stimulating cAMP accumulation in rat myocytes. Thus, it seems possible that NE provided less than the full β-AR response in the experiments of Xiao and colleagues.

A second methodologic point involves the use of PDE inhibitors. Xiao et al (39) measured agonist-dependent cAMP accumulation in the absence of a PDE inhibitor. Experiments without PDE inhibitors are surely more physiologic; however, the interpretation of such experiments is difficult, for several reasons. In the absence of a PDE inhibitor, agonist-induced increases in myocyte cAMP are modest. For β_1-ARs they are 50%–200% vs 5- to 20-fold, without and with a PDE inhibitor, respectively, and the results may be influenced by dynamic changes in the rates of both formation and breakdown of cAMP (including the α_1-AR effect mentioned above). Thus, experimental data from the two disparate protocols ($\pm$ PDE inhibitor) may not be strictly comparable. Nevertheless, it is worth noting that β_2-AR agonists fail to induce more than a trivial increase in cellular cAMP in the context of protocols that reveal a 10- to 20-fold increase in cAMP levels in response to β_1-AR agonists (i.e. in the presence of a PDE inhibitor) (40, 41).

The failure to detect a β_2-AR–stimulated increase in cAMP accumulation in adult cardiomyocytes cannot be attributed to the simultaneous activation of stimulatory (G_s) and inhibitory (G_i) pathways by agonist-activated β_2-ARs. Despite disagreements between various laboratories as to whether β_2-ARs elevate cAMP levels, there is a consensus that PTX-sensitive G proteins play no role in this process. Zhou et al (45) reported that the β_2-AR–stimulated increases in cAMP are identical in control and PTX-treated cells. Steinberg et al (46) reported that β_2-ARs do not promote cAMP accumulation even in cells pretreated with PTX. Thus, issues related to β_2-AR activation of cAMP accumulation remain unresolved in the literature. Accordingly, it seems premature to dismiss a parsimonious interpretation of the data, namely that the capacity of β_2-ARs to support contractile function is via a cAMP-independent mechanism in adult cardiomyocytes (47). In fact, a cAMP-independent pathway for inotropic support by β_2-ARs, involving intracellular alkalinization and sensitization of the contractile apparatus, has been described by Jiang & Steinberg (47). However, a distinct pathway for β_2-AR action raises an equally interesting and puzzling question: What mechanism(s) could allow for robust stimulation of adenylyl cyclase by β_1-ARs while confining the actions of β_2-ARs, expressed in the same cell at levels sufficient to provide functional inotropic support, to a cAMP-independent effector mechanism?

Caveolae

Until recently, the prevailing concept was that receptors, G proteins, and effectors were freely mobile in the plasma membrane. However, this simple "random collision model" is inadequate to explain the experimental results highlighted in the previous sections. Several laboratories have initiated studies to determine whether compartmentation to membrane subdomains such as caveolae could constitute a mechanism to confer higher-order regulation to receptor signaling in cardiomyocytes. The current notion is that caveolae represent a plasma membrane compartment with distinct lipid and protein composition that sequesters and regulates the function of diverse signaling pathways (48). Two seemingly contradictory functions have been attributed to caveolae. Some workers suggest that caveolae serve as a scaffold to preassemble membrane-bound oligomeric complexes to facilitate the efficient and rapid transmission of signals. Such a mechanism would be particularly important for sympathetic regulation of cardiomyocyte function, where any delays in signaling from β-ARs to adenylyl cyclase (due to low, potentially limiting, levels of the adenylyl cyclase enzyme) would be poorly tolerated. Alternatively, others have provided evidence that caveolae dampen signaling, at least in part through interactions with caveolins, the principle structural proteins that coat the internal surface. Caveolins comprise a multigene family of at least three immunologically distinct 21- to 24-kDa isoforms that serve as marker proteins for this organelle. Caveolin-1 and caveolin-2 are widely expressed in similar tissues; caveolin-3 is the muscle-specific isoform. Domain mapping studies identify a cytosolic membrane-proximal region (designated the caveolin-scaffolding domain) in caveolin-1 and caveolin-3 that interacts with putative caveolin-binding motifs in a wide range of signaling molecules. Among these are G protein α subunits, the catalytic domains of certain adenylyl cyclase isoforms, G protein-coupled receptor kinase 2 (GRK2), and PKA. Caveolin negatively regulates the activation state of heterotrimeric G proteins and can serve as a general "kinase inhibitor" for many signaling enzymes. Most of the experiments were performed in the in vitro context. There is still only limited information regarding the functional role of caveolae or caveolin-3 in the physiologic context of transmembrane signaling in the cardiomyocyte.

Most studies that have implicated caveolae as a mechanism to localize signaling in cardiomyocytes have focused on G protein–coupled receptors (GPCRs), with several receptors identified in caveolae either at steady state or following ligand-induced activation. Over 10 years ago, Raposo et al (49) provided the earliest evidence that a GPCR localizes to caveolae by showing that β_2-ARs endogenous to cultured human epidermoid A431 cells segregate to nonclathrin-coated invaginations following incubation with a monoclonal antibody to the receptor and anti-mouse IgG gold. The focus of subsequent literature turned to agonist-dependent β_2-AR redistribution to clathrin-coated vesicles, a receptor sequestration mechanism (50). The discrepancy between the results of the initial study and subsequent literature has not been resolved, but differences in cell type, the method used to

engage the β_2-AR (ligand vs antibody), and the properties of the native vs heterologously expressed β_2-AR could be contributory. Until recently, there was little information on the localization of β_2-ARs in quiescent cells and almost no information on the subcellular distribution of β_1-ARs. However, Schwencke et al (51) recently reported that β_2-ARs heterologously expressed in COS-7 cells cofractionate and copurify with caveolin; Ostrom et al (52) showed similar caveolae targeting for β_1-ARs heterologously expressed in neonatal rat cardiomyocytes. In contrast, Rybin et al (53) recently showed that the subcellular distributions of native cardiomyocyte β_1- and β_2-ARs are distinct. β_1-ARs partition between caveolae, noncaveolar cell surface membranes, and internal membranes; the vast majority of the myocyte's β_1-ARs are excluded from caveolae at rest. In contrast, β_2-ARs reside exclusively in caveolae isolated from quiescent cardiomyocytes. Moreover, the high degree of β_2-AR localization to caveolae is not unique to cardiomyocytes (which contain only a minor β_2-ARs population); β_2-ARs on the surface of cardiac fibroblasts also are confined to caveolae. These studies suggest that differences in β_2-AR expression across the range of endogenous levels do not alter the fidelity of its targeting to caveolae. Drastic differences in the relative densities of β_1- vs β_2-ARs in caveolae (which contain all the β_2-ARs and only a fraction of the β_1-ARs) and the remainder of the plasma membrane (which contains only β_1-ARs) have not previously been appreciated and will need to be incorporated into concepts of the stoichiometry of elements in the cAMP signaling pathways as a mechanism to regulate the transmission of signals (54). Such differences could contribute to the apparent compartmentation of β_1- and β_2-AR responses discussed above.

Domain-specific differences in the stoichiometry of individual elements in the signaling cascade could impact significantly on the efficiency and specificity of signal transduction. For example, targeting to caveolae could provide a mechanism to enhance the efficiency of β_2-AR signaling. The current dogma is that β_2-ARs inherently couple "more efficiently" to the activation of adenylyl cyclase than do β_1-ARs. This has been attributed to structural differences in the receptor's third intracellular loop (55). However, the spatial proximity of β_2-ARs with adenylyl cyclase in caveolae (see below) provides an additional mechanism to facilitate receptor/cyclase coupling. Recent studies also show that β_2-ARs exit from caveolae following ligand activation (53). Hence, the thesis that β_2-ARs compartment to caveolae in quiescent cells fully accommodates the prevailing paradigm for clathrin-dependent endocytosis of β_2-ARs following activation by agonist. This paradigm describes the properties only of the β_2-AR. A similar analysis has not been reported for β_1-ARs. In this context, the subcellular distribution of β_1-ARs is not detectably altered by agonist (53). It is possible that the mode of regulation for β_1-ARs (the predominant β-AR subtype in cardiomyocytes) may differ from that described for the β_2-AR.

Finally, Lasely et al (57) recently published evidence that the adenosine A1 receptor subtype also is highly concentrated in caveolae and exits on stimulation by agonist ligand. Although the authors did not explore the functional significance of this event, they speculated that translocation out of caveolae (away from

other signaling molecules that concentrate in this compartment) could explain the rather trivial actions of A1 receptor stimulation on myocyte contractility. However, this simple explanation is not convincing because β_2-ARs, which display a similar agonist-induced translocation out of caveolae, exert pronounced effects on signaling pathways and contractile function in cardiomyocytes (37, 38).

Immunocytochemical studies over a decade ago also identified muscarinic cholinergic receptors (mAChRs) in caveolae of A431 cells (58). More recent efforts to probe the subcellular localization of M_2-mAChRs were performed in cardiomyocytes. Here, Feron et al (59) reported that M_2-mAChRs are excluded from caveolae in resting cells but appear in caveolae 15 min following activation by ligand. Several functional consequences of M_2-mAChR-caveolar interactions have been proposed. First, targeting to caveolae is believed to be necessary to initiate specific downstream signaling cascades. The generation of nitric oxide (NO) has been proposed as an obligatory (yet controversial) step in M_2-mAChR–mediated inhibition of cardiac contractile function, particularly following β-AR stimulation (60, 61). NO is generated by endothelial nitric oxide synthase (eNOS), which is reported to be quantitatively and specifically associated with caveolae as a consequence of cotranslational myristoylation and subsequent palmitoylation. The current model holds that eNOS activity is markedly attenuated through its association with caveolin (62). eNOS becomes activated following agonist-induced activation of M_2-mAChRs and their dynamic targeting to caveolae; activation of eNOS leads to increased cytosolic cyclic GMP and changes in inactivation of L-type calcium channels and myofilament calcium sensitivity that are critical for parasympathetic regulation of the heart (60).

Second, targeting to caveolae may provide a plausible explanation for differences in M_2-mAChR–β-AR subtype interactions at the level of cAMP accumulation (in the context of previously described differences in cell surface partitioning of β_1- and β_2-ARs). The well-recognized phenomenon of accentuated antagonism reflects the high degree of sensitivity of the β_1-AR–cAMP pathway to inhibitory modulation by M_2-mAChRs. The similar cell surface membrane distributions of β_1-AR and M_2-mAChRs would be permissive for such interactions. Note, however, that the Steinberg laboratory reported that β_2-AR stimulation of cAMP accumulation in neonatal rat myocytes is not susceptible to inhibitory modulation by M_2-mAChRs (56). The targeting of cardiomyocyte β_2-ARs to caveolae, a site distant from most of the cell surface M_2-mAChRs, provides a plausible explanation for the absence of a β_2-AR–M_2-mAChR interaction at the level of cAMP formation.

An additional function for caveolae might be as a platform for the assembly of proteins required to propagate or terminate signaling. There is a growing literature that places components of the signal transduction pathway downstream from the receptor in caveolae, but few studies have extended the analysis to cardiomyocytes. G protein α subunits were one of the earliest signaling molecules identified in caveolar fractions. Lisanti and colleagues (89) reported that G protein α subunits localize to caveolae, where they are maintained in the inactive

GDP-liganded conformation owing to an interaction with caveolin. The single available analysis of the membrane distribution of G protein subunits native to cardiomyocytes shows that $G\alpha_{i/o}$ proteins are highly concentrated in cardiomyocyte caveolae, whereas $G_{\alpha s}$ proteins and $\beta\gamma$ subunits localize to caveolae, but also are abundant on the remainder of the cell surface membrane (53). Data from a variety of cells including cardiac myocytes identify adenylyl cyclase activity or immunoreactivity in caveolae (52, 53, 64, 65). There is evidence that localization to caveolae provides a mechanism to tonically inhibit enzyme activity; release of adenylyl cyclase from caveolae by treatment with cyclodextrin, a membrane-impermeable cholesterol-binding drug that disrupts the functional integrity of the very cholesterol-enriched caveolar membranes, augments adenylyl cyclase activity (53, 66). Two possible inhibitory mechanisms have been proposed. First, it is possible that adenylyl cyclase activity is tonically repressed in caveolae through an interaction with caveolin. Consistent with this idea are data showing that adenylyl cyclase coimmunoprecipitates with caveolin and that peptides based upon the scaffolding-domain of caveolin inhibit the catalytic activity of cardiac isoforms of adenylyl cyclase (53, 65, 67). However, because some of the cardiac adenylyl cyclase isoforms are calcium sensitive, an alternative calcium-dependent local inhibitory mechanism has been proposed.

Fagan et al (66) recently presented evidence that type VI adenylyl cyclase and capacitative calcium entry channels colocalize in caveolae and that this constitutes a local mechanism to tonically inhibit enzyme activity. However, other potential scenarios are possible because cardiomyocyte caveolae are enriched in molecules that regulate calcium (Ca^{2+}-ATPase) (68), and caveolae have been identified as the initiation sites for hormone-dependent calcium waves, possibly because they are enriched in components of the receptor signaling complex, lipid precursors of inositol-1,4,5-triphosphate, and/or inositol-1,4,5-triphosphate receptor-like proteins (69, 70). Finally, it should be noted that adenylyl cyclase has been localized, along with G protein α subunits and components of the L-type calcium channel, along T-tubule membranes (71–73). This is not necessarily in conflict with the notion that these molecules localize to caveolae, as caveolin-3 transiently associates with T-tubules during muscle development and may be involved in early development of the T-tubule system (74); the possibility that immature caveolar structures and elements of the T-tubular system continue to associate requires further study.

There is evidence that G protein-coupled receptor kinases (GRKs) and the catalytic subunit of PKA specifically interact with caveolin through their consensus caveolin-binding motifs and that this interaction leads to the tonic inhibition of their catalytic activity (75, 76). This could represent a novel mode of regulation for these signaling moieties. However, the functional importance of this inhibitory mode of GRK regulation in the heart is uncertain, as only a relatively minor fraction of total cellular GRK and PKA catalytic subunit immunoreactivity is recovered in the caveolar fraction of resting cardiac myocytes (53). Rather, the RII regulatory subunit of PKA is enriched in caveolae; high concentrations of RII in caveolae would effectively increase local concentrations of the PKA II holoenzmye and could serve to promote the phosphorylation of proteins in the

vicinity of this structure. In contrast, RI is largely excluded from caveolae. This isoform-specific localization would be consistent with the provocative but as yet unproven hypothesis that PKAI and PKAII phosphorylate distinct spectra of target proteins and subserve different functions in the heart, as a result of their distinctive subcellular locations.

Although effectors for GPCRs are enzymes and channels, studies to date have focused exclusively on effector enzymes in caveolae. However, recent studies indicate that Shaker-like potassium channels reside in caveolae and are regulated by the local lipid microenvironment (77). With this first example of a voltage-sensitive ion channel in caveolae comes the speculation that channel localization to caveolae may be a more general regulatory mechanism that could be important in excitable tissues such as the heart.

PKA and A-Kinase Anchoring Proteins

One way to ensure that the cAMP signaling pathway is activated in the proper subcellular location at the right time is to produce gradients of cAMP from an imbalance between the opposing actions of appropriately localized and spaced adenylyl cyclase and phosphodiesterase enzymes. Another way to orchestrate cAMP signaling is to maintain local pools of PKA in an inactive form through their association with A-kinase anchoring proteins (AKAPs). AKAPS comprise a family of >30 structurally distinct proteins named according to their apparent molecular weights on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Each AKAP has a conserved R subunit binding motif and separate targeting sequences that direct the PKA holoenzyme-AKAP complex to discrete subcellular locations (structural proteins, membranes, or cellular organelles) (78). Some AKAPs (such as AKAP79, the first AKAP to be cloned) also act as scaffolds. The interaction of AKAP79 with PKA, protein kinase C (PKC), and protein phosphate 2B (PP2B) represents a mechanism to assemble and tether these enzymes in proximity to the plasma membrane. Early studies demonstrated that this tethering is critical for the coordinate regulation of the phosphorylation state (and hence the activity) of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid/kainate–type glutamate receptors in hippocampal neurons (78, 79). Recent studies also place β_2-ARs in this complex and implicate AKAP79 as a targeting mechanism to promote agonist-dependent β-AR phosphorylation and facilitate β_2-AR activation of the extracellular-signal-regulated kinase (ERK) cascade (79). A similar function has been described for AKAP250 (or gravin), which coordinates the interactions between β-ARs, PKA, PKC, and PP2B that are critical for normal agonist-induced desensitization, resensitization, and internalization of the β-AR (80, 81). Other AKAPs have been identified as nucleation centers for combinations of kinases (PKA, Rho-dependent kinase, protein kinase N) and phosphatases (PP1, PP2A) at various intracellular sites (centrosomes, Golgi) (78–81).

However, AKAPs are not merely passive carriers of these enzymes: Binding to AKAPs leads to functionally important changes in the activity of these enzymes (inhibition for the catalytic subunit of PKA or PKC; stimulation for PP1).

Currently, there is relatively limited information on AKAP expression or function in cardiomyocytes. Gao et al (82) have demonstrated that a peptide corresponding to the AKAP RII binding motif can function as an anchoring inhibitor peptide and can inhibit PKA-dependent regulation of L-type calcium channel activity in dissociated adult ventricular cardiomyocytes. These workers have also showed that either AKAP79 or AKAP15/18 reconstitutes PKA regulation of calcium channels activity when expressed with calcium channels in HEK293 cells (82). This result is believed to be more physiologically relevant for AKAP15/18 because it (rather than AKAP79) is detected in cardiomyocytes. Adult rat ventricular cardiomyocytes also express AKAP100, which is unusual in that it targets RII to multiple intracellular compartments (nucleus, intercalated discs, and T-tubule/junctional sarcoplasmic reticulum) (83). Other AKAPs target RII to a single class of intracellular membrane. This has been speculated to reflect the actions of auxiliary proteins that might provide additional targeting information or other factors and requires further study (83).

Protein Kinase C and Receptors for Activated C-Kinases

PKC constitutes a family of related serine-threonine kinases that play fundamental roles in functions that range from the modulation of cardiomyocyte contraction to regulation of cardiomyocyte growth. Cardiomyocytes express several isoforms of PKC; the most widely detected are the calcium-sensitive PKCα, the novel PKCδ and PKCε, and the atypical PKCλ (84–86). On the basis of evidence that several PKC isoforms are detected in caveolar membranes at steady state or following their activation by phorbol esters (87–91), and that the muscle-specific caveolin-3 isoform selectively inhibits the kinase activity of PKCα [but not PKCε (91)], Rybin et al (68) examined whether caveolae may represent a signaling module for PKC isoforms in the heart. Their studies showed that phorbol-ester–sensitive PKC isoforms α, δ, and ε are not detected in caveolae isolated from quiescent cardiomyocytes but are recruited to caveolae following activation with phorbol myristate acetate (PMA). Endothelin induced a similar translocation of the α and ε isoforms to caveolae, which suggests that this mechanism is physiologically relevant. Further studies identify multiple components of the ERK cascade, including A-Raf, c-Raf-1, mitogen-activated protein kinase kinase (MEK), and ERK, in cardiomyocyte caveolae under resting conditions. Levels of these proteins in caveolae are not altered by PMA treatment, but translocation of phorbol ester–sensitive PKC isoforms to caveolae leads to a marked activation of this local ERK cascade. These studies identify cardiomyocyte caveolae as meeting places for activated PKC isoforms and their downstream target substrates. The ramifications of this localization have yet to be worked out. By analogy to the role of caveolae/caveolin in the control of mitogenesis in proliferation-competent cells (92), the control of PKC-ERK signaling pathways by caveolae is anticipated to be critically important in the context of the cardiomyocyte hypertrophic growth response. In addition, PKC localization to caveolae could modulate the activation of adenylyl cyclase (93). One difference

between the cardiac adenylyl cyclase isoforms is their response to PKC: PKC inhibits type VI adenylyl cyclase but stimulates type V adenylyl cyclase (94–97). The colocalization of PKCs with adenylyl cyclase in caveolae could facilitate an interaction between only certain isoforms of PKC and adenylyl cyclase to achieve a higher degree of signaling specificity.

The multiple PKC isoforms expressed in cardiac myocytes display only limited substrate specificity in vitro. However, data suggest that individual PKC isoforms subserve distinct roles in vivo in intact cardiomyocytes. Studies using conventional and confocal microscopy suggest that specificity may be achieved by spatial localization because individual PKC isoforms can be distinguished by their distinct subcellular distributions, both before and following activation by agonist. For example, in neonatal rat ventricular myocyte cultures, PKCβII associates with fibrillar structures at rest and translocates to the perinucleus and cell periphery on activation. In the same cells, PKCε localizes to the nucleus and perinucleus at rest and translocates to cross-striations (putative contractile elements) and cell-cell contacts after activation (98). These observations led Mochly-Rosen & Gordon (99) to propose that PKC isoform specificity is guided by interactions with a family of receptors for activated C-kinases (RACKs), proteins that display specific and saturable binding only to the activated conformation of PKC isoforms and that act as targeting signals. To date, two RACKs have been cloned and characterized. RACK1 is a PKCβII-selective RACK; its binding site on PKCβII maps at least in part to a portion of the C2 domain (a common sequence in conventional PKC isoforms) and to an additional region in the V5 domain, the only sequence that distinguishes PKCβI and PKCβII. RACK1 colocalizes with PKCβII to the perinuclear areas of neonatal rat cardiomyocytes (100). β'-COP, a coatomer COPI protein necessary for Golgi budding and trafficking of vesicles, has been characterized as a PKCε-selective RACK; β'-COP specifically interacts with a unique sequence (amino acids 14–21) within the V1 region of PKC and colocalizes with PKCε to the Golgi complex (101). Other studies identify PICK1 (protein interacting with C-kinase) as a putative RACK for PKCα in the nucleus; F-actin may have the characteristics of a PKCε or PKCβII RACK (102–104).

Mochly-Rosen & Gordon (99) have put forth the concept that another set of proteins termed RICKs (receptors for inactive C-kinase) anchor inactive conformations of individual PKC isoforms. Although a model that has individual PKC isoforms shuttling from RICKs to RACKs could provide a high level of spatial control, direct experimental validation for components of this model is still lacking. The biological importance of targeting for PKC isoforms is underscored by functional studies that selectively abrogate function by manipulating the subcellular distribution of PKC isoforms with competitive PKC isoform translocation inhibitor peptides derived from RACKS. Specifically, PMA-dependent modulation of L-type calcium currents is blocked by peptides that mimic the RACK binding C2-domain of cPKC isoforms (105). Peptides that mimic the RACK-binding site on PKCε block the inhibition of spontaneous automaticity in neonatal rat cardiomyocyte cultures induced by PMA or α_1-adrenergic stimulation (106), a result

that is somewhat perplexing given reports from another laboratory that activation of α_1-adrenergic receptors increases spontaneous automaticity in this model (107, 108). An analogous approach implicates PKCε as the only PKC isoform that increases cardiomyocyte resistance to ischemic/hypoxic insults (i.e. mediates preconditioning) (109, 110). A similar approach has been applied in vivo to show that expression of a catalytically inactive PKC-derived translocation inhibitor peptide interferes with the association of PKC and its anchoring protein, that it does not alter PKC expression levels, and that it is sufficient to interfere with normal myocardial growth in the perinatal period (111).

RACK1's binding activity is not confined to PKC. RACK1 also binds to the cytoplasmic domains of the integrin β subunit and the SH2 domain of pp^{60}Src (112, 113). The association of RACK1 with integrins is enhanced in cells treated with PMA to activate PKC, but the precise role for PKC isoforms in this process has not been identified (112). The interaction of RACK1 with Src leads to a decrease in Src kinase activity (in vitro and in intact cells) (113). RACK1 also inhibits the tyrosine kinase activity of other Src family members such as Yes. In contrast, RACK1 does not inhibit several serine/threonine kinases (PKC, CKII) and the interaction between RACK1 and PKC enhances substrate phosphorylation by PKC (99). On the surface, this suggests that RACK1 functionally resembles caveolin. Both act as membrane-anchored scaffolding proteins that spatially and functionally restrict the activity of various mitogenic kinases and/or phosphatases. The analogy is intriguing, as overexpression of either RACK1 or caveolin-1 leads to a similar inhibitory effect on cell growth rate (113, 114).

CONCLUSIONS AND PERSPECTIVES FOR THE FUTURE

Compartmentation of cAMP signaling in cardiac cells seems well established by available data. A straightforward explanation of the data requires the existence of a fixed spatial relationship of the components of the response pathway (adenylyl cyclase, cAMP, PKA, PDE, and substrates for PKA, and very likely including some hormone receptor and G_s/G_i proteins and phosphoprotein phosphatases). The molecular mechanisms by which this compartmentation occurs are still being established but are becoming known and generally take the form of specific binding or anchoring sites at crucial locations in the sarcolemma and within the cell. A picture is emerging (Figure 1): β-adrenergic receptors, G proteins, and adenylyl cyclases target to caveolae, where their activities may be regulated by local differences in the stoichiometry of signaling components or by interactions with caveolin; certain PKC isoforms associate with caveolae; PKAs are anchored by AKAP proteins in specific locations; PKA substrates may be localized near the AKAPs in fixed macromolecular complexes such as the sarcolemma, the sarcoplasmic reticulum, the contractile machinery, or the glycogen particle; and isoforms of PDE are tethered at specific subcellular locations. The spatial domain of diffusible cAMP may be limited by placement of PDEs or by intracellular

structures. The result is that the signal activated by a hormone-G_s interaction is channeled to a specific subcellular region. Superposed on this spatial organization are the advantages of a multiplicity of isoforms of adenylyl cyclase and PDE, each differentially regulated (115). For instance, different adenylyl cyclase isoforms may be activated or inhibited by Ca^{2+}, PKC, PKA, and $\beta\gamma$ complexes and not all forms are equivalently inhibited by α_i. PDEs differ in specificity and affinity for cyclic nucleotides and may be differentially stimulated or inhibited by Ca^{2+}, cyclic GMP, cAMP, PKA, and other protein kinases. In our view, the spatial organization of the components of the cAMP pathway, coupled with the differential control of individual isoforms of adenylyl cyclase and PDE, could provide for compartmentation of signaling, for cross talk (from systems imposing regulatory control on ACs and PDEs), and for local and dynamic regulation of responsiveness. There is, clearly, still much to be learned about the details by which compartments are established and maintained, but in the end, Ted Rall will be satisfied.

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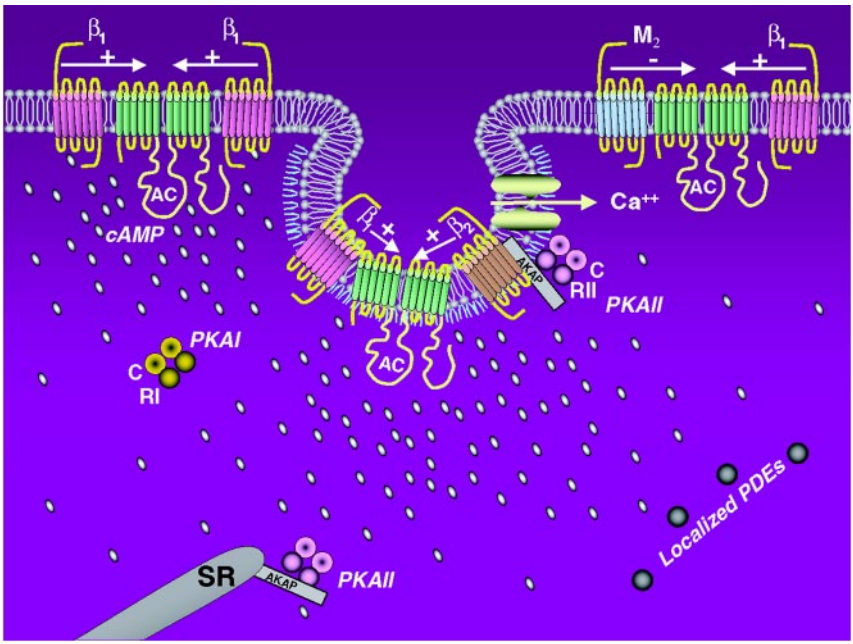

Figure 1 Compartmentation of the β-Adrenergic Response in Quiescent Cardiac Myocytes.

The caveolae structures are depicted as cave-like indentations of the sarcolemma coated with caveolin. This cartoon, in which the small droplets emanating from the membrane represent cyclic AMP molecules, schematizes the following recent results and speculations: (*a*) β_1-adrenergic receptors (β_1-ARs) (the predominant β-AR subtype in cardiomyocytes) reside in both caveolar and noncaveolar membranes. (*b*) The minor population of β_2-ARs is confined to caveolae. (*c*) Adenylyl cyclase is a target for β-ARs in caveolae and noncaveolae membranes. (*d*) M_2-mAChRs are excluded from caveolae: This could facilitate parasympathetic modulation of the β_1-adrenergic response (upper right) and prevent cholinergic modulation of the β_2-adrenergic response. (*e*) RII resides in caveolae. Whether this targeting requires interaction with an AKAP (as demonstrated at other sites) and leads to regulation of local Ca^{2+} channels requires further study. (*f*) Localization of PDEs could produce an area relatively free of cyclic AMP (bottom right) but the cell biology of this process is less well defined at present. (*g*) Localization of adenylyl cyclase and PDE could produce gradients of cyclic AMP as one mechanism to localize signaling, as shown. Other processes predicted to localize the phosphorylation of key intracellular proteins include localization of PKA substrates and the isoform-specific regulation and localization of adenylyl cyclases, PDEs and phosphoprotein phosphatases.