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Rennolds S. Ostrom
Chapman University, rostrom@chapman.edu

Amy S. Bogard
University of Tennessee

Robert Gros
University of Western Ontario

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Choreographing the adenylyl cyclase signalosome: sorting out the partners and the steps

Rennolds S Ostrom[1], Amy S. Bogard[1], Robert Gros[2,3,4], Ross D. Feldman[2,3,4]

Department of Pharmacology[1]
College of Medicine
University of Tennessee Health Science Center
Memphis, Tennessee
Departments of Medicine[2] and of Physiol
and
ogy & Pharmacology[3],
Schulich School of Medicine & Dentistry,
and Vascular Biology Research Group[4],
Robarts Research Institute
University of Western Ontario
London, Canada

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Corresponding Author:
Ross D Feldman, MD
Robarts Research Institute.
100 Perth Dr.
PO Box 5015, Stn B
London, ON, Canada
N6A 5K8
Tel: (519) 931-5717
Fax: (519) 931-5222
Email: feldmanr@lhsc.on.ca

ABSTRACT

Adenylyl cyclases are a ubiquitous family of enzymes and are critical regulators of metabolic and cardiovascular function. Multiple isoforms of the enzyme are expressed in a range of tissues. However, for many processes the adenylyl cyclase isoforms have been thought of as essentially interchangeable, with their impact more dependent on their common actions to increase intracellular cAMP content regardless of the isoform involved. It has long been appreciated that each subfamily of isoforms demonstrate a specific pattern of "upstream" regulation, i.e., specific patterns of ion dependence (e.g., calcium-dependence) and specific patterns of regulation by kinases (PKA, PKC, raf). However, more recent studies have suggested that adenylyl cyclase isoform-selective patterns of signaling are a wide-spread phenomenon. The determinants of these selective signaling patterns relate to a number of factors, including: i) selective coupling of specific adenylyl cyclase isoforms with specific GPCRs, ii) localization of specific adenylyl cyclase isoforms in defined structural domains (AKAP complexes, caveolin/lipid rafts) and iii) selective coupling of adenylyl cyclase isoforms with specific downstream signaling cascades important in regulation of cell growth and contractility. The importance of isoform-specific regulation has now been demonstrated both in mouse models as well as in humans.

Adenylyl cyclase has not been viewed as a useful target for therapeutic regulation, given the ubiquitous expression of the enzyme and the perceived high risk of off-target effects. Understanding which isoforms of adenylyl cyclase mediate distinct cellular effects would bring new significance to the development of isoform-specific ligands to regulate discrete cellular actions.

INTRODUCTION

The adenylyl cyclases are a ubiquitously expressed family of enzymes that catalyze the generation of cyclic adenosine monophosphate (cAMP) from adenosine triphosphate (ATP). Adenylyl cyclases regulate a broad range of cellular functions (Cooper et al., 1995, Hanoune and Defer, 2001, Patel et al., 2001). These enzymes are critical effectors for a number of G protein–coupled receptors (GPCRs). Adenylyl cyclase activation has been suggested to be the rate-limiting step in the GPCR signaling cascade (Ostrom et al., 2000).

The adenylyl cyclases have been extensively and perhaps even exhaustively studied. Since their initial descriptions in the 1960's more than 20,000 papers have been published that include the search terms adenylyl/adenylate cyclase or cyclic AMP, including almost 700 reviews. Further, the field of adenylyl cyclases/cAMP biology has been the basis of 3 Nobel Prize awards include those of Earl Sutherland (1971), Ed Fischer/Ed Krebs (1992) and Al Gilman/Marty Rodbell (1994). Thus, in 2011 it might be reasonable to ask whether there is anything new that a reader could expect to glean from yet another review in this very well-tilled field of research. In this short review we would like to focus on two aspects of cAMP/adenylyl cyclase biology that are perhaps less appreciated but critical for the understanding of this crucial second messenger system.

The basis of this brief review will be to discuss and dispel a widely held but probably untenable belief about the basic workings of this system, viz. that because of the ubiquity of adenylyl cyclases and the very common effects of all isoforms of the family, the development of therapeutic strategies based on isoform-selective regulation is a "fool's errand". In contrast to this widely-held belief, the major focus of this review will be to highlight that:

1) The effects mediated by adenylyl cyclase isoforms are critically dependent on the isoform-selective “signalosomes” in which they are situated, and
2) Regulation of specific isoforms would be expected (and do) have significant impact on cardiovascular and respiratory regulation

ADENYLYL CYCLASE ISOFORMS AND ISOFORM-SPECIFIC REGULATION

Adenylyl cyclases comprise a family of nine membrane-bound isoforms grouped into 3 major subfamilies, comprising: Group 1: AC1, AC3, AC8, Group 2: AC2, AC4, AC7 and Group 3: AC5, AC6 (Patel et al., 2001). Additionally, AC9 has been characterized as a distinct (and atypical) isoform(Premont et al., 1996)(Sunahara and Taussig, 2002) with restricted expression, and a soluble adenylyl cyclase has been characterized that is the predominant form in mammalian sperm (Wuttke et al., 2001). Each isoform has a specific pattern of tissue/organ distribution and a specific pattern of regulation by G proteins, calcium/calmodulin, and protein kinases (Thomas and Hoffman, 1996, Harry et al., 1997, Wang and Brown, 2004). For example, Raf kinases regulate AC2, AC5 and AC6, whereas they do not regulate AC1 (Ding et al., 2004) (Table 1).

The distribution of adenylyl cyclase isoforms varies between tissues (Cooper, 2003). Further, the effects of adenylyl cyclase activation in regulating several cellular functions, including cell growth, qualitatively differ between tissues (Lee et al., 2001, Wong et al., 2001).

Although there are multiple G protein-dependent mechanisms regulating the function of adenylyl cyclase, it has been believed that the consequence of activation of any of the isoforms is singular (i.e., that cAMP generation mediates the same effect regardless of the isoform involved in the process). However, recent studies have suggested that there are many important examples of isoform-specific signaling especially in regulation of cardiovascular and respiratory function.

These relate in part, to the organization of specific adenylyl cyclase isoforms in distinct cellular compartments and (perhaps consequently) association of specific adenylyl cyclase isoforms with specific G protein coupled receptor/G protein complexes as well as with particular downstream effectors.

ADENYLYL CYCLASE COMPARTMENTATION MODELS

Adenylyl cyclase isoforms have been localized in several structural cellular compartments. The best characterized are their association with caveolar and lipid raft microdomains and in complexes with A-kinase anchoring proteins (AKAPs)(Dessauer, 2009).

Specific receptors, adenylyl cyclase isoforms and downstream effectors are targeted to lipid rafts and/or caveolae, creating discrete signaling compartments that allow common signaling pathways to generate a variety of situation-specific responses. A number of studies have demonstrated AC isoform-specific coupling to specific GPCR's, and this coupling reflects the co-localization of receptors and given AC isoforms in lipid rafts. Overexpression of AC6 in rat neonatal myocytes enhances cAMP production in response to the ß-adrenergic receptor (ßAR) agonist isoproterenol without affecting response to stimulation of other $G_{\alpha s}$-coupled receptors such as adenosine A_2, histamine H_2 and glucagon receptors (Ostrom et al., 2000). This selective coupling is seemingly explained by the observation that AC6 colocalizes with ßAR in caveolae/lipid rafts in rat neonatal ventricular myocytes but other GPCR are excluded from these domains. AC6 more efficiently couples to $ß_1$AR than $ß_2$AR in these cells, likely due to 4-fold higher expression of $ß_1$AR and movement of $ß_2$AR out of caveolae following activation (Ostrom et al., 2001).

In human bronchial smooth muscle cells, ßAR preferentially signals via activation of AC6, which coexists with $ß_2$AR in lipid rafts (Bogard et al., 2011). In these same cells EP_2R act through ßγ-stimulable, and calcium insensitive, cyclases (AC2 and AC4) that are expressed exclusinvely in non-raft fractions of the plasma membrane. Thus, ACs appear to have isoform-specific localization with respect tor lipid rafts and non-raft domains and this feature is consistent across many cell types (Fagan et al., 2000, Ostrom et al., 2000, Ostrom et al., 2002, Smith et al., 2002, Ostrom and Insel, 2004). Such compartmentation in plasma membrane microdomains appears responsible, in part, for the observed selective coupling between GPCR and specific adenylyl cyclase isoforms.

Notably, preferential coupling of specific adenylyl cyclase isoforms with specific receptors may be regulatable- adding an additional level of complexity. In uterine smooth muscle, α_2 adrenoceptors switch from being adenylyl cyclase inhibitory to stimulatory during mid-term pregnancy (Zhou et al., 2000). Subsequent studies demonstrated that upregulation of AC2 and the consequent switch in α_2 adrenoceptors effects on adenylyl cyclase activity being predominantly mediated through this isoform being the most likely responsible mechanism (Zhou et al., 2007). Whether this shift parallels a change in compartmentation of either receptor or adenylyl cyclase isoforms remains to be determined.

In addition to placing specific AC isoforms and receptors in close proximity, lipid rafts appear to play a role in cyclase regulation by capacitative calcium entry (CCE). AC6 is inhibited by CCE but not calcium from intracellular stores or ionophore-mediated entry (Cooper et al., 1995). The fast Ca^{2+} chelator BAPTA is able to block CCE impact on cyclase activity, but EGTA, a chelator with similar affinity but slower binding rate, has no effect on cAMP production (Fagan et al., 1998). The lack of EGTA effect suggests AC is located near sites of CCE.

Disrupting lipid rafts removes CCE regulation of AC6 without affecting CCE itself, and these features return upon lipid raft restoration (Fagan et al., 2000). Lipid rafts seem important for association of calcium–sensitive cyclases and sites of CCE, while the cytoskeleton does not appear to play a role since disruption of the cytoskeleton by a number of compounds does not affect the ability of CCE to inhibit Ca-sensitive AC (Fagan et al., 1998).

Studies of numerous cell types make clear that AC isoforms are stratified as either lipid raft localized or non-raft localized (Ostrom and Insel, 2004). One then wonders how ACs are localized in these respective lipid domains. Protein-lipid interactions, protein-protein interactions and post-translational modifications are all likely mechanisms. This question has been examined using a number of different approaches. Two separate studies have revealed the importance of the intracellular C1 and C2 domains in targeting adenylyl cyclase to lipid rafts. Crossthwaite et al. made several chimeric proteins combining various portions of raft localized AC5 and non-raft AC7 and found that the intracellular, and not transmembrane domains, direct localization to lipid rafts (Crossthwaite et al., 2005). Thangavel et al. created a series of AC6 fragments and truncated proteins (Thangavel et al., 2009). C1 and C2 fragments of AC6 localize to bouyant fractions and co-immunoprecipitate with caveolin-1 (Thangavel et al., 2009). These studies indicate that the transmembrane domains do not play a role in targeting cyclase to lipid rafts, implying that interactions with membrane lipids are probably not as important as protein-protein interactions for raft localization of these isoforms.

One protein that is known to localize to caveolae and act as a scaffolding molecule for various signaling proteins is caveolin. Caveolin-1 does not appear to be responsible for anchoring cyclases in lipid rafts, since cardiac fibroblasts isolated from cav-1 knockout mice displayed the same expression and localization of cyclases (Thangavel et al., 2009). In addition, HEK-293 cell

lines that do not express caveolin proteins still target overexpressed AC6 to lipid rafts (Thangavel et al., 2009).

Although it has been demonstrated that the intracellular domains are enough to target cyclase fragments or chimeric proteins to lipid rafts, preventing glycosylation on extracellular loops also stops AC8 from localizing to lipid rafts (Pagano et al., 2009). It is possible that glycosylation facilitates interaction with proteins that are involved in targeting molecules to lipid rafts. Interestingly, the non-raft localized form of AC8 is still regulated by CCE, and (as with native AC8) this regulation is eliminated by MßCD-mediated cholesterol depletion in a reversible manner. Thus, intact lipid rafts are required for CCE regulation of AC activity even when the cyclase is not located in them.

The balance of data indicate that lipid rafts bring together a subset of signaling molecules and may facilitate coupling of specific AC isoforms with certain GPCR's and other signaling proteins, helping to direct specific responses.

Another way cells can bring signaling molecules together for specific pathways and regulation is through A Kinase Anchoring Proteins (AKAPs). All AKAPs have a protein kinase A (PKA) binding motif that binds the regulatory subunit of PKA, but otherwise they are quite variable (Dessauer, 2009). They can be targeted to the plasma membrane or membranes of organelles such as golgi, mitochondria or nucleus. AKAPs act as scaffolds and bring together specific combinations of kinases and their substrates as well as other components involved in a wide array of signaling pathways. Such scaffolding is important for rapid signal transduction as well as rapid termination of signaling.

AKAPs have recently been shown to interact with cyclases in an isoform-specific manner. The first isoform-specific AC-AKAP interactions were determined only a few years

ago (Bauman et al., 2006). Current research is focused on further uncovering specific AC-AKAP associations and investigating how participation in AKAP complexes contributes to the regulation of AC.

So far a conserved AC-binding domain has not been found across AKAPs. Of the sites determined to bind AC, there is little similarity among AKAPs, even when they bind the same AC isoform (Piggott et al., 2008, Kapiloff et al., 2009). Different AC isoforms can bind different locations on the same AKAP. The N terminus of AC2 interacts directly with amino acids 808-957 of the AKAP Yotiao, but AC1, AC3 and AC9 do not interact with this site although they are bound by Yotiao (Piggott et al., 2008). Known AKAP-AC interactions include mAKAP with AC2 and AC5 (Kapiloff et al., 2009), AKAP79/150 with AC2, 3, 5, 6, 8, and 9 (Efendiev et al., 2010), and AKAP9/Yotiao with AC1, 2, 3, and 9 (Piggott et al., 2008).

AKAPs can mediate regulation of cAMP-PKA signaling in a number of ways either through direct interaction or bringing various components together in a complex. Interaction of AC8 with AKAP 79/150 appears to decrease the cyclase's sensitivity to Ca^{2+} (Willoughby et al., 2010). Yotiao acts only as a scaffold with no effect on activity of AC1 and AC9 but has an inhibitory effect on AC2 and AC3 (Piggott et al., 2008). The scaffolding of cyclases and PDEs, kinases and phosphatases, and kinase-sensitive receptors/cyclases allows tight regulation of signaling cascades. For example, AKAP79/150 interacts with PKA, $ß_2$AR, and several isoforms of AC including AC5 and AC6 (Bauman et al., 2006). Once activated, PKA can phosphorylate a number of substrates including ßAR (causing desensitization, G protein switching, receptor translocation) and AC (PKA phosphorylation inhibits AC5 and AC6), preventing further signaling via feedback inhibition. Other kinases such as PKC can also be

anchored by AKAPs and regulate cyclases in an isoform-specific manner. PKC phoshorylation enhances activity of AC1, 2, 3, 5, and 7, but inhibits AC6 (Table 2).

The specifc AKAP-AC interactions that are defined and the role they play in cardiovascular physiology and pathophysiology has recently been reviewed (Efendiev and Dessauer, 2011). Yotiao is one AKAP whose importance in the heart has been recognized due to complications associated with mutations of the KCNQ1 subunit of the slow outward potassium channel that prevents its interaction with Yotiao. When Yotiao with its associated proteins is unable to bind the potassium channel, the normal PKA and PP1 phosphorylation and dephosphorylation of the channel is disrupted and the duration of the action potential is altered (Marx et al., 2002). The resulting arrhythmias can be lethal.

Thus, ACs are permanent residents of specialized lipid microdomains where receptors may selectively and dynamically participate in assembled singaling complexes. AKAPs are key members of such signaling complexes based on their interaction with specific AC isoforms, and bring with them PKA as well as a number of other scaffolded signaling partners. Paralleling these structural studies, there is increasing evidence of “functional” consequences of adenylyl cyclase compartmentation into isoform-specific adenylyl cyclase “signalosomes” linking these enzymes with either specific GPCR/G protein complexes and/or specific adenylyl cyclase/downstream effector complexes. However, it should be emphasized that the link between the evidence for these “structural” compartments with any specific functional adenylyl cyclase signalosome remains to be established.

ADENYLYL CYCLASE ISOFORM-SPECIFIC ASSOCIATION WITH DOWNSTREAM EFFECTORS

Although, it has become increasingly clear that there is adenylyl cyclase isoform-specific linkage with "upstream" regulators of enzyme function, the consequence of adenylyl cyclase activation was seen as singular, i.e., an increase in intracellular cyclic AMP concentrations and activation of its downstream effectors, Exchange Protein Activated by cAMP (Epac) and PKA (Cooper et al., 1995, Hanoune and Defer, 2001, Patel et al., 2001). However, more recent evidence has suggested that the linkages between adenylyl cyclase and its proximate effectors (Epac and PKA) and more distal responses (growth and contractility) might also be isoform-specific (Gros et al., 2006). This has perhaps been most clearly demonstrated in vascular smooth muscle cells and with the use of adenoviral constructs expressing representative isoforms from the subfamilies of adenylyl cyclase, i.e., AC1 and AC3, AC2, AC5, and AC6. Following expression of the isoforms to equivalent levels of both protein content and stimulated catalytic activity (at least as assessed in *absence* of phosphodiesterase activity, see below), isoform-specific differences in both growth regulatory and cytoskeletal reorganization processes could be demonstrated (Gros et al., 2006).

AC6 has been shown to have an isoform-specific effect in regulation of cellular contractile responses. Assessing adenylyl cyclase-mediated cytoskeletal reorganization in vascular smooth muscle cells, only expression of AC6 was associated with an enhanced response. This paralleled the observation that only AC6 gene transfer enhanced adenylyl cyclase-mediated PKA activation as assessed by Vasodilator Stimulated Phosphoprotein (VASP) phosphorylation. Further, in the presence of *uninhibited* endogenous phosphodiesterase activity, only AC6 expression enhanced cAMP accumulation. Because the AC6 effect on cytoskeletal reorganization was paralleled by an AC6-selective uncoupling of cAMP synthesis and breakdown and an AC6-selective effect on

phosphorylation of the PKA substrate, VASP, we hypothesized that the AC6 isoform may participate in molecular complexes devoid of phosphodiesterase but enriched in PKA/VASP as well as in intermediates in cytoskeletal rearrangements (Gros et al., 2006). Thus differential coupling of adenylyl cyclase isoforms with specific cell functions *in this setting* appeared to be best explained by cellular compartmentation of AC6 in a domain distinct from phosphodiesterase effects (at least relative to the other isoforms tested).

In regards to growth regulatory effects, expression of AC1 resulted in significantly greater inhibition of growth vs. both control cells as well as those expressing the other representative adenylyl cyclase isoforms. These isoform-selective growth regulatory effects paralleled the selective actions of AC1 expression to increase adenylyl cyclase-stimulated ERK activation vs. the effects seen with expression of the other isoforms tested. Further, a direct association between ERK and AC1 (and AC3) could be shown, suggesting that these proteins were part of a structural signalosome complex resulting in more efficient isoform-selective regulation of cell growth. The details of this structural complex are yet to be determined. Notably, in these studies, gene transfer of AC1/AC3 was NOT associated with any detectable increase in cAMP concentrations when assessed in the presence of *uninhibited* endogenous phosphodiesterase activity. (Gros et al., 2006) These finding could be explained by the efficient compartment-specific channeling of the product of adenylyl cyclase activity (i.e., cAMP) in these AC1/3 domains, not detectable in whole cell cAMP assays. However, these data might also suggest that the actions of AC1 and AC3 were a consequence of their association with ERK and were cAMP-independent.

Recent studies by Gao *et al*. support the hypothesis that adenylyl cyclase isoforms can have physiological effects independent of their cAMP generating function (Gao et al., 2011). Expressing a catalytically inactive AC6 mutant in cardiac myocytes, Gao *et al*. found these cells had reduced cellular hypertrophy and apoptosis when challenged with phenylephrine. These responses were similar to those observed when wild type AC6 was overexpressed. It was

hypothesized that in this model, AC6 facilitates formation of a signalasome between discreet signaling proteins regulating cell growth. Whether these effects are AC6 isoform-specific in this cell model, analogous to the AC1/3-specific effects on cell growth demonstrated in vascular smooth muscle cells, remains to be determined. However, in aggregate, these studies highlight the need to further understand the signaling complexes formed with specific AC isoforms.

ISOFORM-SPECIFIC REGULATION OF ADENYLYL CYCLASE FUNCTION: GENETIC APPROACHES

As noted above, isoform-specific regulation of adenylyl cyclase has been shown in several contexts. However, the functional consequences of such isoform-specific regulation have only been more recently appreciated. Based on knockout mouse models, genetic disruption of specific AC isoforms has been associated with specific phenotypical defects. AC5 disruption is associated with increased longevity and protection against stress (Yan et al., 2007) but Parkinson-like motor dysfunction (Iwamoto et al., 2003). Genetic disruption of AC3 impairs male fertility and spermatozoon function (Livera et al., 2005) and anosmia (Wong et al., 2000). However, whether any of these phenotypes was truly isoform-specific or more generally related to the decrease in stimulated adenylyl cyclase activity in the tissues in which these specific isoforms predominated could not be determined. Further, the significance in humans of the isoform-specific phenotypes seen in animal models has only begun to be explored.

Perhaps the most illustrative approach in examining the impact of isoform-specific regulation of adenylyl cyclases in humans has utilized missense human genetic AC6 variants. The A674S variant of AC6 has been shown to be expressed in whites with an allelic frequency of approximately 6% (Gros et al., 2005). In cell systems, expression of the variant results in enhanced adenylyl cyclase activity and adenylyl cyclase-mediated cytoskeletal reorganization

(Gros et al., 2007). Further, mononuclear leukocytes from subjects expressing the A674S variant possessed increased adenylyl cyclase activity and adenylyl cyclase mediated cellular retraction. These cellular indices of enhanced adenylyl cyclase function based on expression of this variant paralleled increased potency of ß-adrenergic receptor-mediated vasodilation (Gros et al., 2007). Hemodynamically, younger healthy subjects expressing the variant demonstrated increased systolic blood pressure, heart rate, cardiac output and increased plasma rennin activity (Hodges et al.). This phenotype supports the hypothesis that physiological regulation of a specific isoform (AC6) results in the expression of a hyperkinetic hemodynamic phenotype. Whether those subjects demonstrating genetic variants of other AC isoforms, such as those of the AC1/AC3, will display alterations in regulation of cellular growth remains to be determined.

CHALLENGES TO PROGRESS IN THE ELUCIDATION OF ADENYLYL CYCLASE SIGNALASOMES.

Efforts to define the signalosomes for each AC isoform are still in the early stages. ACs are notoriously difficult genes and proteins to work with due to their size, complexity and resistance to purification. ACs express at low levels in cells and the available tools for studying the proteins, such as antibodies, are of poor quality. ACs have sensitive tertiary structures that are required for normal activity and regulation, yet they also have long half lives, making siRNA knockdown troublesome. Methods for measuring AC activity have advanced in recent years with the development of Epac-based biosensors, including versions that can be targeted to subcellular domains. However, these methods of measuring cAMP still lag the fast and accurate spatial detection of calcium fluorophores and suffer from the need to exogenously express the

sensor. Overcoming one or more of these methodological shortcomings will be required for even incremental advances in our understanding of AC signalasomes.

SUMMARY

Interest in adenylyl cyclase regulation has waned in recent years, perhaps in part related to the perspective that the enzyme might not be a useful target for therapeutic regulation given its ubiquitous expression and multifunctional importance. However, recent studies have demonstrated that adenylyl cyclase isoforms are compartmentalized both structurally and functionally. These findings raise the potential for development of isoform-specific adenylyl cyclase ligands which may have both tissue and functional specificity as therapeutic agents. Understanding which isoforms of adenylyl cyclase mediate which cellular effects is bringing new significance to the development of isoform-specific ligands to regulate discrete cellular actions.

Table 1:

Isoform-specific Regulation of Adenylyl Cyclases

Regulator	Effect	AC subtype	References
G protein			
Gs	Stimulation	All Isoforms	(Iyengar, 1993)
Gi	Inhibition	ACI, AC5, AC6; not AC2	(Taussig et al., 1993a) (Federman et al., 1992) (Chen and Iyengar, 1993) (Taussig et al., 1994)
Gßγ	Inhibition	AC1, AC5, AC6	(Taussig et al., 1993b) (Bayewitch et al., 1998)
	Stimulation	AC2, AC4	(Tang and Gilman, 1991) (Gao and Gilman, 1991)
		Conditional AC5, AC6	(Gao et al., 2007)
Forskolin	Stimulation	All Isoforms (including AC9)	(Onda et al., 2001) (Premont et al., 1996) (Cumbay and Watts, 2004)
Calcium/Calmodulin			
Ca^{2+}/CaM	Stimulation	AC1, AC3, AC8	(Tang et al., 1991) (Choi et al., 1992) (Cali et al., 1994)
Ca^{2+}	Inhibition	AC5, AC6	(Yoshimura and Cooper, 1992) (Katsushika et al., 1992)
Kinase Regulation			
PKC	Stimulation	AC1, AC2, AC3, AC5, AC7	(Jacobowitz et al., 1993) (Jacobowitz and Iyengar, 1994) (Bol et al., 1997) (Kawabe et al., 1994) (Watson et al., 1994)
	Inhibition	AC6	(Lai et al., 1997)
PKA	Inhibition	AC5. AC6	(Iwami et al., 1995) (Chen et al., 1997)
CaM Kinase	Inhibition	AC1. AC3	(Wayman et al., 1996) (Wei et al., 1996)
Raf kinase	Stimulation	AC2, AC5, AC6	(Ding et al., 2004)

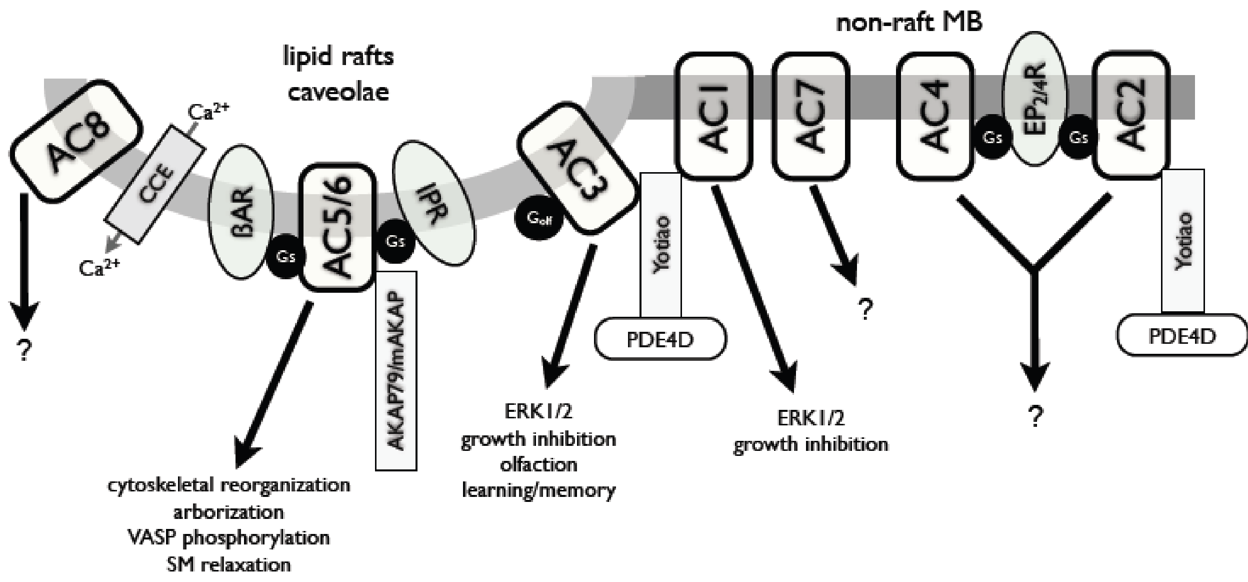

Figure: Schema of paradigms for isoform-specific adenylyl cyclase compartmentation

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