Regulation by Ca^{2+} -Signaling Pathways of Adenylyl Cyclases

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Interplay between the signaling pathways of the intracellular second messengers, cAMP and Ca^{2+} , has vital consequences for numerous essential physiological processes. Although cAMP can impact on Ca^{2+} -homeostasis at many levels, Ca^{2+} either directly, or indirectly (via calmodulin [CaM], CaM-binding proteins, protein kinase C [PKC] or $\text{G}\beta\gamma$ subunits) may also regulate cAMP synthesis. Here, we have evaluated the evidence for regulation of adenylyl cyclases (ACs) by Ca^{2+} -signaling pathways, with an emphasis on verification of this regulation in a physiological context. The effects of compartmentalization and protein signaling complexes on the regulation of AC activity by Ca^{2+} -signaling pathways are also addressed. Major gaps are apparent in the interactions that have been assumed, revealing a need to comprehensively clarify the effects of Ca^{2+} signaling on individual ACs, so that the important ramifications of this critical interplay between Ca^{2+} and cAMP are fully appreciated.

OVERVIEW

Cyclic AMP (cAMP) and calcium (Ca^{2+}) are arguably the prototypical second messengers that control cellular homeostasis. Whereas, for instance, nitric oxide and cyclic GMP may serve essential functions in a number of physiological situations, cAMP and Ca^{2+} are the only truly ubiquitous second messengers. Significantly, it also happens that each of the mammalian adenylyl cyclases (ACs), which are the synthetic sources of cAMP, are potentially regulated by some aspect of the Ca^{2+} -signaling pathway—either directly by Ca^{2+} and/or calmodulin (CaM) or indirectly by CaM kinase (CaMK), protein kinase C (PKC),

or calcineurin (CaN), all of which are potentially activated either when $[\text{Ca}^{2+}]_i$ is increased, or as a result of stimulation of the phospholipase C (PLC) pathway (reviewed in Sunahara et al. 1996; Willoughby and Cooper 2007; Sadana and Dessauer 2009). In addition $\beta\gamma$ subunits of G-proteins liberated in response to $\text{G}\alpha_q$ -coupled receptors can potentially regulate six of the nine membrane-bound AC species (reviewed in Sunahara et al. 1996; Willoughby and Cooper 2007; Sadana and Dessauer 2009). This susceptibility of cAMP production to regulation by the Ca^{2+} -signaling pathway may reflect a remnant control by Ca^{2+} over the presumed newer second messenger cAMP, a developmental sophistication or

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convergent evolution.¹ Whatever the origin of this interaction it is important to consider that Ca^{2+} is never elevated without a possible consequence—either positive or negative—for cAMP levels emanating from any of the ACs. Conversely, it is also noteworthy that cAMP itself impacts on Ca^{2+} -elevation at numerous levels—ranging from direct effects of cAMP on hyperpolarization-activated cyclic nucleotide-gated (HCN) channels and cyclic nucleotide-gated (CNG) channels, to effects of protein kinase A (PKA) and exchange protein directly activated by cAMP (EPAC) on numerous aspects of Ca^{2+} -signaling, including inositol trisphosphate (InsP_3) receptors (reviewed in Straub et al. 2004), voltage-gated Ca^{2+} channels (VGCCs) (reviewed in Dai et al. 2009), etc., so that non-linearity and great complexity is to be the expected norm for the concentration profile of both messengers. An extension of this interaction may be that targets of these second messengers respond to not readily discernible integrals of their respective concentrations and certainly not to gross elevations or declines in the levels of the messengers at some cumulative time-point (which tends to be the experimentalists' approach). This notion elaborates on the proposal made almost 30 years ago, by Howard Rasmussen, of Ca^{2+} and cAMP as “synarchic” messengers (Rasmussen 1981). He, along with Michael Berridge, pointed out that the two systems were rarely independent but were often antagonistic, sometimes synergistic or occasionally redundant (Berridge 1975). Obviously, at the time that Rasmussen and Berridge were discussing synarchic messengers, there was no appreciation of the molecular identities or the multiplicities of any of the components and interactions between the two pathways at numerous early steps. Furthermore, the spatial and temporal complexity of which we are now aware was unknown, and so resolving the problem (or indeed understanding the potential) arising

from the integration of these two ubiquitous second messengers is now infinitely more complex and challenging.

Consequently, if we are to seek to understand the role played by cAMP (or Ca^{2+}) it becomes essential to be able to think about both messengers in equivalent temporal and spatial dimensions. Given the additional developing recognition that cAMP signaling and ACs are highly organized within cells, it seems important to acknowledge that we really know very little about the detailed control by Ca^{2+} or cAMP of cellular processes. Until recently the types of evidence to be gathered to implicate cAMP in a process—actually first promulgated by Sutherland and Rall (Robison et al 1971) were (1) the hormone should stimulate AC in membranes, (2) the hormone should affect cAMP levels in intact cells, (3) inhibition of phosphodiesterase (PDE) should mimic the effect of putative cAMP-linked hormones, and (4) exogenous cAMP should mimic the effect of putative cAMP-linked hormones.² In the light of current knowledge we must now recognize that these are naïve, and in some cases, impossible conditions to fulfill, for reasons that will be expanded on in this article.

Against this backdrop, the major purpose of this review is to address the impact of Ca^{2+} -signaling on each of the mammalian ACs (1) this requires a serious assessment of the evidence for how all of the various potentially Ca^{2+} -regulated ACs are actually regulated as a consequence of activation of Ca^{2+} -signaling pathways, (2) an assessment of their physiological role in terms of their susceptibility to Ca^{2+} -regulation, (3) to summarize what is known about the spatial constraints that may be in place to ensure or preclude this regulation, which will include summarizing what is known of cAMP compartments (these should be viewed to be both dynamic and regulatable, in the manner

¹Not only the synthesis, but also the degradation, of cAMP can be affected by $[\text{Ca}^{2+}]_i$ as a consequence of the activation of one of the members of the phosphodiesterase family, PDE1. This enzyme is not widespread but it can influence cAMP levels where it is expressed.

²Application of exogenous cAMP to cells is not dissimilar to applying ionophore or sustained high K^+ depolarization to mimic the effect of physiological elevation of Ca^{2+} ; all of the potential spatial and temporal information is cast aside. Such an experiment will only apparently “work” if the read-out is so gross that the limitations are not apparent.

of, for instance, focal adhesion complexes), and (4) to argue for an assessment of changes in these second messengers in real time and comparable spaces. The major emphasis will be on the first point because this has not been seriously evaluated in recent years; the other points have received much more recent attention (reviewed in Hanoune and Defer 2001; Willoughby and Cooper 2007; Sadana and Dessauer 2009).

REGULATORY SUSCEPTIBILITIES OF ACs

Nine mammalian membrane-localized ACs have been identified. They share a common, complex 12-membrane-spanning architecture interspersed with two “Walker A” ATP binding motifs that associate to form the catalytic domain (Fig. 1).

Their similarity to the ATP binding cassette superfamily of transporters has been noted and discussed in the still unresolved context of whether they might function as transporters (Krupinski et al. 1989; Willoughby and Cooper 2007). Their largely conserved catalytic domains (C1a and C2a) has led to the conclusion, based on the crystal structure analysis of an AC5C1a/AC2C2a couple, that activation is achieved by promoting an open conformation of this domain (Tesmer et al. 1997; Tesmer et al. 1999; Mou et al. 2009). There is also a modicum of evidence that ACs may at least dimerize, if not

assemble in higher order structures, and that the complex transmembrane architecture may permit or indeed assist such oligomerization, which has been speculated to provide an additional ability of the ACs to form associations with other transmembrane proteins (reviewed in Cooper and Crossthwaite 2006).

Based largely on sequence analysis and relatedness, dendrogram presentation of the nine membrane-bound ACs has facilitated their division into four families: 1, 3, and 8; 2, 4, and 7; 5 and 6; and 9 (Fig. 2).

Studies expressing the cloned lead family members (but not all species, see later) in vitro suggested a pattern of regulatory susceptibilities that concurred with this familial organization as displayed in Table 1. This represents a consensus between earlier and recent reviews that reflects the widely accepted regulation of these enzymes.

A cursory examination of Table 1 reveals that all of the nine membrane-bound ACs are potentially regulated as a consequence of the activation of Ca²⁺-signaling pathways—either directly by Ca²⁺, or Ca²⁺-bound CaM, slightly less directly by CaMK or CaN, or indirectly by PKC, or by βγ subunits of G-proteins released by Gα_q-linked GPCRs (Fig. 3). We have noted this potential interaction previously (Cooper et al. 1995; Willoughby and Cooper 2007) and proposed that it formed a major opportunity

Figure 1. General structural domains of the nine membrane-bound ACs. Each of the nine membrane-bound ACs consist of two transmembrane clusters (TM1 and TM2) each consisting of six membrane-spanning domains. TM1 and TM2 are joined by an intracellular loop containing the C1a and C1b regions. Following TM2 is a long intracellular tail, containing the C2a and C2b regions before the carboxyl terminus.

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Figure 2. Phylogenetic tree of the nine membrane-bound ACs. The sequences of the nine membrane-bound ACs, from five species (human, rat, mouse, dog, and cow) were analyzed for relatedness and a phylogenetic tree was constructed using the Phylogeny.fr server (Castresana 2000; Guindon and Gascuel 2003; Edgar 2004; Anisimova and Gascuel 2006; Chevenet et al. 2006; Dereeper et al. 2008). The branch length is proportional to the number of substitutions per site.

for harmonizing the activities of the two systems in a realization and extension of the earlier discussions of Rasmussen and Berridge.

Because of the potential importance of these interactions, a major purpose of this review is to assess how convincing it is that any of these modes of regulation are actually used—particularly in any physiological context. We therefore performed a detailed review of the literature, and then assessed the evidence in a rather objective but unweighted manner, for the forms of

regulation outlined in Table 1. An obvious caveat for this style of analysis is that on a rigorous critical review, the findings from one original research paper may outweigh the conclusions presented in multiple, less robust experimental studies. Nevertheless, here, we are merely looking for a consistency and reproducibility in the existing evidence for the different forms of regulation of ACs by Ca^{2+} -signaling pathways. The literature was categorized based on the experimental design of the study, and thus designated

Table 1. The “consensus” regulation of ACs by the Ca²⁺-signaling pathway.^a

Isoform	Type of regulation			
	Ca ²⁺	CaMK/CaN	PKC	Gβγ
AC1	Activation (CaM)	Inhibition (CaMKIV)	No Effect/Activation	Inhibition
AC2	No Effect		Activation	Activation
AC3	Activation (CaM)	Inhibition (CaMKII)	No Effect/Activation	No Effect/Inhibition
AC4	No Effect		Activation	Activation
AC5	Inhibition		Activation	No Effect/Activation
AC6	Inhibition		Activation/Inhibition	No Effect/Activation
AC7	No Effect		Activation	No Effect/Activation
AC8	Activation (CaM)		No Effect	Inhibition
AC9		Inhibition (CaN)	Inhibition	No Effect

Some ACs have two effects listed for a particular form of Ca²⁺ regulation—this is because of differing conclusions drawn from the two reviews from which the table was compiled.

^aThe table lists the regulation of each of the nine membrane-bound ACs by Ca²⁺, either directly or indirectly via CaMK, CaN, PKC, or Gβγ. This consensus view of AC regulation was compiled from two recent and comprehensive reviews: Willoughby and Cooper (2007) and Sadana and Dessauer (2009), and the conclusions do not differ significantly from earlier summaries.

Figure 3. Regulation of ACs by Ca²⁺. Ca²⁺ can directly, and indirectly regulate the nine membrane-bound ACs. Submicromolar [Ca²⁺] can directly regulate AC, and some ACs specifically respond to Ca²⁺ derived from capacitative Ca²⁺ entry (CCE) from store-operated Ca²⁺ channels (SOCCs). Ca²⁺ can also regulate AC by binding calmodulin (CaM), and the Ca²⁺/CaM complex can then affect AC activity. Ca²⁺-bound CaM can also activate Ca²⁺/calmodulin-activated kinase (CaMK) and calcineurin (CaN), both of which may regulate AC. More indirectly, Gβγ subunits from Gα_q linked receptors can also regulate AC activity. In addition, Gα_q can activate phospholipase C (PLC), which converts phosphatidylinositol 4,5-bisphosphate (PIP₂) to diacylglycerol (DAG) and inositol trisphosphate (IP₃). DAG activates protein kinase C (PKC), which can also modulate the activity of AC; IP₃ binds to and activates its receptors (IP₃R) on the endoplasmic reticulum (ER), thereby releasing Ca²⁺ from the ER stores into the cytoplasm. This emptying of the ER Ca²⁺ stores triggers extracellular Ca²⁺ entry by SOCCs.

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as conducted in either overexpression or endogenous systems. Following this primary classification, the data was further defined as either binding- or phosphorylation-based, biochemical or pharmacological evidence.

In this analysis we draw attention to the level of proof that has been achieved and highlight the need for clarification of these issues to begin to understand this most critical aspect of cellular signaling.

REGULATION OF ACs BY Ca^{2+} -SIGNALING PATHWAYS

Direct Regulation by Ca^{2+}

The nine AC isoforms are classically grouped according to the ability of Ca^{2+} to regulate their activity. It has been accepted that submicromolar Ca^{2+} via CaM activates AC1, AC3, and AC8, whereas submicromolar Ca^{2+} alone inhibits AC5 and AC6, but has no effect on AC2, AC4, AC7, or AC9. The forms of evidence for Ca^{2+} regulation of each AC are detailed in Table 2.

Inhibition of ACs by Ca^{2+} : AC5 and AC6

The paradigm for Ca^{2+} -mediated inhibition of AC5 and AC6 in the submicromolar range³ (independently of CaM) is supported largely by biochemical studies of membrane preparations in overexpression systems (Guillou et al. 1999; Hu et al. 2002). Indeed the crystal structure of a high affinity Ca^{2+} -pyrophosphate (PPi) complex with the catalytic domain of AC5 has recently been presented (Mou et al. 2009). Much of the evidence for Ca^{2+} inhibition of AC6 comes from experiments conducted with whole cell overexpression systems, although there are also a good number of papers reporting modulation of AC6 activity by Ca^{2+} in an endogenous setting (Boyajian et al. 1989; Garritsen et al. 1992; Yu et al. 1993; Grunberger et al. 2006; Tang et al. 2008). Although the substantiation of Ca^{2+} inhibition of the highly

similar AC5 is less extensive (six studies in overexpression systems, and three in endogenous systems), it is uniform in its contention. Thus, there is good evidence for the inhibition of both AC5 and AC6 by Ca^{2+} , and the conclusion can be considered uncontentious both in vitro and in vivo.

Direct Activation of ACs by Ca^{2+} /CaM: AC1, AC3, and AC8

The most evidence for Ca^{2+} regulation of AC activity exists for the stimulation of AC1 and AC8 via CaM. The majority of this evidence comes from overexpression systems, using purified proteins, membrane preparations, or whole cells. Despite this, the direct binding of CaM to AC1 or AC8 has only been shown a few times for each enzyme (Vorherr et al. 1993; Gu and Cooper 1999; Masada et al. 2009). More importantly, and almost uniquely, there exists for these well-studied ACs, evidence of Ca^{2+} /CaM regulation in endogenous systems; much of this work was facilitated by the generation of AC1 and AC8 knockout mice, and the abundant expression of these isoforms in brain. However, a majority of this evidence still relies on experiments conducted in isolated membranes, with only two (AC1) or three (AC8) studies using intact primary cells (Villacres et al. 1998; Watson et al. 2000; Cioffi et al. 2002; Trubey et al. 2006; Wang et al. 2007). Nevertheless, there is robust evidence for the activation of both AC1 and AC8 by Ca^{2+} /CaM and this regulation can be considered established both in vitro and in vivo.

Far fewer studies have addressed Ca^{2+} /CaM regulation of AC3; some evidence suggests that Ca^{2+} /CaM activates AC3, although the effect is nothing like as pronounced as for AC1 and AC8, and is also conditional on AC3 activation. Although the effect is supported in endogenous systems (Mamluk et al. 1999; Hoffert et al. 2005), the available evidence is not as uniform as it is for Ca^{2+} /CaM activation of AC1 and AC8. In fact one study reports only minimal AC3 activation with high concentrations of Ca^{2+} (above the submicromolar concentrations that affect AC1, AC5, AC6, and AC8), and AC3 activation does not occur in the intact cell in

³All ACs are inhibited by Ca^{2+} in the submillimolar concentration range; this is believed to be because of a simple competition between Ca^{2+} and Mg^{++} at the catalytic site (Cooper 1991).

Table 2. Evidence for direct regulation of ACs by Ca²⁺.^a

Isoform	Action	Overexpression systems			Endogenous systems		Conclusions
		Binding	Biochemical evidence	Pharmacological evidence	Biochemical evidence	Pharmacological evidence	
AC1	Binding + CaM						Activation
	Activation		+++	+++	+++	+	
AC2	No Effect		++			+	No effect
AC3	No Effect		+	+			?
	Activation		+	+	+	+	
AC4	No Effect		+				No effect
AC5	Inhibition		++	+	+	+	Inhibition
AC6	Activation			+			Inhibition
	Inhibition		++	+++	+	+	
AC7	No Effect		+				No effect
AC8	Binding + CaM						Activation
	Activation		+++	+++	+++	++	
AC9	No Effect		+	+			No Effect

The accepted paradigm for Ca²⁺ regulation of each AC is listed in the first column, followed by the type of evidence provided in the literature: + 1–2 papers, ++ 3–5 papers, and +++ >6 papers. Overexpression systems refer to generic cell lines overexpressing recombinant proteins, and endogenous systems refer to primary cell lines or tissues endogenously expressing the AC of interest. Binding-based studies include coimmunoprecipitation or pull-down experiments; biochemical evidence refers to experiments conducted with membrane preparations, and pharmacological evidence refers to experiments conducted using intact cells. The conclusions that can be reasonably drawn following this analysis are listed in the final column: larger type indicates a uniform conclusion from >2 papers, smaller type indicates either a uniform conclusion from ≤2 papers or contradictory evidence substantially outweighed by the conclusion, ? indicates contradictory evidence that prevents a conclusion. (In this and other tables, “papers” refers to original experimental research papers that reported an effect, and not to the number of subsequent citations.)

^aThe table details the volume and type of evidence in the literature for direct regulation of ACs by Ca²⁺, or by Ca²⁺/CaM.

response to capacitative Ca²⁺ entry (CCE) unlike the case for AC1 and AC8 (Fagan et al. 1996).

AC2, AC4, AC7, and AC9 Are Not Directly Regulated by Ca²⁺

The extent of verification of the Ca²⁺-insensitivity of the remaining ACs is fairly even. A few highly cited papers, and one conducted in an endogenous setting (Feinstein et al. 1991; Lustig et al. 1993; Guillou et al. 1999; Hu et al. 2002) report no effect of Ca²⁺ on membrane preparations overexpressing AC2. Similarly, only one (though highly cited) study (Gao and Gilman 1991) details no effect of Ca²⁺ on membrane preparations overexpressing AC4. The lack of effect of Ca²⁺ on AC7 is again reported by only one paper (Crossthwaite et al. 2005), with the study conducted in membrane preparations

overexpressing AC7. For AC9, the evidence is derived from two studies in overexpression systems, one of which is also highly cited (Premont et al. 1996). Thus although the evidence presented in these studies is uniform, it is sparse and predominantly derived from membrane preparations of overexpression systems. Nevertheless it agrees with the “inferred” view.

Indirect Ca²⁺ Regulation via CaMKII, CaMKIV, and CaN

Ca²⁺ can also indirectly, via CaM, regulate ACs via the protein kinases CaMKII, CaMKIV, or the protein phosphatase, CaN (Table 3). Of the nine membrane-bound AC isoforms, only AC1, AC3, and AC9 have been studied in any detail in terms of their regulation by these Ca²⁺/CaM dependent proteins.

Table 3. Evidence for CaMKII, CaMKIV, or CaN regulation of ACs.^a

Isoform	Action	Overexpression systems			Endogenous systems			Conclusions
		Phos.	Biochemical evidence	Pharmacological evidence	Phos.	Biochemical evidence	Pharmacological evidence	
AC1	Phos. (CaMKIV)	+						No Effect (CaMKII)
	No Effect (CaMKII)			+				Inhibition (CaMKIV)
	Inhibition (CaMKIV)		+	+				
AC2								
AC3	Phos.	+			+			Inhibition (CaMKII)
	Inhibition (CaMKII)			++			++	
AC8	No Effect (CaMKII and CaMKIV)			+				No Effect (CaMKII and CaMKIV)
AC9	Activation (CaMKII)			+				Activation (CaMKII)
	Inhibition (CaN)			++			+	Inhibition (CaN)

The accepted paradigm for regulation of each AC is listed in the first column, followed by the type of evidence provided in the literature: + 1–2 papers, ++ 3–5 papers, and +++ >6 papers. AC4, AC5, AC6, and AC7 do not appear on this table, as no evidence can be found for their regulation by CaN, or CaMK. Overexpression systems refer to generic cell lines overexpressing recombinant proteins, and endogenous systems refer to primary cell lines or tissues endogenously expressing the AC of interest. Phosphorylation-based studies include ³²P-incorporation experiments, biochemical evidence refers to experiments conducted with membrane preparations, and pharmacological evidence refers to experiments conducted using intact cells. The conclusions that can be reasonably drawn following this analysis are listed in the final column: large type indicates a uniform conclusion from >2 papers, smaller type indicates either a uniform conclusion from ≤2 papers or contradictory evidence substantially outweighed by the conclusion. Phos., indicates phosphorylation.

^aThe table details the volume and type of evidence in the literature for regulation of ACs by CaMKII, CaMKIV or CaN.

Table 4. Evidence for PKC regulation of ACs.^a

Isoform	Action	Overexpression systems			Endogenous systems			Conclusions
		Binding or Phos.	Biochemical evidence	Pharmacological evidence	Binding or Phos.	Biochemical evidence	Pharmacological evidence	
AC1	No Effect			+				?
	Inhibition			+				
AC2	Phos.	++ P			+ P			Activation
	Activation		+++	+++			+	
AC3	Activation			+			+	Activation
AC4	Binding				+ B			?
	No Effect		+	+				
	Activation			+				
	Inhibition		+					
AC5	Phos.	+ P						Activation
	No Effect			+				
	Activation		+	++			+	
AC6	Binding or Phos.	+ B,P						Inhibition
	No Effect			+				
	Inhibition		++	+				
AC7	Phos.	+ P						Activation
	Activation		+	++			+	
AC8								
AC9	Inhibition			+				Inhibition

The accepted paradigm for PKC regulation of each AC is listed in the first column, followed by the type of evidence provided in the literature: + 1–2 papers, ++ 3–5 papers, and +++ >6 papers. Overexpression systems refer to generic cell lines overexpressing recombinant proteins, and endogenous systems refer to primary cell lines or tissues endogenously expressing the AC. Binding- or phosphorylation-based studies include coimmunoprecipitation, pull-down or ³²P-incorporation experiments; biochemical evidence refers to experiments conducted with membrane preparations, and pharmacological evidence refers to experiments conducted using intact cells. The conclusions that can be reasonably drawn following this analysis are listed in the final column: large type indicates a uniform conclusion from >2 papers, smaller type indicates either a uniform conclusion from ≤2 papers or contradictory evidence substantially outweighed by the conclusion, ? indicates contradictory evidence. B, indicates binding evidence, Phos. or P, indicates evidence of phosphorylation.

^aThe table details the volume and type of evidence in the literature for regulation of ACs by PKC.

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Inhibition of AC1 and AC3 by CaMKs

Most evidence exists for the inhibition of AC3 by CaMKII; in both overexpression (Wei et al. 1996), and endogenous systems (Wei et al. 1998), CaMKII has been shown to phosphorylate AC3 at Ser¹⁰⁷⁶ thus inhibiting cAMP synthesis. However, further support for this assertion solely depends on the use of CaMKII inhibitors and concurrent demonstration of AC3 expression. Nevertheless, the uniformity of reports does suggest that AC3 is inhibited by CaMKII. Much less evidence exists for the inhibition of AC1 by CaMKIV; only one paper (in an overexpression system) shows phosphorylation of AC1 by CaMKIV, and subsequent inhibition of Ca²⁺/CaM-stimulated AC1 following CaMKIV overexpression (Wayman et al. 1996). The same study also shows a lack of effect of CaMKII on AC1, and no effect of CaMKII or CaMKIV on AC8-stimulated cAMP.

AC9: Activation by CaMKII and Inhibition by CaN

Five studies in whole cells overexpressing AC9, and one in primary rat anterior pituitary corticotrophs (Antoni et al. 2003), report inhibition of this AC by CaN. All of these studies depend on the use of CaN inhibitors, combined with AC9 expression. Only one study has provided evidence for CaMKII-mediated potentiation of cAMP generated following stimulation of AC9, which again relied on the use of CaM and CaMKII inhibitors (Cumbay and Watts 2005). Thus although there is conformity within these studies, suggesting activation of AC9 by CaMKII but inhibition via CaN, the extent of this support is quite sparse.

Indirect Regulation by PKC

The elevation of PKC activity is one consequence of stimulation of PLC by GPCRs, in addition to the production of InsP₃ and Ca²⁺-mobilization (Fig. 3). Much evidence exists for PKC activation of AC2, although the majority of this evidence comes from either membrane preparations or whole cells overexpressing the enzyme, with activation of PKC induced by

phorbol esters and then often precluded using PKC inhibitors. Importantly there are also four studies, one from tissue endogenously expressing AC2 (Chakrabarti et al. 1998), that show increased phosphorylation of the AC in response to PKC activation (Jacobowitz and Iyengar 1994; Zimmermann and Taussig 1996; Böhl et al. 1997a; Böhl et al. 1997b). There is less, and conflicting, evidence for PKC activation of AC4; all conducted in overexpression systems, two studies show no effect (Jacobowitz et al. 1993; Zimmermann and Taussig 1996), one shows inhibition (Zimmermann and Taussig 1996) and another stimulation (Marjamäki et al. 1997), depending on the coactivator used. However one study shows binding of PKC to AC4 in an endogenous setting (Rhim et al. 2006). For PKC activation of AC7, there is only a small amount of evidence, although the reports are all consistent, and there is good evidence for both phosphorylation of AC7 in an overexpression system (Nelson et al. 2003), and activation of endogenously expressed enzyme (Haslauer et al. 1998; Antoni et al. 2003; Lariviere et al. 2007). Thus although a detailed review of the literature clearly confirms the prevailing view of activation of both AC2 and AC7 by PKC, the evidence regarding PKC-activation of AC4 is contradictory and does not permit a concrete conclusion.

The Remaining AC Isoforms

The Ca²⁺-inhibitable AC5 and AC6 have also been studied in terms of their PKC susceptibility. The evidence is conflicting for the regulation of both ACs by PKC, despite data showing binding and/or phosphorylation of the enzymes (Kawabe et al. 1994; Iwami et al. 1995; Lai et al. 1997; Lin et al. 2002; Rhim et al. 2006). Consequently, a detailed review of the literature does not allow a simple conclusion regarding the regulation of AC5 or AC6 by PKC.

Even less evidence addresses the PKC regulation of AC1, AC3, AC8, and AC9. Two different studies report conflicting effects of PKC on AC1 (Jacobowitz et al. 1993; Yoshimura and Cooper 1993). A similar paucity of evidence exists for activation of AC3 by PKC, despite consistency

of these reports, and additional evidence for PKC activation of AC3 in an endogenous setting (Mamluk et al. 1999). There are no reports of an effect of PKC on AC8, and only two studies report PKC-mediated inhibition of AC9 (Cumbay and Watts 2004; Cumbay and Watts 2005). Although the volume of reports is sparse, it appears likely that PKC can activate AC3, but inhibits AC9. The conflicting nature of the evidence regarding PKC regulation of AC1, and the lack of reports regarding potential regulation of AC8, prevent any conclusions regarding the PKC regulation of these two ACs.

Indirect Ca²⁺ Regulation via Gβγ

The ACs classically designated as PKC-activated, AC2, AC4, and AC7, are also defined by their conditional activation by Gβγ subunits. Similarly, the group of ACs classically activated by Ca²⁺/CaM (AC1, AC3, and AC8), are concurrently defined by Gβγ-mediated inhibition. A detailed review of the literature regarding Gβγ regulation of ACs is summarized in Table 5.

ACs Conditionally Activated by Gβγ: AC2, AC4, and AC7

The most evidence for Gβγ regulation of AC, in terms of both volume of studies and citation record, refers to the activation of AC2 by Gβγ subunits. There are 23 studies in isolated membranes or whole cells overexpressing AC2 that document this activation, and some of these are very frequently cited (Tang and Gilman 1991; Federman et al. 1992). An additional four studies show direct Gβγ binding to AC2, and this has also been reported in endogenous expression systems (Wang et al. 2005; Wang and Burns 2006). In stark contrast, there is much less evidence for the activation of AC4 by Gβγ subunits, with only two studies conducted in overexpression systems (Gao and Gilman 1991; Marjamaki et al. 1997). However, evidence for both binding and activation in an endogenous setting also exists (Belevych et al. 2001; Wang et al. 2005; Wang and Burns 2006). The scant evidence for AC7 activation by Gβγ subunits consists of one well-cited study in whole cells

overexpressing the enzyme (Yoshimura et al. 1996). Thus there is a substantial volume and consistency of studies reporting activation of AC2 by Gβγ subunits, and this conclusion appears to hold for AC4 and AC7, despite a much narrower evidence base.

Inhibition of AC1, AC3, and AC8 by Gβγ

The second-most verified Gβγ-mediated regulation of AC, is the inhibition of AC1 by Gβγ subunits. Eleven studies document this inhibition in overexpression systems, in addition to one that reports the direct binding of Gβγ to the enzyme (Weitmann et al. 2001). Furthermore there is a highly cited paper that shows inhibition of ACs by Gβγ in membranes from brain (Chen et al. 1995), although this does not distinguish between AC1 and AC8. Apart from this study, only one other documents AC8 inhibition by Gβγ subunits, which was conducted in whole cells overexpressing the enzyme (Steiner et al. 2006). The evidence for AC3 regulation by Gβγ is conflicting and sparse; two studies in overexpression systems have reported no effect (Tang and Gilman 1991; Marjamaki et al. 1997), whereas another reports inhibition (Diel et al. 2006). There is no evidence from endogenous systems. Thus although the inhibition of AC1 by Gβγ represents a robust paradigm, Gβγ mediated inhibition of AC8 is less substantiated, and no firm conclusions can be made regarding the regulation of AC3 by Gβγ subunits.

Effects of Gβγ on AC5, AC6, and AC9

There is conflicting evidence for an effect of Gβγ subunits on both AC5 and AC6, with no effect, activation and inhibition all reported. Binding of Gβγ subunits to AC5 and AC6 has been reported in both overexpression (Sadana et al. 2009) and endogenous systems (Gao et al. 2007), although the latter involved a yeast-two hybrid screen of a mouse brain cDNA library. The most highly cited papers report the activation of AC5 by Gβγ subunits (Avidor-Reiss et al. 1996), and no effect of Gβγ on AC6 (Premont et al. 1992); however, these reports are

Table 5. Evidence for G $\beta\gamma$ regulation of ACs.^a

Isoform	Action	Overexpression systems			Endogenous Systems			Conclusions
		Binding	Biochemical evidence	Pharmacological evidence	Binding	Biochemical evidence	Pharmacological evidence	
AC1	Binding	+						Inhibition
	Inhibition		+++	+			+	
AC2	Binding	++				+		Activation
	Activation		+++	+++				
AC3	No Effect		+	+				?
	Inhibition		+					
AC4	Binding					+		Activation
	Activation		+	+			+	
AC5	Binding	+			+			?
	No Effect		+					
	Activation		+	+				
	Inhibition			+				
AC6	Binding	+			+			?
	No Effect		+					
	Activation		+					
	Inhibition			+				
AC7	Activation			+				Activation
AC8	Inhibition			+		+		Inhibition
AC9	No Effect		+	+				No Effect

The accepted paradigm for G $\beta\gamma$ regulation of each AC is listed in the first column, followed by the type of evidence provided in the literature: + 1–2 papers, ++ 3–5 papers, and +++ >6 papers. Overexpression systems refer to generic cell lines overexpressing recombinant proteins, and endogenous systems refers to primary cell lines or tissues endogenously expressing the AC of interest. Binding-based studies include coimmunoprecipitation or pull-down experiments; biochemical evidence refers to experiments conducted with membrane preparations, and pharmacological evidence refers to experiments conducted using intact cells. The conclusions that can be reasonably drawn following this analysis are listed in the final column: large type indicates a uniform conclusion from >2 papers, smaller type indicates either a uniform conclusion from ≤ 2 papers or contradictory evidence substantially outweighed by the conclusion, ? indicates contradictory evidence.

^aThe table details the volume and type of evidence in the literature for regulation of ACs by G $\beta\gamma$.

contradicted by an additional five studies. Although the reports for no effect of Gβγ subunits on AC9 activity are consistent, only two studies have shown this, both in overexpression systems (Premont et al. 1996; Cumbay and Watts 2005). Hence no concrete statements can be made regarding the effects of Gβγ on regulation of AC5 or AC6. In contrast, there appears to be no effect of Gβγ on AC9.

OVERALL SUMMARY OF EVIDENCE FOR THE REGULATION OF Ca²⁺-SIGNALING PATHWAYS

Table 6 summarizes the evidence for direct and indirect regulation of ACs by Ca²⁺ emanating from our assessment of the literature.⁴ It seems clear that AC5 and AC6 are inhibited by Ca²⁺, AC1 and AC8 are activated by Ca²⁺/CaM, AC2 is activated by PKC and Gβγ, and AC1 is inhibited by Gβγ. Further, it is likely that AC3 is conditionally activated by Ca²⁺/CaM and activated by PKC but inhibited by CaMKII, AC5 is activated by PKC, AC6 is inhibited by PKC, AC7 is activated by PKC, and AC9 is inhibited by CaN. The regulation of the remaining ACs by Ca²⁺, PKC and Gβγ remains uncertain.⁵

The initial classification of the nine membrane-bound ACs into subgroups based on sequence similarities, while initially useful and constructive on a gross scale, is perhaps too vague for the amount and type of evidence present in the literature, at least in terms of their susceptibility to regulation by Ca²⁺.⁶ Thus although AC1, AC3, and AC8 are activated to varying extents by Ca²⁺/CaM, AC1 is also inhibited by Gβγ subunits, and AC3 is inhibited by CaMKII and activated by PKC. Similarly,

although both AC5 and AC6 are inhibited by Ca²⁺, they have opposing regulation by PKC (AC5 is activated, whereas AC6 is inhibited). Even more surprisingly, although AC2 is activated by PKC and Gβγ, only AC7 (but not AC4) is also activated by PKC, and neither AC4 nor AC7 appears to be activated by Gβγ.

An additional confounding factor in this analysis is the likely sensitivity of the ACs to particular isoforms of the regulating proteins, specifically in the case of regulation by PKC and Gβγ subunits. It would not be surprising for an AC to be differentially regulated by distinct isoforms of PKC, or combinations of Gβγ subunits. Thus some of the more conflicting evidence compiled here may be resolved by clarifying the precise PKC and Gβγ entities that were used, because studies using unidentified PKC or Gβγ preparations can obviously be ambiguous. Additionally, the background cell type will be a critical determinant in susceptibility of the AC isoforms to Ca²⁺-signaling pathways. This will not only influence the available complement of regulatory proteins, including varied PKC isoforms or Gβγ subunit combinations, but may also dictate the AC response to a particular stimulus because of differential compartmentalization of Ca²⁺-signaling pathway components. Again, resolution of the effects of cell type on the regulation of AC activity may further illuminate the analysis conducted here.

Nevertheless, although the current state-of-the-art classification of the nine membrane-bound ACs, based on amino acid sequences, does generally apply on a gross scale, it becomes significantly less concrete, and quite sparse, when examined in detail. Consequently there is a strong case, given the potential importance of the regulation, for a systematic and controlled analysis of each AC.

PHYSIOLOGICAL ROLES FOR THE Ca²⁺-DEPENDENCY OF ACs

Of course, even when we are convinced that these enzymes may be regulated as outlined above *in vivo* is there any evidence that this regulation is used or is an important part of the physiological role of these ACs?

⁴We intended to be relatively comprehensive in this review, although we have missed some studies, but hopefully not with any unrepresentative consequences.

⁵In some cases the ACs have not been studied in sufficient detail, whereas in other cases the situation is indeed far from certain based on conflicting studies, as outlined in the literature review.

⁶It should be recognized that the classification of the ACs into families based on sequence similarity leaves ample room for significant differences between members of the same family.

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Table 6. Evidence-based summary of the regulation of ACs by the Ca^{2+} -signaling pathway.^a

Isoform	Type of regulation			
	Ca^{2+}	CaMK/CaN	PKC	Gβγ
AC1	Activation (CaM)	–	?	Inhibition
AC2	No Effect		Activation	Activation
AC3	?	Inhibition (CaMKII)	Activation	?
AC4	–		?	–
AC5	Inhibition (Direct)		Activation	?
AC6	Inhibition (Direct)		Inhibition	?
AC7	–		Activation	–
AC8	Activation (CaM)	–		–
AC9	–	Inhibition (CaN)	–	–

Large text indicates strong support for the regulation, small text indicates consistent but weaker support, – indicates that although uniform, the limited evidence available prevents concrete conclusions, and ? indicates that the available evidence is conflicting and prevents any robust conclusions.

^aThe table shows the effects of Ca^{2+} , CaM, CaMK, CaN, PKC, and Gβγ on the nine membrane-bound ACs that can be robustly supported by the available literature.

AC1 is exclusively neuronal in its disposition and so whether it is regulated in an endogenous setting is not simple to resolve. Knock-out studies suggest a clear role in various models of learning and memory, addiction, stress-responses, and pain. In particular, AC1 knockout mice show decreased Ca^{2+} /CaM stimulated AC activity in the hippocampus and cerebellum, which correlates with reduced long-term potentiation (Wu et al. 1995; Storm et al. 1998). These studies clearly indicate an essential physiological role for AC1, but its Ca^{2+} -sensitivity is best attested to by the exuberant axon model, in which appropriate pathfinding does not occur in the absence of neuronal excitability, Ca^{2+} -entry or in AC1 knockout mice (Nicol et al. 2007). The latter study strongly suggests that stimulation of AC1 by Ca^{2+} does occur and that it is essential for the effects of cAMP in that context. A knock-in AC1 that was not regulated by Ca^{2+} /CaM would cement these conclusions, if it was expressed appropriately.

AC8 knockout mice have a clear array of defects, with a predominant impairment in learning and memory; this is also associated with decreased Ca^{2+} /CaM stimulated AC activity in the hippocampus, hypothalamus, thalamus, and brainstem (Schaefer et al. 2000). So, clearly these enzymes are important; however,

whether their sensitivity to Ca^{2+} is important in a physiological context remains to be absolutely shown.

Other studies examining the effect of AC5 and AC6 knockout have shown definite physiological consequences, but none of these can be directly attributed to the Ca^{2+} -inhibibility of these enzymes (reviewed in Sadana and Des-sauer 2009). AC5 knockout mice have altered pain responses, attenuated motor function and altered cardiac function. It had been speculated that the predominance of AC5 and AC6 in cardiac tissue could contribute significantly to the rhythmicity of sympathetic control of inotropy. Specifically, the AC5 knockout mice have decreased left ventricular ejection fraction, and attenuated baroreflexes and this is associated with a loss of acetylcholine mediated $\text{G}\alpha_i$ inhibition of AC activity, and a reduced Ca^{2+} -mediated inhibition of cAMP (Okumura et al. 2003). AC6 knockout mice show decreased left ventricular function, and this is also associated with a decreased Ca^{2+} -mediated inhibition of cAMP (Tang et al. 2008).

There are also physiological effects of AC3 knockout; the mice do not show intermale aggressiveness or male sexual behaviors, and this is associated with decreased cAMP in response to pheromones (Wang et al. 2006). Thus, although

there are definitive consequences of knockout of AC1, AC3, AC5, AC6, and AC8, apart from AC1, whether the physiological effects shown are caused by the Ca²⁺-dependency of the ACs remains to be determined.

FUNCTIONAL COMPARTMENTALIZATION OF Ca²⁺ AND cAMP SIGNALING

From the foregoing it is reasonably clear that AC1 and AC8, and AC5 and AC6 are stimulated and inhibited respectively by Ca²⁺-rises in vitro and in the intact cell. However, and of even more regulatory significance, they are also highly discerning for the form of Ca²⁺-rise to which they will respond.

AC Regulation by Ca²⁺ Entry

Elsewhere, and in detail, we have summarized how CCE triggered by either GPCR-linked agonists or by passive store depletion regulates each of the directly Ca²⁺-regulatable ACs, either when expressed heterologously or when endogenously expressed (Cooper 2003; Willoughby and Cooper 2007). In our lab we have tended to use CCE that is triggered by passive store depletion rather than by agonists, but in various studies carbachol (CCh), bradykinin, or thyrotropin-releasing hormone (TRH) have also been used (Boyajian et al. 1991; Wachten et al. 2010; Willoughby et al. 2010). However because the consequences associated with agonist-triggered store depletion via PLC stimulation, such as PKC activation or the liberation of $\beta\gamma$ subunits of G-proteins, can have confounding additional effects on various AC species (as outlined above) the results of passive store depletion seem more straightforward.

Despite regulation by CCE, these enzymes are extremely unresponsive to ionophore mediated Ca²⁺-entry. Early reports of responses to high concentrations of ionophore and external CaCl₂ were likely due directly to the triggering of CCE that is caused by store permeabilization and a subsequent CCE component of the Ca²⁺-rise. A large body of data not reviewed here has established the dependence on CCE (Willoughby and Cooper 2007). Such is the

dependence of the Ca²⁺-sensitive ACs on CCE that we and others have used CCE-induced changes in cAMP as a measure for separating CCE from other Ca²⁺-entry processes (Shuttleworth and Thompson 1999; Martin and Cooper 2006) and further, to study potential candidates that participate in CCE, such as Orai and STIM1 (the heterologous expression of which leads to increased regulation of these ACs by CCE; Martin et al. 2009).

This ability of the ACs to be regulated by specific forms of physiological Ca²⁺-rise strongly suggests a selective affinity of the enzymes for the immediate environments of Ca²⁺ channels if not an actual affinity for Ca²⁺ channel subunits. In fact in addition to CCE, these ACs can also be regulated by voltage-gated Ca²⁺-entry, though this topic has been far less well studied (Chetkovich et al. 1991; Yu et al. 1993; Fagan et al. 2000), and overexpression of CNG channels can promote the inhibition of AC6 by Ca²⁺-entry (Fagan et al. 1999). The properties of Ca²⁺ channel subunits to form heterogeneous (and poorly understood) associations, e.g., various TRPC1/TRPM1/Orai combinations, or the various combinations of α , β , γ , and ϵ subunits possible in VGCCs, may also allow such subunits to associate with other membrane-inserted proteins (such as ACs), which are themselves capable of multivalency (Cooper and Crossthwaite 2006). Nevertheless, no credible associations between ACs and putative channel components have yet been revealed.

One puzzle implicit in the reliance of Ca²⁺-regulatable ACs on CCE is the nature of the Ca²⁺ signal to which these ACs respond. In vitro (steady-state) studies suggest a susceptibility to concentrations of Ca²⁺ in the just submicromolar range; however significantly higher concentrations would be anticipated in the immediate vicinity of Ca²⁺ channels. We have speculated elsewhere on this apparent anachronism (Willoughby and Cooper 2007). The simplest reconciliation may be that ACs in intact cells specifically respond to the kinetic upstrokes in Ca²⁺ concentrations that occur at near-by channels—not to the steady state levels that are subsequently achieved.

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AC Regulation by Agonist-Triggered Ca^{2+} Release

This mode of Ca^{2+} rise causes a small stimulation of both AC1 and AC8 when they are heterologously expressed. This is a fraction of the effect of CCE in the case of AC8 but almost equal to the effect of CCE in the case of AC1 (Masada et al. 2009). There is also evidence from endogenous systems that AC8 is stimulated by Ca^{2+} release mediated by TRH in GH3B6 cells (Wachten et al. 2010) but the effect of entry was not explored in that study.

From the foregoing it is clear that both the Ca^{2+} -sensitive ACs, and the machinery for Ca^{2+} -entry must be organized in such a way as to ensure the specific regulation of the ACs. Whether there is any compartmentalization or organization with regard to regulation of ACs by PKC, CaMK, CaN, or G $\beta\gamma$ subunits has never been addressed, but there is no reason to imagine that such organization may not occur. Below we outline some of our speculations on the molecular mechanisms or organizational constraints necessitated by the precise Ca^{2+} -regulation of ACs.

NATURE OF THE COMPARTMENTS FOR Ca^{2+} AND cAMP

Implicit in the regulation of cAMP by Ca^{2+} -signaling pathways is a degree of organizational constraint; we are now beginning to learn something of the elements of these constraints. Although it is well known that compartmentalization is intrinsic to Ca^{2+} , the rapid diffusion of Ca^{2+} makes these compartments extremely transient. Nevertheless at sites of Ca^{2+} -release or Ca^{2+} -entry, steep declines in concentration from extremely high to ambient can be both predicted and observed. Imaging studies have revealed sparks, sparklets, scintillas, puffs, or blinks (reviewed in Berridge 2006; Rizzuto and Pozzan 2006). Although Ca^{2+} -buffering can also play a significant theoretical role in the diffusion of Ca^{2+} , few practical consequences of buffering by proteins such as calbindin have been shown. There is of course gross compartmentalization of Ca^{2+} within ER stores and the mitochondria. In addition, assembly of

Ca^{2+} -entry complexes in response to store depletion, such as the puncta formed by Orai, and STIM1 (along with other elements, e.g., TRPC1) show a degree of subcellular compartmentalization. Direct compartmentalized signaling by Ca^{2+} is obviously a major regulatory device in situations such as vesicle release sites at synapses, etc. Based on the foregoing discussion of the selective regulation by Ca^{2+} -entry of Ca^{2+} -sensitive ACs, it can be anticipated that ACs cluster at such locations.

Compartmentalized signaling occurs at a different level for cAMP. As with channels, the source of cAMP—in this case the ACs—is the first opportunity for compartmentalization of the second messenger. PDEs are a further early opportunity to impact on these compartments, because the presence or absence of a PDE will impact heavily on the diffusion range of cAMP (reviewed in Fischmeister et al. 2006; Zaccolo 2006; Houslay 2009). Ca^{2+} -stimulated PDE1 is potentially particularly relevant, because in this case Ca^{2+} -rises will also impact on the PDE-limited diffusion of cAMP. Curiously, very few studies have considered the role of PDE1 in controlling cAMP (Evans et al. 1984) but it does appear that for continual activation of PDE1, sustained CCE is required (Goraya et al. 2004).

At a very gross level some ACs are localized in lipid raft domains of the plasma membrane whereas others are excluded (Ostrom et al. 2002; Crossthwaite et al. 2005). However, the issue of lipid rafts had been controversial based on oversimple assumptions and variability between systems. In situations such as the immunologic synapse or postsynaptic densities, clearly stable domains are encountered that differ in their lipid composition from the rest of the plasma membrane. On the other hand dynamically changing lipid inhomogeneities also occur, which can have variably observed half-lives and are variably populated by signaling molecules. Encounters between such rafts may promote or facilitate productive regulatory interactions between resident proteins. These transient associations could potentially promote cAMP hotspots by concentrating, e.g., GPCR and G-proteins or other regulatory factors such

as the elements of the CCE apparatus, with ACs and facilitating their interaction. In the case of the nine membrane-bound ACs, the acutely Ca²⁺-regulated species AC1, AC8, AC5, and AC6 are all localized in rafts, as assessed by a variety of criteria and preparation methods (Fagan et al. 2000; Ostrom et al. 2002; Pagano et al. 2009; reviewed in Patel et al. 2008). In addition, the elements of the CCE apparatus, Orai and STIM1, can also be found in lipid rafts (Pani et al. 2008). However, the conclusion that STIM1 associates with lipid rafts was recently contested in elegant functional studies of CCE in live cells (DeHaven et al. 2009). Furthermore, the studies that originally suggested an essential functional association of Ca²⁺-regulated ACs with lipid rafts, showed only a loss of the regulation of the ACs following cholesterol depletion, with no associated change in CCE (Fagan et al. 2000; Smith et al. 2002). These conflicting studies of the residence or otherwise of the CCE apparatus in lipid rafts underline the difficulties in comparing biochemical and cell biological assessments of raft components.

Nevertheless, there are clear indications that ACs can act as organizers of their own domain. Most of the ACs (i.e., AC1, AC2, AC3, AC5, AC6, AC8, and AC9) bind A-kinase anchoring proteins (AKAPs), and AKAPs can also bind PDEs as well as PKC, CaN, and various channels (reviewed in Dessauer 2009). In the brain, AC5 associates with a complex including AKAP79, PKA, and AMPA receptors to facilitate glutamate-stimulated AC5 activity (Efendiev et al. 2010), and AC2 associates with Yotiao (AKAP9) which inhibits the AC (Piggot et al. 2008). In the heart AC5 associates with mAKAP β and PKA, which attenuates AC5 activity (Kapiloff et al. 2009). In pancreatic and neuronal systems, AC8 associates with AKAP79, which limits the sensitivity of the AC to stimulation by CCE (Willoughby et al. 2010). AC8 also associates with protein phosphatase PP2A, which is itself a scaffolding protein (Cooper and Crossthwaite 2006). Signaling hubs that are built around the ACs ensure fidelity in cAMP signaling, and, in the case of the neuronal system, ensure that Ca²⁺ very efficiently regulates the AC that organizes the scaffold. Other data also suggest that ACs can

interact with the cytoskeleton and thereby potentially regulate cellular dynamics (reviewed in Patel et al. 2008). In this context it is interesting that emptying of Ca²⁺ stores by treatment with 1 mM TPEN (an intra-ER Zn²⁺/Ca²⁺-chelator) or 5 μ M ionomycin results in increased cAMP accumulation in a colonic cell line. Sequestration of free Ca²⁺ within the ER, or extrusion of Ca²⁺ from the ER in these cells (which do not express a Ca²⁺-stimulable AC1 or AC8) resulted in a slow increase in cAMP accumulation, independently of CCE (i.e., the effect occurred in the absence of extracellular Ca²⁺) and cytosolic Ca²⁺ (thereby excluding direct effects of Ca²⁺ on AC activity), but in a manner and time scale that appeared to coincide with STIM1 translocation (Lefkimmatis et al. 2009; Roy et al. 2010). The time course of this response does not match the rapid effects of extracellular Ca²⁺ on agonist- or thapsigargin-store depleted cells expressing either Ca²⁺-stimulable or Ca²⁺-inhibitable ACs; nor were such Ca²⁺-independent effects on AC activity seen during store-depletion before the addition of extracellular Ca²⁺ in such systems (Fagan et al. 1998; Willoughby and Cooper 2006). An intriguing mechanism is thus suggested whereby the gross cellular architectural changes that occur following treatments with high concentrations of TPEN and ionomycin, which include STIM1 translocation to the plasma membrane, leads to increased cAMP accumulation. Conceivably the increased cAMP accumulation results from a relief from an undefined inhibitory constraint, e.g., between AC and the cytoskeleton or plasma membrane, that accompanies the major cellular transitions that are associated with store depletion and STIM1 translocation. A similar slow effect, presumed to reflect disinhibition of ACs, is seen when cells are deprived of cholesterol (Fagan et al. 2000; Pagano et al. 2009), thereby disrupting lipid rafts and their associated protein complexes.

EXPLORING AC ACTIVITY AT THE SINGLE CELL LEVEL

Given the susceptibility of most of the nine membrane-bound ACs to regulation by various aspects of Ca²⁺-signaling as discussed up to

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now, along with the essential compartmentalization associated with this regulation, it seems essential to adopt very discerning methods in order to establish whether or how these enzymes are regulated. In addition, given that Ca^{2+} -levels can oscillate in a nonhomogenous manner from cell to cell, it is essential to adopt single cell methods to determine whether the various Ca^{2+} -sensitive ACs can actually respond in an oscillatory manner to such Ca^{2+} -signals.

Elsewhere (reviewed in Nikolaev and Lohse 2006; Willoughby and Cooper 2008), methods have been described that allow the measurement of cAMP in single cells; these are all based on the natural targets of cAMP, i.e., PKA, EPAC, and CNG and HCN channels, and they all have specific advantages and applications. In addition modification of these sensors to allow targeting to specific AC microdomains has facilitated the beginning of an explicit exploration of these microenvironments (Wachten et al. 2010). Such methods are also potentially particularly powerful in dynamic situations, e.g., in moving cells.

cAMP oscillations had been predicted based on the exposure of Ca^{2+} -sensitive ACs to oscillations in Ca^{2+} concentrations (e.g., in cardiac tissue; reviewed in Cooper et al. 1995). Examples of cAMP oscillations have now been described in a number of systems by multiple single-cell cAMP imaging methods (Gorbunova et al. 2002; Landa et al. 2005; Dyachok et al. 2006; Willoughby and Cooper 2006), and a variety of mechanisms for the oscillations in cAMP have been advanced. It is still not clear whether cAMP oscillations generate functional consequences in a physiological setting, although one study clearly indicates that cAMP oscillations derived from Ca^{2+} -stimulation of AC1 are required for exuberant axonal pathfinding (Nicol et al. 2007).

FUTURE DIRECTIONS

This review of the regulation of ACs by activation of Ca^{2+} -signaling pathways has been surprising from a few viewpoints. Whereas it seems clear that AC1, AC8 and AC5, and AC6 are directly regulated by Ca^{2+} /CaM and Ca^{2+} ,

respectively, both in vitro and as a result of meaningful elevations in intracellular Ca^{2+} , the regulatory influence of the Ca^{2+} -signaling pathway on the other ACs, particularly in terms of the role of PKC, G $\beta\gamma$ subunits, CaN, CaMKII, and CaMKIV, is far from established. This is not to suggest that the evidence is unconvincing, just that the subject has received so little attention. Given the potential importance of these interactions and the undoubted potential magnitude of the effects, this is a striking oversight in signaling awareness. It would seem extremely worthwhile to attempt to fill these gaps. Obviously, if we do not comprehensively appreciate how these enzymes are regulated, we cannot begin to contemplate their potential physiological significance. The problem is compounded by the growing awareness of the complexity of compartmentalized signaling and the extreme paucity of information on the organization and cellular distribution of, for instance, PKC. Nevertheless, the relevant tools are available in terms of both single cell techniques and intracellular targeting of probes; the philosophical will may understandably be lacking given the magnitude of the information that is likely to be uncovered. The comfortable naïveté that accompanies two-dimensional portrayals of signaling cascades is of course troubled by confronting the likely complexity of real cell signaling. Against this background, it seems essential to engage with these issues and to develop further conceptual and experimental frameworks for addressing these interactions. Whereas a “systems” approach conjures up images of the mechanistically unimaginative, the true and considerable dimensions of the problem may demand at least a flirtation with such approaches (Xu et al. 2010). In any event there is little doubt that these two crucial systems are critically intertwined, that there are highly significant consequences for this interaction, and we do need to address and respond to this complexity.

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