REVIEW ARTICLE

Regulation and organization of adenylyl cyclases and cAMP

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Adenylyl cyclases are a critically important family of multiply regulated signalling molecules. Their susceptibility to many modes of regulation allows them to integrate the activities of a variety of signalling pathways. However, this property brings with it the problem of imparting specificity and discrimination. Recent studies are revealing the range of strategies utilized by the cyclases to solve this problem. Microdomains are a consequence of these

solutions, in which cAMP dynamics may differ from the broad cytosol. Currently evolving methodologies are beginning to reveal cAMP fluctuations in these various compartments.

Key words: adenylyl cyclase, calcium, cAMP, G-protein, protein kinase A (PKA), raft.

INTRODUCTION

The ubiquity of cAMP signalling in nature is associated with the evolution of three broad families of synthetic enzymes [1]. Organisms as diverse as *Paramecium*, *Euglena*, *Dictyostelium* and man have all developed the ability to convert ATP into cAMP [1,2]. Invasive micro-organisms, such as *Vibrio cholerae*, *Bordetella pertussis* and *Bacillus anthracis*, have also exploited the dependence of higher animals on this second messenger, either by synthesizing their own secreted form of the cyclases or various classes of activators of the enzyme in order to subvert the host's physiology. Cloning of mammalian adenylyl cyclases (ACs) had identified the currently known nine ACs by the mid 1990s. Their highly complex membrane topologies and duplicated catalytic domain are a striking structural fingerprint (Figure 1). The complex topologies of the ACs are a sharp contrast with guanylate cyclases, which catalyse a very similar reaction, but use only one transmembrane domain, although both enzymes dimerize to form a catalytic domain. {A soluble AC (sAC) has also been cloned that is directly stimulated by bicarbonate both *in vivo* and *in vitro* in a pH-independent manner. Although the initial isolation of sAC was from spermatozoa, sAC is also expressed in other bicarbonate-responsive tissues [3].}

Mammalian ACs vary significantly outside of their conserved catalytic domains [4]. This diversity underlies a broad range of individual regulatory susceptibilities. Immediately striking is the ability of ACs to be regulated by other signalling pathways, particularly Ca^{2+} . Although detailed structural information is now available on the catalytic region of AC, the features conferring individual regulatory properties are emerging slowly. Discrete subcellular targeting, as well as higher order assemblies of AC molecules, can occur. Both of these properties may play a role in the type-specific regulation of some ACs. It is also clear that PDEs (phosphodiesterases), the other side of the equation that establishes cellular cAMP levels, and PKA (protein kinase A/cAMP-dependent protein kinase), the enzyme that mediates most of the actions of cAMP, are similarly compartmentalized and involved in protein–protein associations [5–8]. A consequence of this situation is the likelihood that the concentration of cAMP will differ in the microdomain where it is synthesized from the broad

cytosol into which it can diffuse. New methods to measure cAMP in single cells are being developed that support this prediction. This review summarizes the current status of the major regulatory influences on AC and focuses on some of the mechanisms that facilitate this regulation. The compartmentalization of cAMP, which these factors lead to, and how it is observed is also considered.

DIVERSITY AND DISTRIBUTION OF ACs

The first mammalian AC was cloned in 1989 [9], following purification of the major enzyme activity from bovine brain. Eight more species were added over the next six years. Subsequently a soluble enzyme, sAC [3], which is not responsive to G-proteins or other regulators of the membrane-bound enzymes was discovered. Although this multiplicity of species was an initial surprise, different regulatory patterns coupled with selective tissue expression suggested underlying physiological rationales, which are still being unravelled. The specific patterns of expression of the ACs have been largely established; in some cases at the cellular level by elegant immunohistochemical analyses, in other cases at a more gross level by *in situ* hybridization or by PCR, as discussed in detail elsewhere [10–16].

Although most tissues and indeed many cell types express more than one AC species, some selective concentrations are notable. For instance, all of the Ca^{2+} /calmodulin-stimulated species are restricted to neuronal and secretory tissue (including hippocampus, cerebellum, pancreatic acinar and islet cells [17–19]). In addition, the Ca^{2+} -inhibited species, AC5, is at its highest levels in striatum and cardiac tissue [20–22] (see Table 1). As discussed below, subcellular localization probably provides a key device whereby differently regulated ACs can respond selectively within a single cell type.

REGULATORY PROPERTIES

Regulation by G-proteins

The discovery of multiple species of AC was quickly followed by the realization that these enzymes were multiply and uniquely

Abbreviations used: AC, adenylyl cyclase; $[\text{Ca}^{2+}]$, intracellular $[\text{Ca}^{2+}]$; CCE, capacitative Ca^{2+} entry; CNG, cyclic-nucleotide-gated; EGF, epidermal growth factor; PDE, phosphodiesterase; PKA, protein kinase A/cAMP-dependent protein kinase; AKAP, PKA-anchoring proteins; PKC, protein kinase C; PLC, phospholipase C; PSD, post-synaptic density; sAC, soluble AC; Tm, transmembrane domain; Trp, transient receptor potential.

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Figure 1 Structure of AC

ACs can be divided into 5 major domains: the N-terminus (Nt, red); the first Tm cluster (Tm1, black cylinders), the first cytoplasmic loop (C1, light blue), the second transmembrane cluster (Tm2, black cylinders) and the second cytoplasmic loop (C2, green). The C1 and C2 regions are further subdivided into the highly conserved catalytic C1a and C2a regions, which dimerize to form the catalytic site (stippled areas) and the less conserved C1b and C2b domains.

regulated. An early surprise was the fact that G-protein subunits exerted type-specific effects on ACs. This observation actually resolved two conflicting proposals for the mechanism of the inhibition of AC by G-proteins, which had been based on reconstitution assays of purified G-protein subunits with crude membrane sources of AC. The first proposal had posited that the inhibition of AC by G-proteins was due to the release of $\beta\gamma$ subunits from G_i/G_o , resulting in the chelation of $G\alpha_s$ and de-stimulation, as it were, with the $G_i\alpha/G_o\alpha$ subunit serving an indirect role as a repository of $\beta\gamma$ subunits. The second proposal held that $G_i\alpha$ subunits directly inhibited AC [23,24]. When defined AC species were used by over-expression in insect cell membranes, it turned out (fortunately) that both models could be correct, at least *in vitro*, and indeed were a subset of an even greater range of G-protein effects on cyclases. Thus some cyclases, e.g. AC5, are directly inhibitable by $G_i\alpha$ and some cyclases, e.g. AC2, are not; some species, e.g. AC1, are inhibited by both $\beta\gamma$ subunits and by $G_i\alpha$. Some cyclases are actually stimulated by $\beta\gamma$ subunits, e.g. AC2 and AC7 [25,26] (Table 1). (Whether $\beta\gamma$ subunits actually ever inhibit any AC by the mechanism originally proposed for G_i , i.e. chelation of α_s , is still not clear.)

Beyond the very simple level of how different cyclases are regulated by G-protein subunits, much remains to be discovered on the selectivity of individual cyclases for specific G-protein α and $\beta\gamma$ subunits. Selectivity is indicated from *in vitro* assays, but whether this is mirrored in actual cell systems is uncertain. For instance, purified AC5 and AC6 can be inhibited by all three $G_i\alpha$ subunits, whereas AC1 can also be inhibited by $G_o\alpha$ [27]. Similarly, selectivity for a $\beta_5\gamma_2$ combination over $\beta_1\gamma_2$ has been demonstrated in the activation of purified AC2 [28,29]. Participation by $G\alpha_{olf}$ in cyclase regulation may also extend beyond olfactory tissue. HEK 293 cells transfected with constitutively activated $G\alpha_{olf}$ (where $Gln^{214} \rightarrow Lys$) show a preferential activation of AC1 and AC8 over $G_s\alpha$ [30].

Some effort has now been undertaken to determine whether these more refined modes of regulation actually occur in native systems, since reconstitution studies, even between 'defined' components, must inevitably be subject to caveats regarding how the concentrations of reactants relate to the effective concentrations in native systems and whether there is in fact access be-

Table 1 Distribution and regulation of AC isoforms

Most abundant tissue distribution is indicated (see the text for reference details). *, Response to submicromolar $[Ca^{2+}]$; ?, not tested; %, inhibited by calcineurin.

Isoform	Distribution	Ca^{2+} *	$G\beta\gamma$	$G_i\alpha$
1	Brain	Stimulation	Inhibition	Inhibition
2	Lung, brain	None	?	None
3	Olfactory epithelia, pancreas	Uncertain	No effect	?
4	Widespread	None	Stimulation	None
5	Heart, striatum	Inhibition	No effect	Inhibition
6	Heart, kidney, widespread	Inhibition	No effect	Inhibition
7	Widespread	No effect	Stimulation	None
8	Brain, pancreas	Stimulation	?	Inhibition
9	Pituitary, widespread	None%	?	?
sAC	Testis, widespread	No effect	No effect	No effect

tween these components in native systems. [Even the term 'defined' must be used carefully in over-expression systems. We are never sure that the over-expression of one protein is without consequence for the expression of other proteins in the cell, with consequent accommodations for the outcome and interpretation of such experiments. This reservation can also be applied to knock-out or knock-in experiments (see below)]. In cerebral cortical tissue slices there had been a longstanding synergistic effect of α_1 - and β_1 -adrenergic receptors on cAMP accumulation [31], which could be reflective of a $\beta\gamma$ effect on AC2. In rat olfactory bulb a clear stimulation of AC2 or AC4 appears to arise from muscarinic receptor liberation of $\beta\gamma$ subunits [32]. Furthermore, μ -opiate receptors expressed in HEK 293 cells inhibit co-expressed AC5, but stimulate co-expressed AC7 [33]. This latter effect is muted by co-expression of $G_i\alpha$ (from transducin, as a $\beta\gamma$ 'sink'). The selective involvement of $G\alpha_{olf}$ in AC5 stimulation is indicated by the ablation in the striatum of $G\alpha_{olf}$ -deleted mice of the stimulatory effects of adenosine A2- and dopamine D1 receptors [34].

Regulation by Ca^{2+}

Stimulation

Shortly after the discovery of calmodulin as a Ca^{2+} -dependent regulator of PDE, it was recognized that AC activity in membranes prepared from brain tissue could also be stimulated by calmodulin. More correctly, of course, this was a stimulation by background Ca^{2+} that was mediated by calmodulin. The concentrations of Ca^{2+} eliciting this stimulation *in vitro* are those that correspond to those measured in the intact cell with Ca^{2+} -sensitive dyes upon activation of the PLC (phospholipase C) pathway or activation of voltage-gated Ca^{2+} channels [35]. As mentioned above, the first AC cloned was the Ca^{2+} /calmodulin-stimulated species, AC1, shortly followed by the similarly regulated AC3 and AC8 [36,37].

The Ca^{2+} /calmodulin-stimulated ACs differ in their sensitivities to Ca^{2+} *in vitro*. In fact, AC3, which is most closely related to AC1 and AC8, is far from being proven to be unambiguously stimulated by Ca^{2+} /calmodulin [38]. In one report, AC3 was not stimulated by Ca^{2+} /calmodulin in the absence of other effectors, but was stimulated by Ca^{2+} /calmodulin in the presence of either 5-guanylimidodiphosphate or forskolin *in vitro*. The EC_{50} value for free $[Ca^{2+}]$ of AC1 and AC3 are 0.05 μM and 5.0 μM Ca^{2+} respectively [39]. AC1 and AC8 can both be stimulated by Ca^{2+} /calmodulin in the absence or presence of forskolin with EC_{50} values of approx. 0.1 μM and 0.5 μM [38].

Figure 2 Responses of ACs to Ca^{2+}

Representation of the range of responses evoked by Ca^{2+} in representative tissues that express Ca^{2+} -stimulatable, -insensitive or -inhibitable AC. Note that regardless of the response to Ca^{2+} at submicromolar concentrations, all cyclases show inhibition at supramicromolar concentrations [40].

Inhibition

Ca^{2+} also inhibits the activity of AC in many tissues. This inhibition takes two forms: either low affinity which is seen in all tissues, or both low and high affinity, which is seen in selected tissues such as cardiac, anterior pituitary, striatum, and a variety of cell lines [22,40–43]. Inhibition of AC by Ca^{2+} does not require calmodulin (see below). The cloning of ACs in the early 1990s yielded what are now distinguished as Ca^{2+} /calmodulin-stimulated (AC1, AC3 and AC8), high affinity Ca^{2+} -inhibited (AC5 and AC6) and Ca^{2+} -insensitive, but PKC (protein kinase C)-stimulated ACs (see below; AC2, AC4 and AC7; Figure 2) [44].

Both the high affinity inhibition by Ca^{2+} and the calmodulin-dependent stimulation of ACs occur at concentrations that are achieved in cells upon the physiological elevation of $[\text{Ca}^{2+}]_i$ (intracellular $[\text{Ca}^{2+}]$), as measured by fura 2. Considerable effort has been put into proving that hormones that elevate $[\text{Ca}^{2+}]_i$ can inhibit cAMP-accumulation cyclases by this mechanism (e.g. [45,46]). One alternate mechanism was the stimulation of PDE activity by an elevation of Ca^{2+} in 1321 glioma cells [47]. However, a considerable body of data has now accumulated from a variety of systems which shows that the anticipated stimulation and inhibition of these cyclases occurs as a direct result of the elevation of $[\text{Ca}^{2+}]_i$ [18,19,48–56].

Regulation by capacitative Ca^{2+} entry

One of the more remarkable properties of Ca^{2+} -sensitive ACs is their regulation by CCE (capacitative Ca^{2+} entry), i.e. the entry of Ca^{2+} that is triggered by depletion of Ca^{2+} stores. A component of establishing the Ca^{2+} sensitivity of these enzymes *in vivo* was by assessing their responses to CCE. This simple, but readily interpretable, measure of Ca^{2+} sensitivity circumvents many of the steps required to exclude other mechanisms when a hormone is used to elevate $[\text{Ca}^{2+}]_i$. When expressed in HEK 293 cells, putatively Ca^{2+} -sensitive ACs give rise to the anticipated changes in cAMP levels in response to CCE [57]. (AC3 is not stimulated by CCE [38]). Remarkably, however, when regulation of these cyclases, either endogenously or exogenously expressed, by CCE

is compared with other means of elevating $[\text{Ca}^{2+}]_i$, [i.e. (i) release from intracellular stores, (ii) ionophore-mediated entry through the plasma membrane and (iii) arachidonic acid-mediated entry], only CCE is effective [38,58,59]. Such selectivity has since been confirmed in a number of other systems [19,51,52,55,60]. The available evidence suggests a very close apposition between Ca^{2+} -sensitive ACs and CCE channels [58,61,62]. The basis for this co-localization is explored below.

Regulation by PKC

Another consequence of activating the phosphoinositide signalling pathway, PKC activation, stimulates the Ca^{2+} -insensitive ACs, AC2, AC4 and AC7. The most common means of activating transfected ACs was by phorbol esters (e.g. [63,64]). Levin and Reed [65] identified a small region near the C-terminus as the site of action of PKC on AC2. Effectively, therefore, all cyclases are potentially subject to regulation by one or other arm of the PLC pathway. Although, regulation by $[\text{Ca}^{2+}]_i$ clearly does occur, fewer studies have addressed PKC regulation in response to hormones. However, in pancreatic islets a clear stimulation of cAMP accumulation is due to the activity of muscarinic and cholecystokinin receptor activation of PKC [18] and in HL-60 cells by P2Y_{11} purinoceptors [66]. In one study, a selective role for PKC δ has been seen in activating AC7 [67].

Type-specific regulation

The cyclases can be broadly subclassified based on their relatedness, which correlates with their responsiveness to Ca^{2+} and PKC. However, even within subclasses, the cyclases are sufficiently different to allow for some individual regulation. Selected examples are briefly outlined below. Further examples will undoubtedly be uncovered.

Regulation of AC6 by PKC

It seems as though both arms of the phosphoinositide signalling pathway are targeted to inhibit AC6 activity [68]. Chern and colleagues have shown that PKC activation inhibits AC6 activity through phosphorylation of the N-terminus [69]. N-terminal deletion of AC6 (1–86) or mutation of Ser¹⁰ → Ala reduced the PKC-mediated inhibition and protein phosphorylation. More recent studies show a complete loss of PKC-mediated inhibition of AC6 by mutating two of the three potential phosphorylation sites in the N and C1 region (Ser¹⁰, Ser⁵⁶⁸, and Ser⁶⁷⁴) or a single mutation in the C2 region (Thr⁹³¹) [70].

Regulation of AC5 and AC6 by PKA

Iyengar and colleagues have shown that a component of hormone-induced desensitization of the AC system occurs at the level of the cyclase molecule itself, rather than at the level of the receptor, where PKA-mediated phosphorylation reduces responsiveness [71]. This finding was consolidated by Ishikawa and colleagues, showing that PKA directly phosphorylates AC5, resulting in reduced activity [72].

Receptor-tyrosine-kinase modulation of AC5 and AC6

EGF (epidermal growth factor) increases AC activity and elevates cAMP accumulation in cells expressing rat and canine AC5, but not those over-expressing AC1, AC2 or AC6. The EGF stimulation

requires $G_s\alpha$, a finding that is consistent with previous results concerning EGF effects on cardiac AC (presumably AC5) [73,74].

Receptor-tyrosine-kinase activation with insulin-like growth factor-1 and tyrosine phosphatase inhibition with sodium orthovanadate increased AC6 activity and serine phosphorylation. Mutation of serine residues at positions 603 and 608 or 744, 746, 750 and 754 in the C1 region attenuated both the tyrosine-kinase-mediated enhancement of enzyme phosphorylation, as well as the sensitization of function [75].

NO

NO donors inhibit hormone- and forskolin-stimulated AC activity in purified plasma membrane preparations from N18TG2 neuroblastoma cells, which, as judged by Northern blot analysis, predominantly express AC6. Reducing agents reverse the inhibition, which implicates a cysteine residue(s) as the target for NO and an S-nitrosylation as the underlying modification [76,77]. In one comparative study of AC1, AC2, AC5 and AC6 expressed in insect cells, this effect was selective for AC5/AC6 [78], although an earlier report had indicated that AC1 (expressed in HEK 293 cells) could also be inhibited [79]. The full significance of the effects of NO on cyclases remains to be determined, although it could provide a basis for the old Yin and Yang hypothesis, which proposed that cGMP levels went up as cAMP went down (given that NO stimulates guanylate cyclase), in addition to mechanisms relying on cGMP stimulation of cAMP PDE activities (see below) [80].

Calcineurin and AC9

AC9 is not a very active cyclase; it is somewhat unresponsive to forskolin and activity overall is rather low. Nevertheless, uniquely, Ca^{2+} /calcineurin inhibits AC9 over the same Ca^{2+} concentration range at which Ca^{2+} stimulates AC1 via calmodulin. These calcineurin effects were deduced by the use of calcineurin inhibitors [81–83].

Calmodulin kinase and AC3

Although $[Ca^{2+}]_i$ when elevated by CCE does not affect AC3 in transiently transfected HEK 293 cells [38], treatment of HEK 293 cells stably expressing AC3 with ionophore causes a profound inhibition of cAMP accumulation [84]. This effect reflects an apparently unique sensitivity of AC3 to inhibition by calmodulin kinase II. The inhibition is antagonized by an inhibitor of calmodulin kinases. Co-expression of constitutively activated calmodulin kinase II completely inhibits isoprenaline-stimulated AC3 activity [85]. Mutagenesis of a calmodulin kinase II consensus site (Ser¹⁰⁷⁶ → Ala) in AC3 greatly reduces Ca^{2+} -stimulated phosphorylation and inhibition of AC3 *in vivo* [86].

Membrane potential

The initial discovery that ACs superficially resembled ion channels prompted speculation on whether they might also function as ion channels or transporters [9]. This speculation was supported by the observation that a hyperpolarization-activated K^+ efflux appeared to directly regulate AC activity in *Paramecium in vivo*. [87]. Support for a similar phenomenon in cerebellar neurons came from the observation that AC activity was synergistically stimulated by depolarization with high KCl and β -adrenergic receptor activation in the absence of extracellular Ca^{2+} [88]. A later study showed that this effect was due to the entry of

Na^+ ions through L-type channels in the absence of Ca^{2+} , with no dependence on membrane potential other than the depolarization required to open L-type channels. Gramicidin, which generates pores that are permeable to monovalent cations, and concurrently eliminates the membrane potential, caused a similar stimulation by extracellularly applied Na^+ [89]. At present, the complex membrane topology remains a tantalizing puzzle, whose function may lie in its affiliations with the ATP-binding cassette superfamily and may yet reveal associations with other membrane proteins (see below).

STRUCTURAL PROPERTIES

Catalytic domain

A remarkable feature of ACs is the fact that the catalytic domains, which reside in the cytosol, can be expressed independently of the rest of the molecule. This has allowed the expression of the considerable amounts of protein necessary for the determination of its crystal structure. This structure has revealed the atomic basis of AC catalytic activity ([90–92], but see [92a]). Despite no obvious sequence similarity, AC is strikingly similar at the structural level to DNA polymerase. This is understandable in light of the fact that both enzymes catalyse a rather similar reaction: the formation of an intramolecular 3'–5' diester bond in the case of AC and an intermolecular 3'–5' diester bond in the case of the DNA polymerase [91]. Both enzymes catalyse the attack of the 3' hydroxy of ribose on the phosphate of a nucleoside triphosphate. The common structural motif is a 'palm' domain comprising a $\beta\alpha\beta\beta\alpha\beta$ motif. Two invariant aspartic acid residues that co-ordinate two catalytic metal ions occur at conserved topological locations within this motif (Figure 3, left-hand panel). The C_{1a} and C_{2a} domains of AC also contain such a motif, but only C_{1a} bears the analogous aspartic acids. Mutation of the two aspartic acids in the C_{1a} of AC not surprisingly eliminates catalytic activity. Indeed mutation of nearby amino acids, which impinge on the ability of those aspartates to co-ordinate metal ions, also has severe consequences [93].

Although both are presumably occupied by Mg^{2+} in the cell, the specificity of the two metal sites for Mn^{2+} or Zn^{2+} , which have the same charge and similar ionic radii, probably reflects the number and stereochemistry of the co-ordinating ligands. Accordingly, Zn^{2+} , which readily accommodates four ligands, preferentially binds at site A, whereas Mn^{2+} , which prefers octahedral coordination, binds at site B. Mn^{2+} is an activator of AC, whereas Zn^{2+} is a low affinity inhibitor [91]. As discussed below this latter site may be of most relevance to high affinity inhibition by Ca^{2+} .

Fine structural information is also available on the mechanism of activation by $G_s\alpha$ and $G_i\alpha$. This has been discussed in detail elsewhere [90,94–97], but in brief, $G_s\alpha$ plays the role of helping the catalytic domain collapse around the substrate, whereas $G_i\alpha$ maintains the catalytic domain in an open conformation.

Structural basis for Ca^{2+} inhibition

Information on the structural determinants for isotype specific regulation is limited, probably due to the number of cyclases, coupled with the number of individual forms of regulation and the paucity of investigators exploring this aspect. However, the mechanism for high affinity inhibition by Ca^{2+} appears to be nearing resolution. AC5 and AC6 uniquely display high affinity inhibition by Ca^{2+} , whether expressed heterologously or in the tissues where they occur at high levels, e.g. striatum, cardiac tissue, anterior pituitary and cell lines derived therefrom [22,40–42]. Studies by Guillou et al. [40] suggested that both high and low

Figure 3 Structure of the catalytic domain of AC and location of amino acid mutations

Left panel: the forskolin-binding site and active site of AC. The active site occurs at the interface of the C1a and C2a subunits of AC, which are represented in dark and light blue respectively. The structure is that of the complex of ACV-C1a and ACII-C2a. Forskolin, shown in stick representation and in red, binds in the cleft that contains the active site. The ATP-analogue (2',3'-dideoxy 5'-ATP; DAD, in yellow) and two Mg²⁺ ions (represented as orange spheres) are bound in the AC active site in this crystal structure. Right panel: location of the mutated amino acids. Ribbon representation of the active site and location of the four 'active-site' mutations. The two Mg²⁺ ions are displayed as orange spheres, and DAD and the amino acids which are mutated are shown in stick representation. Each residue is coloured by atom (yellow, carbon; blue, nitrogen; red, oxygen; pink, sulphur). Each of the four mutations occurs proximal to the Mg²⁺-binding sites. Cys⁴⁴¹ and Tyr⁴⁴² are the two residues just after Asp⁴⁴⁰, which is of prime importance for Mg²⁺ binding and AC activity. The Arg⁴³⁴ → Ser and Phe⁴²³ → Leu mutations are located more distally from the active site. Both residues contact Tyr⁴⁴², suggesting that these mutations might transmit their effects on Mg²⁺-activation through this residue. (Reproduced with permission from Hu, B., Nakata, H., Gu, C., De Beer, T. and Cooper, D. M. (2002), *J. Biol. Chem.* **277**, 33139–33147, © The American Society for Biochemistry & Molecular Biology).

affinity inhibition by Ca²⁺ reflected a competition by Ca²⁺ for activation by Mg²⁺. Chimeras constructed between AC5 and AC2 pointed to the catalytic domain as the region responsible for high affinity inhibition [98]. A series of mutants had been constructed around the catalytic domain of AC5, which differed in their sensitivity to Mg²⁺ [93]. The X-ray crystallography structure showed that these mutants would have been expected to perturb, to varying extents, the ability of the key aspartate residues to chelate Mg²⁺ (Figure 3, right-hand panel). Thus when it turned out that the ability of Ca²⁺ to inhibit these mutant forms was directly correlated to their sensitivity to Mg²⁺, the simplest explanation for inhibition by Ca²⁺ was that it occurs at the catalytic site at some conformation of the Mg²⁺ site(s) [98]. It is slightly surprising that this inhibition should reside in the catalytic domain, since this domain is highly conserved among ACs, regardless of their response to Ca²⁺. However, it should be noted that there are sufficient differences in the immediate environment of the catalytic domain between different species that it would take detailed crystallographic analysis to resolve this issue.

Structural basis for stimulation by Ca²⁺/calmodulin

Two different approaches have been used to identify the calmodulin regulatory sites on AC1 and AC8. For AC1, putative

calmodulin-binding sites were identified based on sequence analysis. A peptide corresponding to part of the C1b region inhibited the regulation mediated by calmodulin and subsequently mutation of some amino acids in this region eliminated the stimulation by Ca²⁺/calmodulin [99,100]. For AC8, calmodulin-binding sites were identified by I¹²⁵-labelled calmodulin binding. Deletions of putative calmodulin-binding sites and peptide competition assays led to the identification of two sites for calmodulin binding, one at the N- and one at the C-terminus. The site at the C-terminus was critical for regulation of AC8 by Ca²⁺/calmodulin in assays *in vitro* [101]. The mechanism proposed for the regulation by calmodulin was a dis-inhibition of the catalytic site upon Ca²⁺ binding to calmodulin. Such a mechanism is commonly encountered with Ca²⁺/calmodulin-regulated targets. It is even possible that both binding sites bind calmodulin co-operatively, rather analogous to the two sites on phosphorylase kinase [102]. Although the sequences of AC1 and AC8 are sufficiently different to permit different modes of regulation by Ca²⁺/calmodulin, it is tempting to seek for a conserved regulatory mechanism that can reconcile these disparate findings. Conceivably, the binding at the N- and C-terminus are the primary binding sites for calmodulin, whereas the sequences in the C1b domain are required to translate the effect into catalytic consequences, since indeed it is a little difficult to envisage a bulky molecule like calmodulin being accommodated

near to the plasma membrane with the bulk of the dimerized catalytic domain in the way.

Quaternary structure

As with other members of the ATP-binding cassette superfamily, a degree of ordering is anticipated for the complex membrane topology of ACs. Irradiation inactivation analysis performed over 20 years ago suggested that the catalytic unit of AC would exist as a dimer [103]. More recent studies have shown that the two transmembrane domains (Tm1 and Tm2), independently of any cytosolic region, are brought together in the endoplasmic reticulum to both promote the association of the two catalytic domains and to allow the delivery of the AC to the plasma membrane [104,105]. It also appears clear that Tm2 is divalent, such that even when binding Tm1, a second Tm2 can associate, which results in full-length dimers [106]. The functional significance of these dimers is not clear, but the possibility has been debated that higher order assemblies of AC may provide one mechanism for ensuring co-localization with critical regulatory partners [106].

COMPARTMENTALIZATION OF ACs

The susceptibility of ACs to multiple modes of regulation raises the possibility that cAMP levels will be elevated globally in response to a wide array of stimuli. This may be desirable in certain situations, e.g. as separate responses to growth factors, neurotransmitters and hormones, reflecting different responses of the organism to changing environmental or other situations. However, mechanisms such as compartmentalization have evolved to sequester advantageous reactants together and to exclude others in order to establish particular regulatory contexts [107]. One example of co-localization of interacting reactants is the association of Ca^{2+} -regulated cyclases with CCE. As mentioned above, in this situation, Ca^{2+} -regulated cyclases respond only to CCE and not to non-CCE, ionophore-mediated Ca^{2+} -entry or release from stores. At least part of this co-localization rests on a residence in lipid rafts. An additional (not necessarily exclusive) layer of co-localization involves scaffolding proteins.

Localization of ACs in caveolae and rafts

The fluid mosaic model for the plasma membrane emphasized the diffusional mobility of membrane lipids and proteins, and the consequent lack of lateral organization of bilayer membranes [108]. This model was a central feature of the 'collision coupling' model of hormone-receptor-G-protein interaction. It is now becoming apparent that cell surface membranes are far more heterogeneous, comprising 'microdomains' of different lipid and protein composition. Such microdomains may be of profound regulatory significance in allowing interacting proteins to be concentrated [109]. These domains can be up to hundreds of nanometres in diameter [110], and it has been estimated that they can contain no more than 15 protein molecules [111], although much remains to be known on their degrees of complexity and homogeneity. The microdomains are commonly termed lipid rafts, of which caveolae are a subset. Their characteristic feature is an enrichment of cholesterol and sphingolipids in the outer leaflet of the bilayer [112,113]. The dominance of saturated and therefore extended hydrocarbon chains in sphingolipids allows the cholesterol to be rendered rather immobile. The regulatory significance of rafts lies in the fact that they may selectively concentrate or exclude proteins. G-protein-coupled receptors, glycosylphosphatidylinositol-anchored proteins, the α subunits

of G-proteins and transmembrane proteins, including AC, are all reported to have been found in rafts [114,115]. Acylation can increase a protein's affinity for rafts, but this is not by itself sufficient for association [116]. Caveolae are an important subset of lipid rafts. These were first identified as cell surface invaginations by Palade and others in the 1950s. Caveolae are formed by polymerization of their characteristic marker protein, caveolin. These proteins also bind cholesterol. The functions of caveolae are still open to debate, but they have been implicated in endocytosis, transcytosis and in organizing signalling molecules [117].

Ca^{2+} -regulated ACs appear to exploit caveolae or rafts as part of their responsiveness to CCE. As discussed above, Ca^{2+} -sensitive ACs are much more selective for CCE rather than Ca^{2+} -entry by other means, or release from stores for their regulation [38,50,52,56–59,62,118–120]. A number of candidate CCE channels, Trp proteins (transient receptor potential proteins), have been found in caveolae [121]; endothelial Ca^{2+} waves originate in caveolin-enriched cell edges [122]. Thus rafts or caveolae might provide a means for concentrating the relevant factors. Rafts are commonly separated from the rest of the plasma membrane by exploiting their buoyancy on sucrose gradients. Endogenously expressed AC6 and transfected AC8 fractionated along with caveolin on such gradients [119]. Similarly, the endogenous AC5/AC6 and β 2-adrenergic receptor of cardiac myocytes co-fractionated with caveolin on such gradients [123,124]. In fact, at the risk of being premature, it appears as though Ca^{2+} -sensitive ACs, either endogenously expressed or transfected, (at least AC3, AC5, AC6 and AC8) seem to be restricted to rafts, whereas Ca^{2+} -insensitive ACs (AC2, AC4 and AC7) are excluded [119,125,126]. Although it is an obvious convenience to concentrate interacting regulatory elements in such microdomains, it is not absolutely clear that the relatively crude means for resolving such domains currently available allow a clear resolution of their roles. For instance, it is conceivable that although regulatory elements co-elute, they are actually not present in the same rafts. Co-immunoprecipitation is commonly used to assess interactions in lipid domains, but here again it is possible that this may be an immuno-isolation of lipid domains, rather than a measure of protein-protein interactions. Nevertheless, there have been suggestions that AC5 is directly localized to caveolae by an interaction with caveolin [127–129].

Another tool that is used to probe the importance of rafts in maintaining regulatory assemblies is methyl- β -cyclodextrin, a compound that extracts cholesterol from the plasma membrane and by such means disrupts rafts. Methyl- β -cyclodextrin treatment perturbed the stimulation of PLC by G_q -linked G-protein-coupled receptors [130]. Similarly, methyl- β -cyclodextrin treatment disrupted the regulation of AC8 by CCE, as well as its buoyancy on sucrose gradients. Restoration of cholesterol re-established the regulation [119]. The possibility has also been considered that the presence of AC8 in caveolae/rafts might have a similar underlying mechanism to that of eNOS (endothelial NO synthase). eNOS is myristoylated and confined to caveolae by binding to caveolin [131]. Upon the elevation of $[\text{Ca}^{2+}]_i$, calmodulin displaces the caveolin and activates the enzyme. Since AC8 also occurs in caveolae/rafts and possesses an N-terminal calmodulin-binding site that is not required for *in vitro* regulation by Ca^{2+} [101], a similar targeting role might be envisaged. It turns out that, although deletion of the N-terminal calmodulin-binding site muted the susceptibility of AC8 to CCE, this did not result in the movement of AC8 from rafts [125]. The possibility should not be discounted, however, that AC8 was moved to a different population of rafts not in association with CCE channels, although it seems more likely that the N-terminus

Figure 4 Co-localization of AC with CCE channels in caveolae and rafts

The selectivity of ACs for the source of Ca^{2+} to which they respond is indicated. Limits to diffusion of high concentrations of Ca^{2+} are illustrated by the blue shading. Ionomycin-mediated transmembrane Ca^{2+} movement is also suggested to originate from non-cholesterol-rich regions of the plasma membrane, since cholesterol incorporation into model liposomes inhibits Ca^{2+} movement [179]. Cholesterol-rich regions are indicated by the presence of yellow spheres. Ca^{2+} -insensitive (Ca-insens) ACs (green) are indicated to be excluded from rafts, whereas Ca^{2+} -sensitive (Ca-sens) ACs (red) occur in rafts along with CCE channels. ER, endoplasmic reticulum; IP3R, inositol 1,4,5-trisphosphate receptor; PM, plasma membrane.

is required for protein–protein interactions involved in the CCE regulation of ACs (Figure 4).

In neuronal tissue, another mechanism for concentrating ACs may be by constraining them to PSDs (post-synaptic densities). PSDs do have unique lipid composition and an ability to concentrate signalling molecules. They also contain scaffolding molecules, such as PSD-95, whose lipid solubility is crucial to their targeting [132–134]. ACs have been observed to be enriched in PSDs in the hippocampus and the dendritic spines of cerebellar granule cells [89,135].

Scaffolding proteins

Apart from the somewhat loose constraints imposed by co-expression in lipid microdomains, a far more organized compartmentalization can be achieved by scaffolding proteins. The best known is the constraint of the cAMP target (PKA) by AKAPs (PKA-anchoring proteins), which are proteins with two important structural motifs; one to bind the regulatory subunits of PKA and the other a targeting domain to bind to another protein or some subcellular organelle [7,136,137]. Although regulatory assemblies, including AC, have been implied, e.g. with the β -adrenergic receptor, L-type Ca^{2+} -channel, protein phosphatase 2A and with K^{+} -channels [138–141], direct binding of ACs to any scaffolding protein has still not been demonstrated. The case for the involvement of PDEs in such assemblies has been made far more clearly, particularly for PDE4. To date, eleven families of PDEs have been identified following purification and by cloning

[142,143]. These PDEs are diversely regulated, like ACs, and much is known about their targeting and inhibition by selective compounds. Perhaps because they are largely soluble or quasi-soluble proteins (without Tm spanning motifs), their domain structure has been clarified. Thus, for instance, calmodulin-binding domains, membrane-targeting domains and β -arrestin-binding sites have all been identified. A comparable degree of detail should be anticipated for the ACs, given that the cAMP-synthesizing enzymes should be no less organized than the degradative enzymes. Additionally Ca^{2+} -signalling complexes such as the InaD system, which include the CCE candidate proteins, Trp and Trpl [144,145], would seem to predict similar organization for Ca^{2+} -targets such as AC, given the close apposition of ACs and CCE channels.

LOCAL cAMP

cAMP microdomains

PKA has numerous targets within the cell, which ideally should be selectively activated in response to differing stimuli. A consequence of the compartmentalization of AC is the likelihood that cAMP produced in the microdomain of a cyclase may not faithfully reflect cAMP within the cytosol of cells. Indeed, evidence that compartments of cAMP existed in myocytes had been advanced 20 years ago. Buxton and Brunton [146] showed that in ventricular myocytes equivalent elevations of cAMP and PKA achieved by PGE1 (prostaglandin E1) and isoprenaline

elicited quite disparate effects on glycogen phosphorylase activation. Later, Jurevicius and Fischmeister [147] showed that local elevations in cAMP modulated the activity of local, but not distant Ca^{2+} -channels; inclusion of PDE inhibitors eliminated the barriers to the diffusion of cAMP. As discussed above, mechanisms are in place for containing cAMP signalling, i.e. with scaffolding proteins for PKA and PDE and PP2A (protein phosphatase 2A) (and presumably AC), along with some supporting role for lipid domains [5,7,148]. Indeed one does not wish to suggest that these are mutually exclusive devices; there are clear examples of scaffolded proteins also having selective lipid associations, e.g. PDE4. Another instance which links both scaffolding proteins and caveolae is the binding and inhibition of PKA by caveolin [149].

Measuring cAMP

Given the experimental evidence and teleological rationalization for the existence of microdomains of cAMP, a number of methods have been developed to resolve cAMP with some spatial and temporal fidelity. Kramer and colleagues have pioneered the use of 'patch cramming'. This elegant method places a patch pipette containing a cAMP- or cGMP-gated ion channel in a membrane patch into a recipient cell, where the channel measures the intracellular messenger locally [150,151]. A somewhat less technically demanding method was by the microinjection into cells of fluorescently labelled PKA subunits, and measuring the changes in fluorescence resonance energy transfer in response to cAMP [152,153]. This method has since been modified to use genetically encoded cyan-fluorescent-protein- and yellow-fluorescent-protein-tagged PKA subunits, so that only transfection of cells is required to deliver the sensor [154]. Valuable information has been derived from both variants of this method [147,155,156]. Serendipitously, although the sensor might only be expected to measure cAMP in the cell cytosol, because it is a modified PKA, it can be bound by AKAPs and evidence of the measurement of cAMP in compartments in myocytes has been obtained [156]. Endogenous CNG (cyclic-nucleotide-gated) channels have also been used to measure cAMP in neurons [157,158]. High concentrations ($> 25 \mu\text{M}$) were encountered, consistent with the presence of a microdomain of high cAMP around the channel. Olfactory CNG channels encoded in adenovirus have been used to measure cAMP in a variety of cell types. Because these channels are non-selective cation channels, either Ca^{2+} influx or Na^{+} current can be used to measure cAMP by fluorimetric or electrophysiological means respectively [159,160]. The expressed channel and the endogenous AC seem to be close, since the modest $[\text{Ca}^{2+}]_i$ rise due to activation of the channel can regulate cAMP accumulation [161]. Application of this methodology provides convincing evidence for regions of high cAMP in the domain of the channel, and in HEK 293 cells, for the close apposition of a PDE4 activity with the channel, and by implication with the cyclase (Figure 5) [159,162]. Each of these methods has limitations, strengths and discrete applications. It seems clear that we are very close to being able to assess cAMP dynamics in discrete cellular domains.

FUTURE DIRECTIONS – OUTSTANDING QUESTIONS

The importance of the susceptibility to specific regulation of AC isoforms in particular physiological contexts is one of the most obvious questions confronting research in this area. There are a few situations where there is a semblance of understanding, but in general this is a huge black box. Reservations about the genetic background or cryptic consequences to gene disruption

experiments notwithstanding, a number of apparently straightforward observations have been made from such studies. For instance, from knockout studies, the Ca^{2+} /calmodulin-stimulated AC1 and AC8 clearly play a key role in the mouse model of learning and memory [163–168]. In addition, anosmia is observed in AC3 knockout mice [169], which correlates with the role predicted for AC3 after its initial discovery in olfactory epithelium [36]. Slightly conflicting findings were encountered in two studies of striatal AC5-knockout mice. In one study, motor activity was not disturbed [170], whereas in another the anticipated motor dysfunction was observed [171], although in both cases hyper-responsiveness of locomotion to dopamine D1 stimulation was observed. Apparently, genetic background can play a significant role in the outcome of some such knockout experiments.

Over-expression is another approach to probe the physiological roles of specific cyclases. For instance, the Ca^{2+} inhibatability of AC6 of pulmonary artery endothelial cells appears to play a key role in the maintenance of endothelial barrier formation [55]. In another study, over-expression of an AC8 in mice increased cardiac contractility [172]. Beyond these few examples, much remains to be learned on the underlying rationale behind the expression of the cyclases in particular contexts.

A major gap in our knowledge of cyclase regulation concerns the targeting mechanisms that are utilized. There seems little doubt that cyclases are targeted to specific parts of the cell, along with other regulators. Nevertheless, we are on the threshold of discovering the details. Doubtless, scaffolding proteins, lipid environments and probably ultrastructural associations are involved.

Much also remains to be known on the structural aspects, at the very fine level, responsible for rendering some cyclases inhibitable, or not, by Ca^{2+} and at the cruder level for type-specific regulation by other factors. At an even cruder level, why do cyclases look like transporters? Do they extrude cAMP? This issue has been considered and apparently discounted, but that ungainly structure remains an abiding puzzle.

Oscillations in cAMP have been predicted to occur as a result of either the feedback between a Ca^{2+} -inhibitable AC and the voltage-gated Ca^{2+} channel that it modulates in cardiac tissue [44,173], or a variety of intersecting regulatory elements [174, 175]. The development of better single cell methods, as described above, may lead to clarification of this issue. Of course, whether or not oscillations in cAMP occur, the major issue, and perhaps even more challenging technically, is whether any aspect of cellular regulatory elements, be they PKA, CNG channels or the EPAC (exchange protein directly activated by cAMP) proteins [176], are capable of responding to such oscillations, rather than simple amplitude changes in cAMP levels.

A final issue, which perhaps raises the greatest technical challenges, are the types of Ca^{2+} signal to which the Ca^{2+} -sensitive cyclases respond. It is clear that in assays *in vitro* they can respond to a constant level of Ca^{2+} , but in the intact cell we have seen that they respond selectively to CCE in non-excitabile cells and to voltage-gated Ca^{2+} -channels in excitable cells. Given their co-localization with these channels, is it possible that they are responding to the spikes of Ca^{2+} that arise upon the opening and closing of ion channels, so that it is not the steady state level of Ca^{2+} , but the occasional bursts in Ca^{2+} to which they respond. Could it be that the binding of Ca^{2+} to calmodulin is initiated by a Ca^{2+} transient, which then results in a transient activation of AC activity? Obviously in a population assay of cAMP accumulation over a protracted time period it is not possible to discern such sensitivities. Preliminary evidence in support of a sensitivity of AC1 to Ca^{2+} transients was provided by Abrams and colleagues [177,178].

Figure 5 Representation of a microdomain

Some of the elements involved in generating a micro-environment of cAMP that differs from the overall cytosol. An internal physical barrier to the diffusion of cAMP is indicated, since higher concentrations of cAMP are reported in the vicinity of the CNG channel than are measured conventionally in the cytosol [159]. This barrier may be comprised of apparatus associated with scaffolding proteins or parts of the endoplasmic reticulum juxtaposed near Ca^{2+} -entry sites and ACs [58]. In addition a PKA-stimulated PDE is proposed to be juxtaposed near the AC [7], resulting in different [cAMP] dynamics in the microdomain (1) compared with the cytosol (2). Insets: y-axis, [cAMP]; x-axis, time in arbitrary units.

Overall the questions facing the cyclase field are daunting from a technical viewpoint. However, the complexity of the questions that can now be phrased reveals, not only how far the field has moved in the short time since the cloning of the mammalian cyclases, but also the range of therapeutic opportunities that present themselves.

I am indebted to Dr Kent Fagan for many valuable discussions and help with an early draft of the manuscript. The work in the D. M. F. C.'s lab is supported by the National Institutes of Health and the Wellcome Trust.

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Received 15 July 2003/7 August 2003; accepted 26 August 2003

Published as Immediate Publication 26 August 2003, DOI 10.1042/BJ20031061