Molecular and cellular requirements for the regulation of adenylate cyclases by calcium

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Abstract

Calcium-sensitive adenylate cyclases provide a key regulatory device for integrating the activities of the two major signalling systems, Ca2+ and cAMP. Recent experiments have brought us closer to understanding the molecular mechanisms whereby Ca2+ either stimulates or inhibits susceptible adenylate cyclases in vitro. However, in the intact cell an additional layer of sophistication is evident whereby Ca2+-sensitive adenylate cyclases are juxtaposed with Ca2+-entry channels, such that the cyclases respond selectively to capacitative Ca2+ entry. Part of this dependency is enforced by the placement of Ca2+-sensitive adenylate cyclases (AC5, AC6 and AC8) in caveolae, from which at least one Ca2+-insensitive adenylate cyclase (AC7) is excluded. However, additional protein–protein interactions are also required to ensure the dependency of these cyclases on capacitative Ca2+ entry. Recent findings in this area and their implications for ‘local cAMP signals’ will be discussed.

Introduction

Mammalian adenylate cyclases vary significantly outside of their largely conserved catalytic domains. This diversity underlies a broad range of individual regulatory properties. Detailed structural information is available on the catalytic region of adenylate cyclase, but the properties conferring individual regulatory properties are only emerging slowly. The type-specific regulation of adenylate cyclases is augmented by higher-order assemblies as well as discrete subcellular targeting. It is also clear that phosphodiesterases – the other side of the equation that establishes cAMP levels – are also diverse, compartmentalized and involved in protein–protein associations [2,3]. A consequence of the organization of cAMP signalling complexes is the likelihood that the concentration of cAMP differs in the microdomains where it is synthesized from the broad cytosol into which it can diffuse. Newly developed methods for the measurement of cAMP in microdomains may allow a dissection of the factors contributing to the organization of these domains.

Diversity and distribution of adenylate cyclases

Since the first mammalian adenylate cyclase was cloned in 1989 [4], eight other species have been identified. Although this multiplicity of species was an initial surprise, different regulatory patterns coupled with selective tissue expression suggested underlying cellular rationales, which are still being unravelled [5]. Although most tissues, and indeed many cell types express more than one adenylate cyclase species, some selective enrichments are striking. For instance, the Ca2+-stimulated species principally occur in neuronal tissue, as well as pancreatic tissue [5,6]. In addition, the Ca2+-inhibited species, AC5 (adenylate cyclase type 5), is at its highest levels in striatum and cardiac tissue [5,7]. In cardiac tissue it has been proposed that the susceptibility to inhibition by Ca2+ of the cyclases (AC5 and AC6) mediating the actions of sympathetic neurotransmission provides a key negative feedback device that contributes to cardiac rhythmicity [7]. Furthermore, knockout experiments have established an important role for the adenylate cyclases that are stimulated by Ca2+ acting by calmodulin (AC1 and AC8) in hippocampal learning and memory [8,9].

Regulation by calcium

Stimulation

Shortly after the discovery of calmodulin as a calcium-dependent regulator of phosphodiesterase, adenylate cyclase activity in membranes prepared from brain tissue was also seen to be stimulated by calmodulin (more correctly, of course, this was a stimulation by Ca2+ in the medium, that was dependent on calmodulin). The concentrations of Ca2+ eliciting this stimulation in vitro correspond to those achieved in the cell cytosol upon activation of the phospholipase C pathway or activation of voltage-gated Ca2+-channels. The first adenylate cyclase cloned was the Ca2+/calmodulin-stimulated species, AC1, shortly followed by the similarly regulated AC3 and AC8 [1].

The Ca2+/calmodulin-stimulated adenylate cyclases differ somewhat in their sensitivities to Ca2+ in vitro. In fact, AC3, while being most closely related to AC1 and AC8 at the amino acid level, is far from being proven to be stimulated
Schematic representation of the three types of response to Ca\textsuperscript{2+} of the cloned adenylate cyclases

Roman numerals correspond to adenylate cyclases (e.g. V is AC5).

Figure 1

Inhibition

Calcium also inhibits the activity of adenylate cyclase in many tissues. This inhibition takes two forms, either low affinity (approx. \( K_i \) value 10–25 \( \mu M \)), which is seen in all tissues, or both low and high affinity (approx. \( K_i \) value 0.2–0.6 \( \mu M \)), which is seen in selected tissues such as cardiac, anterior pituitary, striatum and a variety of cell lines [12]. Inhibition of adenylate cyclase by Ca\textsuperscript{2+} does not require calmodulin (see below). Cloning of the adenylate cyclases in the early 1990s yielded what are now distinguished as Ca\textsuperscript{2+}/calmodulin-stimulated (AC1, AC3 and AC8), high- (and low-) affinity Ca\textsuperscript{2+}-inhibited (AC5 and AC6) and Ca\textsuperscript{2+}-insensitive, but protein kinase C-stimulated (AC2, AC4 and AC7) adenylate cyclases (Figure 1) [1] (AC9, the ninth known adenylate cyclase, is a widely distributed, low-activity enzyme that is inhibited by calcineurin).

Both the high-affinity inhibition by Ca\textsuperscript{2+} and the calmodulin-dependent stimulation of adenylate cyclases occur at concentrations that are achieved in cells upon the physiological elevation of [Ca\textsuperscript{2+}]\textsubscript{free} (intracellular [Ca\textsuperscript{2+}]), as measured by fura-2. Considerable effort has been put into proving that hormones that elevate [Ca\textsuperscript{2+}] can regulate such cyclases by this mechanism – and none other. For instance, hormones that activate phospholipase C can thereby activate protein kinase C, which stimulates the activity of certain cyclases. Furthermore, the elevation of [Ca\textsuperscript{2+}] by carbachol stimulates phosphodiesterase (type I) and thereby inhibits cAMP accumulation in 1321 glioma cells [13]. However, insufficient data have now accumulated from a variety of systems to show that the anticipated stimulation and inhibition of these cyclases does occur as a direct result of the elevation of [Ca\textsuperscript{2+}] [10,14–18].

Regulation by CCE (capacitative Ca\textsuperscript{2+} entry)

One of the more remarkable properties of Ca\textsuperscript{2+}-sensitive adenylate cyclases is their virtual dependence on CCE for their regulation. A side effect of establishing the Ca\textsuperscript{2+} sensitivity of these enzymes in vivo was assessing their responses to CCE, i.e. the entry of Ca\textsuperscript{2+} that is triggered by depletion of Ca\textsuperscript{2+} stores. This simple but readily interpretable measure of Ca\textsuperscript{2+} sensitivity was chosen since it circumvents many of the steps required to exclude other mechanisms when hormone is used to elevate [Ca\textsuperscript{2+}]. When expressed in HEK-293 cells, putatively Ca\textsuperscript{2+}-sensitive adenylate cyclases give rise to the anticipated changes in cAMP levels in response to CCE [16] (AC3, although expressed at adequate levels, is not stimulated by CCE [11]). Remarkably, however, when regulation of these cyclases, either endogenously or exogenously expressed, by CCE is compared with other means of elevating [Ca\textsuperscript{2+}], 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 [11,19,20]. Such selectivity has since been confirmed in a number of other systems [6,14,18,21,22]. The available evidence suggests a very close apposition between Ca\textsuperscript{2+}-sensitive adenylate cyclases and CCE channels [19,23,24].

Structural properties

Structural basis for Ca\textsuperscript{2+} inhibition

A remarkable feature of adenylate cyclases is the fact that the catalytic domains, which are cytosolic, can be expressed independently of the rest of the molecule. This has allowed the expression of considerable amounts of protein, its purification and determination of its crystal structure. Elucidation of this structure has revealed the atomic basis of adenylate cyclase catalytic activity [25–27]. Solving the structure also made it possible to address the structural basis for Ca\textsuperscript{2+} inhibition. Early experiments had eliminated the obvious participation of readily dissociable calmodulin in the inhibition of adenylate cyclases by Ca\textsuperscript{2+}. More recently, chimaeras were made between AC5 and AC2 in an attempt at narrowing down the domains of AC5 that were responsible for its inhibitory sensitivity. Retention of the first catalytic domain of AC5 was sufficient to maintain a Ca\textsuperscript{2+}-sensitive adenylate cyclase [28]. This finding focused attention on the mechanism inferred from the crystal structure. Two aspartate residues play critical roles in co-ordinating two Mg\textsuperscript{2+} ions, which catalyse the nucleophilic attack of the 3′ hydroxyl of ribose on the \( \alpha \)-phosphate of ATP. Mutation of the two aspartic acids in the C\textsubscript{8} domain eliminates activity, as expected. However, mutation of more distal amino acids, which would be expected to impact on the precise orientation of those residues, did not significantly affect Ca\textsuperscript{2+} sensitivity.
aspartyl hydroxyl groups, had a more benign effect of
reducing the affinity of the adenylate cyclase for Mg\(^{2+}\). These
mutations turned out to have decreased sensitivity to Ca\(^{2+}\)
inhibition. The simplest interpretation of these findings is that
both high- and low-affinity Ca\(^{2+}\) inhibition are mediated in
the Mg\(^{2+}\)-binding domain of the catalytic site [28].

**Structural basis for Ca\(^{2+}\) stimulation**

Calmodulin mediates the stimulation of both AC1 and AC8.
Removal of calmodulin by EGTA washes eliminates the
stimulation by Ca\(^{2+}\). Stimulation of AC1 requires amino
acids near the plasma membrane in the C1b region [29,30].
AC8 binds calmodulin at both the N- and the C-terminus.
The latter domain is critical for Ca\(^{2+}\)/calmodulin stimulation
[31]. Deletion of both calmodulin-binding domains from
AC8 results in a super-active enzyme, which suggests that
calmodulin activation of AC8, like other calmodulin targets,
is via a disinhibitory mechanism [31].

**Compartmentalization of adenylate cyclases**

**Localization of adenylate cyclases in caveolae or rafts**

One factor contributing to the dependence of adenylate cyclases
on CCE is their localization in discrete domains
of the plasma membrane [32,33]. Both AC8 transfected in
HEK-293 cells and endogenous AC6 in C6–2B glioma cells
occur in buoyant membrane fractions. Their presence in
these cholesterol-rich domains is essential for their regu-
lation by CCE, since disruption of these domains by the
cholesterol-extracting agent, methyl-β-cyclodextrin, elimin-
ates the regulation. The Ca\(^{2+}\)-insensitive AC7 is excluded
from rafts. However, even though AC8 must be present in
these domains for regulation by CCE, mutants of AC8 can be
generated that remain in rafts, but are insensitive to CCE [34].
Thus, other presumably protein–protein interactions must
also be required. AC5 also occurs in rafts; it has been proposed
in direct association with caveolin [35–37]. The presence of
AC5/AC6 in caveolae in cardiac myocytes is also believed to
play a critical role in β-adrenergic signalling [38,39].

Although it is not clear which protein–protein interactions
ensure the regulation of AC8 by CCE, some analogies with the
endothelial nitric oxide synthase (eNOS) system are
tantalizing. eNOS is apparently recruited to caveolae by
myristoylation of the C-terminus and retained in an inactive
state through an interaction with caveolin. Upon the trig-
nering of CCE, calmodulin displaces caveolin from eNOS,
resulting in activation of the enzyme [40]. In the case of
AC8, there is an apparently non-essential calmodulin-binding
site at the N-terminus (see above). Deletion of this domain
renders AC8 insensitive to CCE, although it is retained within
caveolae. We wonder whether there may be some parallels
between the eNOS and the cyclase systems in this regard.

**Local cAMP**

Isolated reports over the years have suggested that cAMP in
the microdomain of its site of synthesis may not faithfully
reflect cAMP concentrations within the cytosol of cells [41–
43]. As we learn more about the compartmentalization of
adenylate cyclases to sub-domains of the plasma membrane
more physical support for this concept develops. In addition,
the fact that phosphodiesterases are selectively targeted to
different domains of the cell along with CAMP-dependent
protein kinase-analyzing proteins makes an extremely
strong, physical case for CAMP-signalling microdomains [3].
Recent methodologies that are being developed to explore the
dynamics of CAMP in single cells are encountering data that
support this possibility [3,43–49].

**Future directions**

We have learned a lot about the regulation of Ca\(^{2+}\)-sensitive
adenylate cyclases over the last few years – both at the bio-
chemical and cell-biological levels. From a biochemical viewpoint it will be of considerable interest to know what makes
some cyclases subject to high-affinity inhibition by Ca\(^{2+}\) and
others insensitive, given that adenylate cyclases are at their
most conserved in their catalytic domains. However, there
are significant differences in the amino acids surrounding the
catalytic aspartates between different isoforms. Ideally, crys-
tal structure comparisons of Ca\(^{2+}\)-sensitive and Ca\(^{2+}\)-
insensitive forms will reveal the underlying mechanisms. It
will also be of considerable interest to determine the role of
the N-terminal binding of calmodulin by AC8 – whether, for
instance, it plays a recruiting role for calmodulin or, perhaps,
duces a conformation in the N-terminus that allows inter-
actions with scaffolding proteins. Newly emerging studies
suggest that higher-order assemblies of adenylate cyclase
molecules can occur. This propensity may be enlightening
in terms of identifying partners in signalling complexes. The
mechanism underlying the apposition of cyclases with CCE
channels remains a burning question, whose solution may
yet provide unsuspected insights into CCE organization
[50]. Continued application of single-cell methods to the
measurement of CAMP is expected to provide novel insights
into the dynamics of the second messenger near the plasma
membrane. It was predicted some time ago with rather con-
servative modelling, and again more recently, that the inter-
action between Ca\(^{2+}\) and Ca\(^{2+}\)-sensitive adenylate cyclases
could yield CAMP oscillations [51,52]. If the current technolo-
gies support these predictions, the next challenge will be
to develop strategies to search for potential targets of CAMP
spikes.

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**References**
