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Physiological Roles for G Protein-Regulated Adenylyl Cyclase Isoforms: Insights from Knockout and Overexpression Studies

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Adenylyl cyclase · G protein · Cyclic AMP · Calcium · Synaptic plasticity · Cardiac function · Olfaction

Abstract
Cyclic AMP is a universal second messenger, produced by a family of adenylyl cyclase (AC) enzymes. The last three decades have brought a wealth of new information about the regulation of cyclic AMP production by ACs. Nine hormone-sensitive, membrane-bound AC isoforms have been identified in addition to a tenth isoform that lacks membrane spans and more closely resembles the cyanobacterial AC enzymes. New model systems for purifying and characterizing the catalytic domains of AC have led to the crystal structure of these domains and the mapping of numerous interaction sites. However, big hurdles remain in unraveling the roles of individual AC isoforms and their regulation in physiological systems. In this review we explore the latest on AC knockout and overexpression studies to better understand the roles of G protein regulation of ACs in the brain, olfactory bulb, and heart.

Introduction

The control of second messengers involves a complex system of proteins, many or all of which are independently regulated. One of the most highly studied signal transduction pathways is the intricate control of cyclic AMP (cAMP) generation. Biochemical and genetic evidence points to roles for cAMP in a vast number of biological systems, including but not limited to oogenesis [1], embryogenesis [2], larval development, hormone secretion, glycogen breakdown [3], smooth muscle relaxation [4], cardiac contraction [5, 6], olfaction [7], and learning and memory [8–10]. Adenylyl cyclase (AC) is an ATP-pyrophosphate lyase that converts ATP to cAMP and pyrophosphate. Since the cloning of the first AC isoform AC1 in 1989 [11], there has been much progress in the cloning, characterization, and structural analysis of the individual AC enzymes. Nine mammalian transmembrane ACs are recognized, with a tenth 'soluble' form that has distinct catalytic and regulatory properties resembling the cyanobacterial enzymes [12].

Numerous strategies have been developed to characterize individual AC isoforms. The assignment of regulatory properties to each isoform resulted in large part from expression of full-length AC isoforms in mammalian or insect cells (*Spodoptera frugiperda*, Sf9). The frustration from these systems was the lack of large amounts of pure

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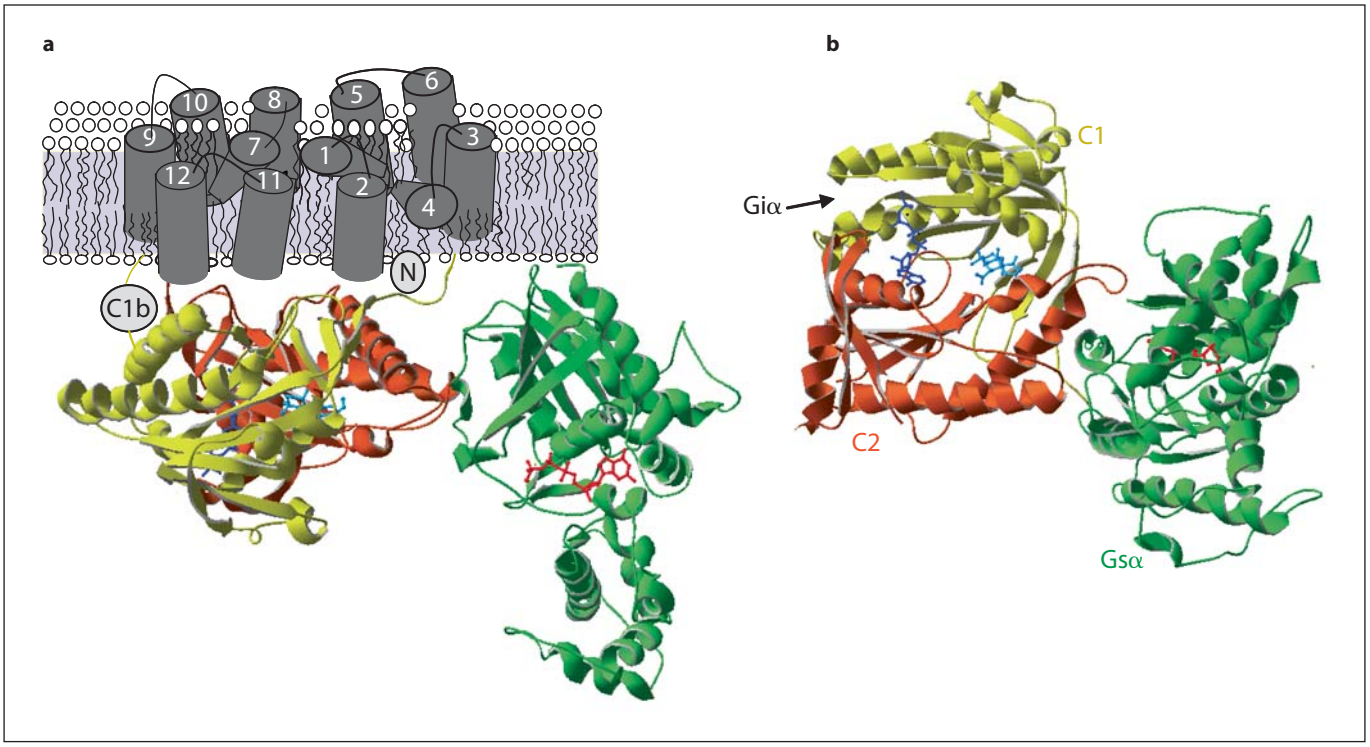

Fig. 1. Structure of adenylyl cyclase. **a** Crystal structure of cytoplasmic domains of AC in complex with GTPγS-Gsα, forskolin (FSK) and P-site inhibitor, 2′5′-dideoxy-3′ATP [100]. Shown are C1 (yellow), C2 (rust), Gsα (green), FSK (cyan), and P-site inhibitor (dark blue). Membrane spans are modeled from the 12-membrane spanning transporters [199]. **b** Alternate view from cytoplasmic side, showing forskolin and catalytic site more clearly. Interaction site of Giα with C1 domain is indicated by an arrow.

protein for detailed biochemical characterization. The expression of the two catalytic domains of AC in *Escherichia coli* largely solved this issue and resulted in sufficient protein for biochemical, kinetic, and structural studies.

Despite the progress made in the identification and biochemical characterization of cellular regulators of ACs, there are many questions that still remain unanswered. One particularly difficult question is, 'Why are there so many isoforms of AC and what roles do individual isoforms serve?' We will briefly review the basic structure, regulation, and tissue distribution of ACs before addressing the physiological roles of AC isoforms in the brain, olfactory neurons, and heart. The major focus will be on the phenotypes of AC knockout and overexpression studies. Although no comprehensive answers are yet available, we will attempt to address the complex issue of why unique regulatory properties of AC isoforms serve specific roles in cAMP biology.

Adenylyl Cyclases: Topology and Structure

Mammalian transmembrane ACs share a similar topology of a variable N-terminus (NT) and two repeats of a membrane-spanning domain followed by a cytoplasmic domain [11]. The overall topology is very reminiscent of the ABC cassette transporter proteins (fig. 1). Pseudosymmetry results from the fact that each of the two cytoplasmic domains (C1 and C2) contain a region of approximately 230 amino acid residues that are roughly 40% identical (C1a and C2a). Together the cytoplasmic domains form the catalytic moiety at their interface, creating a pseudosymmetrical site that is primed for bidirectional regulation as discussed below. The NT and C-terminal portion of the C1 and C2 domains (C1b and C2b) are the most variable regions among the different isoforms and can differ even among species.

The elegance of design, form, and function of AC is clearly seen in the crystal structure of the C1a-C2-Gsα-forskolin complex [13]. The C1a and C2 domains have

Table 1. Regulatory properties of transmembrane adenylyl cyclase (AC) isoforms

AC isoform	G protein regulators		Protein kinases		Calcium	RGS2	Other
	stimulatory	inhibitory	stimulatory	inhibitory			
Group I							
AC1	Gsα	Gα i, z, o, Gβγ	PKCα (weak)	CaMK IV	↑ CaM		PAM
AC8	Gsα	Gβγ			↑ CaM		PP2A
AC3	Gsα	Gβγ	PKCα (weak)	CaMK II	↑ CaM*	↓	
Group II							
AC2	Gsα, Gβγ		PKCα				
AC4	Gsα, Gβγ			PKCα			
AC7	Gsα, Gβγ		PKCα				PAM
Group III							
AC5	Gsα, Gβγ	Gα i, z	PKC (α, ζ)	PKA	↓ free Ca^{2+}	↓	PAM, Ric8a
AC6	Gsα, Gβγ	Gα i, z		PKA, PKC (δ, ε)	↓ free Ca^{2+}	↓	PAM, Snapin
Group IV							
AC9	Gsα			PKC	↓ via calcineurin		

nearly identical tertiary structures, as predicted from their sequence similarities, despite the fact that these structures were solved with a C1 domain from type 5 AC and a C2 domain from type 2 AC. The pseudosymmetry creates two related sites along the domain interface, a substrate-binding site and a related forskolin site. Both pockets are well defined and are structurally related. There are notable differences between the C1a and C2 structures, particularly comparing the regions that play an important role in the binding of Gsα (C2 domain) or in the formation of a P-loop structure that binds pyrophosphate in the active site (C1 domain). It is of note that the active site shares many similarities with DNA polymerases, although the surrounding structures are quite different [14].

The mammalian soluble AC (sAC) has homology to cyanobacterial ACs with several known splice variants [12]. The most studied of these are the full-length (~187 kDa) and the testis splice variant (~50 kDa) that contain tandem C1 and C2 domains which form the catalytic site [15]. The overall structure of the catalytic core is highly conserved with the transmembrane ACs, although there are significant differences in primary sequence [16]. Although sAC is not regulated by G proteins, it is stimulated by calcium and bicarbonate [17–19]. Our discussions of physiological roles for ACs will focus on the transmembrane ACs, although sAC has been implicated in sperm motility, fertilization, and neurite outgrowth of neuronal cells [20–22].

Classification of Isoforms

Membrane-bound ACs are often classified into four different categories based on regulatory properties. Group I consists of Ca^{2+}-stimulated AC 1, 3 and 8; group II consists of Gβγ-stimulated AC 2, 4 and 7; group III is comprised of Giα/Ca^{2+}-inhibited AC5 and 6, while group IV contains forskolin-insensitive AC9 (table 1). Note that although significant sequence homology exists within members of groups II and III, members of group I are more distantly related [12]. This is reflected in the overall regulatory patterns for the various groups as well.

Regulation by Heterotrimeric G Proteins

All isoforms of transmembrane ACs are stimulated by the GTP-bound α subunit of Gs (Gsα). The splice variants of Gs (short, long and XL) [23, 24] activate AC to a similar extent in vitro, although some variations in receptor-mediated activation have been reported [25]. Golf,α is highly homologous to Gsα and also stimulates AC [26]. Golf is mainly expressed in the olfactory system but can be found in other tissues, particularly in striatum [26–29]. In both olfactory neurons and striatum, it is the Golf,α subunit that predominates over Gsα [29]. These G proteins interact with AC mainly through their switch II α helices, which are conforma-

tional sensors for the α-activation state [30]. The major binding site for Gsα on AC is located on the C2 domain in the cleft formed by α2′ and α3′ helices (fig. 1b) [13]. Lesser contacts are observed with the N-terminal segment of C1.

The α subunits of Gi (1, 2, 3), Gz, and Go can inhibit select AC isoforms [31–33]. The calmodulin-stimulated state of AC1 is inhibited by all three of these Gi family members, whereas Gsα- or forskolin-stimulated forms of AC1 are only weakly inhibited or not at all [32]. AC5 and 6 are inhibited by Giα (1, 2, 3) and Gzα, most potently at reduced levels of activation [34]. All other ACs are insensitive to Giα. Mutagenesis studies show that Giα binds to a cleft in the C1 domain [35], analogous to the Gsα-binding site in C2, and acts in opposition to Gsα.

The βγ subunit of heterotrimeric G proteins can be either stimulatory or inhibitory depending on the AC isoform. Gβγ is inhibitory for all group I ACs which include AC 1, 3, and 8 [36–38]. In the case of AC1, inhibition by Gβγ is more potent than that of Giα family members, which would presumably be also contributing Gβγ subunits [32]. When Gβγ is released upon activation of Gs-coupled receptors, the inhibition by Gβγ is reported to negate AC1 and AC8 stimulation by Gsα in some cell types, although Gs stimulation is still synergistic with Ca^{2+}/CAM for AC1 but not AC8 [39].

The hallmark of the AC2 family (or group II) is the conditional stimulation by Gβγ. Gsα-stimulated activity of AC2, 4 and 7 is enhanced by ~5- to 10-fold by Gβγ, although there is no effect of Gβγ alone [36, 40–43]. The binding site of Gβγ on AC2 has been mapped to several regions that include the C2 domain and the PFAHL motif in the C1b domain [38, 44]. It is likely that Gβγ works through a two-site interaction mechanism to regulate AC2 activity [45].

Although overexpression of Gβγ has been reported to lower cAMP levels in cells transfected with AC5 or 6 [46], the direct effect of Gβγ on these isoforms is stimulation. Gβγ binds directly to the NT of AC5 and 6 to increase Gsα-stimulated activity by approximately 2-fold [47]. In fact, it is the Gβγ that is released upon Gs activation by isoproternol that is responsible for the full activation of AC6 [47]. Whereas for AC2 or AC7, it has been traditionally thought that Gβγ released from Gi-coupled receptors could synergistically stimulate AC activity in the presence of activated Gsα, representing a form of crosstalk between G protein-coupled receptors [40].

Other Modes of Regulation

Ca^{2+} and Calmodulin

Calcium-bound calmodulin is an important regulator of the group I ACs. AC1 and AC8 are directly stimulated by calmodulin [48, 49]. The calmodulin-binding sites for AC1 have been mapped to the C1b and C2 domains; whereas the sites on AC8 reside within the NT and C2 domain [50, 51]. AC3 activity is conditionally stimulated by calmodulin, requiring the presence of Gsα or forskolin [48]. However, the more relevant regulation of AC3 in vivo may be a calmodulin-dependent inhibition via regulation by calmodulin kinase II (CaMK II) [52]. CaMK II directly phosphorylates AC3 on ser 1076 and inhibits AC3 activity [53]. This serves as an important feedback mechanism in the olfactory system (discussed in detail later). AC1 is also subject to feedback inhibition via CaMK IV which phosphorylates AC1 within C1b and inhibits calmodulin-stimulated activity [54]. Thus both AC1 and AC3 regulation by calcium-calmodulin is tightly controlled in neuronal signaling. AC8 is not subjected to regulation by either CaMK II or IV [54].

All AC isoforms are inhibited by high, non-physiological concentrations of Ca^{2+}, via competition for magnesium at the active site. However, AC5 and 6 are inhibited by submicromolar concentrations of free Ca^{2+} [55], which may have important physiological implications in generating oscillating Ca^{2+} and cAMP signals [56]. Curiously, these ACs respond primarily to elevations in calcium by capacitative- or store-operated calcium entry (CCE), as opposed to global increases in calcium that may be elicited by ionomycin or agonists that stimulate calcium release from the endoplasmic reticulum [for review see, 57]. Thus, ACs and CCE channels are presumed to be in close proximity on the plasma membrane. Studies using AC6 linked to the fluorescent calcium sensor aequorin are suggestive that this is indeed the case [58]. However, the identity of the CCE channels that are responsible for cAMP regulation has remained elusive. The recent identification of Stim1 and Orai1 make these channels an exciting possibility for regulating AC5 and 6 by calcium [59–62].

Regulation by Protein Kinases

Most ACs are regulated by either protein kinase (PK) A or C. PKA serves as a feedback inhibitor for AC5 and 6 by phosphorylating these isoforms on a serine near the end of the C1b domain [63]. PKC regulation can be either stimulatory or inhibitory. Stimulation by conventional PKCs is often highly synergistic with other forms of regulation;

this is particularly true for AC2 and 5 [64–66]. The novel PKCδ isoform also displays synergy with Gsα in activating AC7 [67, 68]. Atypical forms of PKC can also stimulate AC5 [65]. PKCζ is an atypical PKC which can be stimulated by the product of phosphoinositide-3′ kinases, phosphotidylinositol-3,4,5-triphosphate. Stimulation of ACs by PI3K/PKCζ can produce temporal effects, prolonging cAMP production and creating biphasic time courses to further enhance transcriptional responses [69, 70].

Inhibition by PKC can occur by either conventional or novel PKCs. PKCα inhibits Gsα-stimulated AC4, but has no effect on basal or forskolin-stimulated activity. The novel PKCδ and ε isoforms inhibit AC6 [66, 71]. Not surprisingly, the sites of PKC phosphorylation on ACs differ greatly between these different isoforms. PKC phosphorylation sites within the C-terminus of AC2 stimulate the enzyme [72, 73], whereas phosphorylation of several sites within the NT of AC6 mediate inhibition [74, 75].

The regulation of AC9 by phosphorylation is more complex. At least 12 sites of phosphate incorporation have been detected on AC9 [76]. The mouse AC9 isoform can be inhibited by the calcium-activated protein phosphatase calcineurin (or protein phosphatase 2B) [76–78]. PKC can also inhibit AC9 activity [78–80]; however, it is unknown whether the effects by calcineurin or PKC are direct.

Additional kinases have been reported to regulate ACs. Raf1 can stimulate phosphate incorporation and activity of AC6 via activation of receptor tyrosine kinases [81]. Serines within the C1b region and the loop between membrane spans 8 and 9 are required for activation by Raf1 [82, 83]. Tyrosine kinases such as the EGF receptor can also indirectly stimulate AC5 activity by the phosphorylation of Gsα [84–86]. Phosphatases would also be predicted to regulate AC activity and as such AC8 serves as a scaffold for PP2A [87].

Forskolin and P-Site Inhibitors

Forskolin is a diterpene derived from the root of the plant *Coleus forskohlii* [88]. It highly activates all the membrane-bound AC isoforms except AC9 [89, 90]. A single forskolin molecule binds at the interface of the C1 and C2 domains, in the pocket structurally related to the AC active site [13, 91]. AC9 is missing a key residue within this forskolin-binding pocket that when mutated can restore forskolin sensitivity [92]. AC2, 4, 5, 6, and 7 are synergistically activated by Gs and forskolin, while the effect on AC1, 3 and 8 is additive. Although tantalizing to speculate about, no mammalian forskolin analogs have been identified that might regulate ACs via the forskolin-binding pocket.

P-site inhibitors are adenosine analogs that are typically noncompetitive or uncompetitive with respect to substrate ATP [93–95]. These inhibitors are more potent on activated forms of AC versus the basal state [96–98], and form a product-like transition state with pyrophosphate [13, 99]. More potent inhibitors, such as adenosine 3′-polyphosphates (i.e. 2′,5′ dideoxy 3′ATP), bind in the absence of pyrophosphate since the 3′-phosphates bind in the pyrophosphate pocket, increasing the affinity of these inhibitors by 100- to 1,000-fold [95, 100]. In general, P-site inhibitors are not greatly isoform specific with the exception of AC9, which is largely insensitive to 2′-deoxy-3′-AMP [101, 102].

Additional Regulators

Several additional regulators and binding partners of ACs exist that do not fall into any of the above categories, including the regulator of G protein signaling (RGS2), the protein associated with Myc (PAM), Snapin, Ric8a, and the A-kinase-anchoring protein (AKAP79). RGS2 inhibits the activities of AC3, 5 and 6 [103, 104] and directly interacts with C1 domain of AC5 [105]. The PAM serves to inhibit Gsα stimulation of AC1, 5, 6 and 7 and interacts with the C2 domain of AC5 [106, 107]. Snapin is a member of the SNAP-25/Snare complex that physically interacts with AC6 in hippocampal neurons, preventing PKC-mediated inhibition of AC6 [108]. Ric-8 is a guanine nucleotide exchange protein for heterotrimeric G proteins. Ric-8a-8 binds to AC5 and inhibits activity, possibly via the activation of Gi [109]. Finally, AC5 can also bind to the PKA scaffolding protein, AKAP79, which facilitates PKA-mediated inhibition of AC5 [110]. AKAP79 may link cAMP production more closely with PKA substrates such as the NMDA receptor, bound within the same complex [111]. The regulation of AC5 and 6 by various regulators including PAM, RGS2, Snapin, and nitric oxide has recently been reviewed in depth [112].

Mechanism(s) of Regulation: Interactions with Catalytic Core versus N-Terminus

The key to regulation of AC is the interface between the C1 and C2 domains which forms a single ATP-binding site [13, 91]. Thus, many of the regulators discussed above bind to these domains to modulate enzymatic activity. The relative affinity between these domains is weak in the absence of any activators, as measured by interactions between the purified domains [113–115]. However, both forskolin and Gsα increase the affinity be-

tween the C1 and C2 domains by ~10-fold; a 100-fold increase in affinity is observed upon synergistic stimulation by both regulators [113–115]. Forskolin binds at the interface, thus it is easy to visualize its affects on domain interactions since both C1 and C2 contribute residues for binding. However, Gsα binds at a cleft that is on the opposite side of AC from the catalytic site. Comparisons with an inactive C2 homodimer suggest that Gsα and forskolin may induce a 7° rotation of the two domains with respect to each other [13, 116]. This movement would bring key residues in the active site closer to the 3′-hydroxyl group of ATP, creating a conformation more favorable for catalysis [13]. Binding of Giα to the C1 domain directly opposes the actions of Gsα that is bound to the pseudosymmetical site in C2 [35]. Giα decreases the affinity of the C1 and C2 domains for one another and inhibits catalytic activity [117]. Thus, where Gsα facilitates closure of the active site around ATP, Giα would hinder this change and stabilize a more open inactive conformation. Gβγ interacts with multiple domains of AC2 that would also facilitate conformational changes to cooperatively stimulate the enzyme [45]. In the full-length enzyme the membrane spans bring together the two domains, thus the changes in relative affinity that are observed between the two domains upon stimulation likely represents these conformational changes at the domain interface.

Metal ions such as Ca^{2+} and Mn^{2+} also have effects on the catalytic site. Similar to the mechanism for DNA polymerases, AC catalyzes phosphoryl transfer by a two metal ion mechanism, which generally uses Mg^{2+} [118, 119]. Manganese can bind to the 'B' metal site to stimulate the enzyme, while calcium likely binds to the 'A' metal site to inhibit the enzyme, similar to the inhibition observed with Zn^{2+} [119, 120].

Although the C1 and C2 domains have received much of the attention, the NT clearly interacts with a number of regulators to control activity. The sequence of the NT is highly divergent, even among AC family members, and thus provides additional regulatory specificity. Studies with AC6 suggest that the NT may contact the C1 domain to modulate Giα-mediated inhibition [121]. Phosphorylation of the NT of AC6 by PKCδ and ε serves to also inhibit the enzyme [71, 74, 75], whereas binding of the SNAP25-binding protein Snapin to the NT blocks PKC inhibition [108]. In addition, the NTs of both AC5 and AC6 bind Gβγ to conditionally stimulate the enzyme [47]. The NT of AC5 also interacts with the G protein-exchange factor RiC8a to suppress AC activity, possibly by modulating Gi activity [109]. Finally, the NT of AC8 binds protein phosphatase 2A (PP2A) [87], and forms part of the CaM-binding site to regulate activity [51]. The mechanism(s) for N-terminal regulation of ACs is unknown, but certainly opens another interesting chapter in the complex regulation of these enzymes.

Physiological Roles for Individual AC Isoforms

The question arises as to why multiple isoforms of ACs are needed and what specific functional roles are regulated by each isoform. Tissue distribution defines much of the specificity observed with respect to AC function. Due to the low abundance of AC expression and the poor antibodies available, most of the data for tissue distribution rely on PCR or Northern blotting (table 2). In many cases these patterns of expression have also been verified by functional assays based upon their differential regulatory properties. However, it is clear that most cells express two or more AC isoforms and nearly all AC isoforms are expressed in the brain. Some putative roles for ACs were initially assigned according to localization. For example, AC1 and 8 (primarily expressed in the brain) were ascribed roles in learning and memory; AC3 (most abundant in the olfactory epithelium) in olfaction, and AC5 and 6 (dominant in the heart) for cardiac contractility. Additional complexity arises in the expression patterns of GPCRs, G proteins, and other regulators. Due to a lack of isoform-specific inhibitors, homozygous knockout or overexpression studies of ACs have been used primarily to identify specific isoform functions. Although ACs regulate processes in all tissues, we will focus on recent studies performed in the brain, heart, and olfactory system.

Synaptic Plasticity, Long-Term Potentiation, and Memory

Synaptic plasticity is the ability of specialized connections between two neurons (i.e. synapse) to change strength. Long-term potentiation (LTP) is the long-lasting enhancement in communication between two neurons that results from stimulation. Since neurons communicate via synapses and memory is believed to be stored in synapses, LTP and long-term depression are widely considered the major cellular mechanisms that underlie learning and memory [8, 9]. The hippocampus plays a critical role in the formation of new memories and all of the major synaptic pathways in the hippocampus exhibit LTP, including the perforant, mossy fiber

Table 2. Tissue distribution and physiological functions of individual mammalian isoforms

AC isoforms	Site of expression	Availability of		Physiological functions
		knockout	overexpression	
AC1	Brain, adrenal medulla	Yes		Learning, memory, synaptic plasticity, opiate withdrawal
AC2	Brain, lung, skeletal muscle, heart			
AC3	Olfactory epithelium, pancreas, brain, heart, lung, testis, BAT	Yes		Olfaction, sperm function
AC4	Widespread			
AC5	Heart, striatum, kidney, liver, lung, testis, adrenal, BAT	Yes	Yes[1]	Cardiac contraction, motor coordination, opiate dependency, pain responses
AC6	Heart, kidney, liver, lung, brain, testis, skeletal muscle, adrenal, BAT	Yes	Yes[1]	Cardiac contraction and calcium sensitivity
AC7	Widespread		Yes[1]	Ethanol dependency
AC8	Brain, lung, pancreas, testis, adrenal	Yes	Yes[1]	Learning, memory, synaptic plasticity, opiate withdrawal
AC9	Widespread	Yes[2]		
sAC	Testis and detected in all tissues	Yes		Sperm capacitation, fertilization

Sites of expression for all mammalian isoforms of AC have previously been expertly reviewed in detail [57, 176]. Expression patterns of ACs in developmental stages of mouse brain are found in Visel et al. [137].

[1] Tissue-directed overexpression.

[2] Unpublished results [76].

and Schaffer collateral, although the mechanisms may differ.

Early studies in Drosophila and Aplysia led to the hypothesis that AC is involved in learning and memory. The mutant flies, *dunce* (phosphodiesterase activity deficient) and *rutabaga* (deficient in Ca^{2+}/CAM-stimulated cyclase activity) fail a number of different learning tasks, including learning to avoid a neutral odor [8]. The sequence of the *rutabaga* gene was most closely related to AC1 [122]. AC1 is primarily expressed in the brain, particularly in the hippocampus, neocortex, entorhinal cortex, cerebellar cortex, olfactory bulb and pineal gland [123, 124]. Hence it became a likely candidate for learning and memory in mammalian systems.

To further explore this notion, AC1 knockout mice ($AC1^{(-/-)}$) were generated in 1995 by Wu et al. [10]. Mutant mice had normal growth, motor coordination, and lifespan. No abnormalities were observed at anatomical or morphological levels in the brain, except for a lack of barrel patterning in the sensory motor cortex [125]. However, $AC1^{(-/-)}$ mice had decreased Ca^{2+}-stimulated AC activity (~40–60%) in the cerebellum, cortex and hippocampal regions, in addition to attenuated developmental expression of Ca^{2+}-stimulated activity [10, 126]. The decrease in Ca^{2+}-stimulated cAMP correlated with a decrease in CA1/CA3 hippocampal and cerebellar LTP, and a deficiency in spatial memory [10, 127]. The hippocampal defect was displayed in the early phases of LTP (~50%), suggesting a contribution to synaptic plasticity.

A role for AC1 in learning and memory has been supported by additional studies in cultured anterior cingular cortex neurons, where AC1 is essential for induction of LTP induced by Theta burst stimulation or forskolin [128]. Overexpression of AC1 in the forebrain enhanced recognition memory and LTP due to an enhancement of ERK/MAPK signaling [129].

$AC1^{(-/-)}$ mice also have impaired mossy fiber LTP, although perforant path LTP in the dentate gyrus and long lasting LTP at the Schaffer collateral are normal [130]. The impairment in mossy fiber LTP could be reversed by forskolin, indicating that the abnormality is due to an absence of AC1 activity and not a loss of downstream sig-

naling molecules such as PKA, ion channels, or secretory machinery. However, it is clear that AC1 is not the only AC isoform important in these activities. Other AC isoforms must be present that respond to forskolin to reverse LTP defects. In addition, impairments in hippocampal and mossy fiber LTP are not complete. The most likely candidate to share overlapping functions with AC1 is the Ca^{2+}/CAM-stimulated AC8. AC8 is also highly expressed in numerous brain regions, including the hippocampus, olfactory bulb, thalamus, habenula, cerebellar cortex and hypothalamic supraoptic and paraventricular nuclei [49, 131].

$AC8^{(-/-)}$ mice show decreased Ca^{2+}-stimulated AC activity in the hippocampus, hypothalamus, thalamus, and brainstem, and exhibit little or no mossy fiber LTP [132, 133]. Short-term plasticity is also impaired in $AC8^{(-/-)}$ mice. Double knockouts of both AC1 and AC8 also exhibit a nearly complete loss of mossy fiber LTP [133]. Thus, both AC1 and AC8 contribute to mossy fiber LTP. In addition, AC1 and AC8 are functionally redundant for long-term memory and fear-associated memory formation [134]. The individual AC1 and AC8 knockouts exhibit normal L-LTP and fear-associated memory, but double knockouts were significantly impaired [134]. Infusion of forskolin in the CA1 region of the hippocampus restored normal long-term memory. Either AC1 or AC8 can generate cAMP needed for transcription-dependent long-term LTP [134].

Despite many overlapping functions, there are differences in the pathways that AC1 and AC8 control. AC1, but not AC8, is required for homeostatic plasticity during activity deprivation [135]. $AC8^{(-/-)}$ mice show abnormal anxiety behavior under stress [132]. The latter phenotype may relate to the high expression of AC8, in the thalamus, habenula, and hypothalamus, regions involved in responses to stress. AC1 is not highly expressed in these regions [123]. Thus, AC8 is more involved in synaptic plasticity related to anxiety.

The various forms of hippocampal LTP require increases in Ca^{2+} either postsynaptically through NMDA receptors or presynaptically via voltage-sensitive Ca^{2+} channels. Perforant and Schaffer LTP are dependent on NMDA receptor activation, whereas mossy fiber/CA3 LTP likely relies on presynaptic changes in Ca^{2+} [136]. AC1 and AC8 are expressed both presynaptically and postsynaptically [133]. The increase in Ca^{2+} stimulates AC1 and AC8 to generate cAMP, which in turn activates several signal transduction pathways, including PKA and Erk/MAPK via Rap-1. Erk/MAPK activates Rsk2, the major kinase for CREB. The activation of CREB/CRE transcriptional pathways leads to expression of genes required for LTP and long-term memory.

In summary, AC1 and AC8 are not necessary for survival, but play major roles in learning and memory. Although nearly all AC isoforms are expressed in the brain (AC4 is expressed only in the brain blood vessels) [137, 138], there are clear differences in their physiological functions. Both AC1 and AC8 are members of the Ca^{2+}/CAM-stimulated family of ACs, but display differences in regulatory patterns. AC1 has a 5-fold lower EC_{50} for Ca^{2+} (150 nM) than AC8 (800 nM) and stimulation of AC1, but not AC8, by Ca^{2+}/CAM is synergistic with Gs activation [39]. In addition, AC1 activity is enhanced by PKC [139], which is also activated during LTP [140]. Thus, AC1 is stimulated by multiple routes for stronger synapses and in turn adapted for roles in learning and memory. But it also has several check points like inhibition by CaMK IV and Gi-coupled receptors to keep cAMP levels optimal. AC8 gene expression can be increased by CREB activation [141], but is a low-affinity Ca^{2+} detector with few inhibitory controls [39]. It should be mentioned that AC9 is also highly expressed in the brain, particularly the hippocampus [137]. However, genetic deletion of this isoform is embryonically lethal and thus a true assessment of the role for AC9 in brain function is not available at this time [76].

Synaptic Plasticity Associated with Pain

Pain perception is a complex trait involving peripheral and central processing, and dramatic alterations in neuronal properties induced by inflammation and injury. A clear role for cyclic AMP has been established in the sensitization of nociceptors and pain projection neurons in the spinal cord after noxious stimulation and inflammation [for review see, 142, 143]. However, the AC isoforms that contribute to these processes have only recently been studied.

AC1, but not AC8, knockout mice have significantly reduced behavioral nociceptive (pain transmission) responses of the intermediate and late phases of acute muscle pain (induced by formalin injections) [144]. In AC1 and the AC1/8 double knockouts, chronic muscle inflammatory pain (induced by carrageenan injections) was also significantly reduced but could be rescued by microinjections of forskolin in the spinal cord [144]. In addition, injection of a novel AC1 inhibitor also significantly reduced behavioral responses in both acute and chronic inflammatory muscle pain. Thus, AC1 plays an important

role in acute and chronic muscle pain, although clearly additional ACs are present that can rescue impaired effects.

AC5 also has strong effects on acute and chronic pain responses. AC5$^{(-/-)}$ mice have attenuated pain responses in acute thermal and mechanical pain tests [145]. They display hypoalgesic responses to inflammatory pain and inflammatory visceral pain (induced by injection of sulfate or acetic acid) [145]. AC5$^{(-/-)}$ mice display strongly attenuated mechanical and thermal allodynia (an exaggerated response to normal stimuli) in neuropathic pain models. The question still remains as to where AC isoforms are exerting their effects. Pharmacological studies support the spinal cord as a major site of action [142]; AC1 and 5 are expressed in the spinal cord in addition to AC2, 6, and 8 [146]. Although AC1 and 5 display strong differences in their regulation, both are inhibited by PAM. PAM is upregulated in the spinal cord in response to nociceptive stimulation [146] and produces a sustained inhibition in response to sphingosine-1-phosphate [147], a regulator of neuronal cell survival [148]. In summary, these studies support roles for AC1 and 5, but not AC8 in synaptic plasticity related to different forms of pain.

Excitotoxicity and Neurodegeneration

Excitotoxicity is the pathological process by which nerve cells are damaged and killed by glutamate or similar substances. When NMDA or AMPA receptors are overactivated, neuronal death ensues via an influx of Ca^{2+} leading to apoptosis. Knockout of AC1 significantly attenuated neuronal cell death induced by intracortical injections of NMDA, but deletion of AC8 had no such effect [149]. Thus, Ca^{2+}-stimulated AC1 modulates neuronal responses to excitotoxicity and may serve as a novel target for treatment of neuronal excitotoxicity in stroke and neurodegenerative disease.

Ethanol can also induce neurodegeneration in the brains of neonatal mice, which can be mimicked by NMDA receptor antagonists or potentiators of the GABA receptor. Genetic deletion of AC1, AC8 or both isoforms enhanced ethanol- or phenobarbital-induced neurodegeneration, but not cell death due to hypoxia/ischemia [150]. Therefore AC1 alone controls neuronal death in response to NMDA-dependent excitotoxicity, while both AC1 and 8 may play important roles in neurodegeneration induced by activity blockade in the neonatal brain.

Motor Functions

The striatum is the region of brain important for the planning and programming of voluntary movements, as well as some cognitive functions. These functions involve dopamine receptor signaling. AC1 and 8 have no effects on motor coordination [144]. However, AC5 is highly enriched in the striatum and genetic ablation of AC5 shows a reduction in forskolin-stimulated activity in the striatum (>80%), cerebral cortex (~27%), and cerebellum (40%) [151]. Only 10% of dopamine D1-stimulated AC activity and 16% of adenosine A2A-stimulated AC activity remain in the striatum of AC5$^{(-/-)}$ mice, while the dopamine D2 inhibition of AC activity mediated by Gi is completely absent [138, 151]. General motor behavior is normal in AC5$^{(-/-)}$ mice; however, these mice show loss of neuroleptic responsiveness towards the D2 antagonist class of antipsychotic drugs [151]. Other AC5$^{(-/-)}$ models exhibit Parkinson-like motor dysfunction, displaying abnormal coordination, bradykinesia, and locomotor impairment [138]. Motor coordination can be restored by D2 stimulation, while bradykinesia was largely restored by either D1 or D2 stimulation of residual striatal AC activity. Although AC6 and AC1 (the other Gi-inhibited ACs) are present in the striatum, they cannot fully compensate for AC5 function [138]. AC5 is the physiological isoform coupled to dopamine D2 receptors and plays an important role in the response to antipsychotic drugs. AC5 provides a site of convergence for both D1 and D2 dopaminergic signals and the inhibition of AC5 by Giα is a crucial regulatory property for cAMP-dependent motor control.

Drug Dependence (Morphine, Ethanol)

Morphine

Opiate-induced analgesia is mediated by the activation of Gi-coupled μ, and to a lesser extent by δ-opioid receptors. Their analgesic properties are related to the inhibition of AC, inhibition of voltage-gated Ca^{2+}, and activation of inward rectifying K^+ channels by Gi [152, 153]. The inhibition of AC has been linked to long-term adaptations by opiates. Long-term morphine use causes an upregulation of AC signal transduction components (AC, PKA, or CREB) in regions of the brain associated with drug reinforcement and withdrawal [153]. In cell culture systems, AC supersensitization can be measured after treatment with Gi-coupled ligands (such as morphine, muscarinic agents, and somatostatin). The increase in AC

activity is long-lived, appears to require Gβγ subunits (although not in a direct regulatory role of AC), and is specific for the AC1, 5, 6, and 8 isoforms [154–156].

AC1 and 8 are upregulated by long-term exposure to morphine, and genetic deletion of AC1 and 8 caused a significant reduction in withdrawal behaviors including reduced shakes and forepaw tremors after naloxone injection [157]. The AC1/8 knockout mice had less morphine-induced hyper-locomotion and conditioned place preference; although the latter effect may be due to impairments in learning and memory. CREB activation induced by morphine was not evident in the AC1/8 double knockout mice in the ventral tegmental area. Gene expression patterns after chronic morphine administration in AC1 or 8 knockouts were only partially overlapping in the locus coeruleus (a region critical for opioid withdrawal), providing additional evidence that these AC isoforms have distinct functions during chronic morphine exposure [158]. Ca^{2+}-stimulated cyclases (AC1 and 8) are important mediators of morphine responses but not the only required AC isoforms.

AC5 has also been reported to be an essential mediator of morphine action within striatum [159]. The μ-opioid receptors are at their highest level in the striatum and implicated in reward mechanisms [160]. AC5 is the primary AC effector for μ- and δ-opioid receptors in the striatum, with deletion of AC5 resulting in a loss of opioid-induced inhibition of AC activity in the striatum [159]. All the major behavioral effects of morphine, including locomotor activation, analgesia, tolerance, reward and physiological dependence, and withdrawal symptoms, were attenuated in AC5 KO mice. These behavioral effects were selective for μ- and δ-opioid receptor agonists; κ-dependent locomotor activity was unaffected.

The roles of cAMP in morphine dependence have been further evaluated by overexpression of AC7 in the mouse brain, resulting in the enhancement of acute and chronic actions of morphine [161]. In this model, tolerance to morphine develops more rapidly than in wild-type mice. Thus it is clear that cAMP signaling is important in opioid dependency with AC1 and 8 playing roles in withdrawal, hyper-locomotion, and the learned responses to morphine; whereas AC5 is involved in all major behavioral effects of morphine, including analgesia, locomotor, reward, tolerance, and withdrawal.

Ethanol

As discussed previously, ethanol acts as a neuroactive agent by antagonizing NMDA or potentiating the effects of GABA. Both in drosophila and mice, the sedative effects of ethanol are due to a decrease in cAMP signaling. AC1 knockouts or the knockout of both AC1 and 8 display enhanced sensitivity to the sedative but not ataxic effects of ethanol [162]. The effect on sedation was minimal for the deletion of AC8 alone, but $AC8^{(-/-)}$ had decreased voluntary ethanol consumption that was not observed in the $AC1^{(-/-)}$ mice.

An increase in cAMP (by overexpression of AC7) resulted in high levels of phosphorylated DARPP32 (a dopamine- and cAMP-regulated phosphoprotein) which has been implicated in the motivational effects of ethanol [163]. AC7 is indirectly stimulated 2- to 3-fold by ethanol or morphine, but the role of AC7 in alcohol dependence may be more prominent in platelets rather than in the brain where AC7 is expressed at lower levels (mainly the cerebellar granule layer) [164]. In fact, AC7 activity in platelets has been proposed to be a trait marker for alcoholism [165, 166].

Olfactory Signaling

Odorants interact with G protein-coupled receptors to stimulate AC via Golf. Cylic AMP directly binds to cyclic nucleotide gated channels (CNG) causing an influx of cations (largely Ca^{2+} and some Na^{+}) and a small depolarization of olfactory neurons. Ca^{2+} influx opens Ca^{2+}-activated chloride channels, to further polarize the neuron, triggering an action potential. The olfactory system is composed of two subsystems: the main olfactory epithelium (responsible for odorant detection) and the vomeronasal system (responsible for pheromone detection). Although AC2, 3 and 4 are expressed in the olfactory system, AC3 is the predominant isoform [7]. AC3, Golf, and CNG channels have not been detected in the vomeronasal system; instead AC2 predominates [167].

Genetic deletion of AC3 confirms a role in olfaction [7]. $AC3^{(-/-)}$ mice were initially runts but later gained weight comparable to their wild-type littermates. The initial growth defect is likely due to the fact that $AC3^{(-/-)}$ mice do not detect mouse milk [168]. Deletion of AC3 had major effects on odorant-induced signaling. Responses to cAMP- or IP3-inducing odorants were completely ablated in $AC3^{(-/-)}$ mice as measured by electro-olfactograms, and olfactory epithelial membranes lacked stimulation to the mouse pheromone, 2-heptanone. However, some volatile odorants could be detected by the vomeronasal system, independent of AC3 [169], consistent with a role of AC2 or another AC in this system.

Deletion of AC3 also gave rise to impairments in olfactory-dependent learning and olfaction-based behavioral tests, signifying a critical role for AC3 and cAMP in these processes. In addition, $AC3^{(-/-)}$ mice do not detect mouse urine or pheromones and inter-male aggressiveness and male sexual behavior is absent [168]. AC3 also appears to be responsible for spermatozoa function and male fertility [170]. In general the vomeronasal organ is thought to be responsible for pheromone detection; however, it is clear that the olfactory epithelium and AC3 are also associated with these activities.

Finally, the absence of AC3 perturbed the peripheral olfactory projections in mice and the establishment of mature glomerular [171]. AC3 represents a pivotal element in odorant-mediated axonal guidance, sorting, and identity, and its deletion results in a modified olfactory bulb topographical map and prevents expression of the major axon guidance molecule, neuropilin-1 [172].

AC3 regulatory patterns are adapted for a role in olfaction. AC3 is strongly stimulated by Golf and displays feedback regulation from CaMKII and RGS2. CAMKII is activated in response to increased CNG-dependent Ca^{2+} influx and mediates a rapid feedback inhibition of AC3 [173]. RGS2 negatively regulates odorant-evoked intracellular signaling of olfactory neurons [103], and may give rise to a longer adaptation since it is upregulated in response to cAMP and Ca^{2+} [174, 175].

Cardiac Function

Sympathetic stimulation in the heart leads to an increase in AC activity, resulting in PKA activation and the phosphorylation of numerous effectors including L-type Ca^{2+} channels, phospholamban, and troponin-I. These PKA substrates are involved in cardiac contractility, Ca^{2+} uptake, and cardiac relaxation. Heart expresses all isoforms except AC8 [57, 176] (AC1 is only in the sino-atrial node [177]). AC5 and 6 are the major isoforms expressed in cardiac myocytes and have been the focus of several deletion and overexpression studies outlined below.

Two independent strains of $AC5^{(-/-)}$ mice have been generated with similar decreases in AC activity. The disruption of the AC5 gene leads to decreased basal and stimulated (isoproterenol and forskolin) activity (~35–40%) in cardiac membranes and isolated myocytes [5, 178]. However, differences have been reported between the strains in terms of cardiac function. Okumura et al. [5] reported no change in basal cardiac function in $AC5^{(-/-)}$ (with intravenous isoproterenol), but the isoproterenol-stimulated left ventricular (LV) ejection fraction was significantly decreased [5]. In a second $AC5^{(-/-)}$ model by Tang et al. [178], basal contractile function was increased in isolated perfused hearts, but with decreased sensitivity to a β_1-adrenergic receptor agonist although the maximal levels were unchanged. However, a significant reduction in Gsα protein (~60%) was reported in the latter model [178], whereas no differences in G protein, receptor, or AC levels were reported by Okumura et al. [5].

The greatest effect of AC5 deletion is on parasympathetic regulation of cAMP. Deletion of AC5 results in a complete loss of acetylcholine-mediated (Gi) inhibition and a significant reduction in Ca^{2+}-mediated inhibition of cAMP production [5]. This corresponded with a reduction in the effects of muscarinic agonists on LV ejection fraction and heart rate in $AC5^{(-/-)}$ mice. Baroreflexes were also attenuated. These effects on parasympathetic regulation of cardiac function may partially explain the odd increase in basal heart rates observed in $AC5^{(-/-)}$ mice [5, 178].

Chronic activation of cAMP signaling by overexpression of β-AR, Gsα, or PKA results in cardiomyopathy [179–181]. Thus limiting cAMP under stress conditions should be beneficial. Certainly the use of β-blockers for the treatment of congestive heart failure is consistent with this notion. Similarly, disruption of AC5 under stress conditions (pressure overload by thoracic banding) is protective against heart failure, potentially by increasing Bcl-2 and reducing myocardial apoptosis [182]. AC5 disruption protects against other forms of stress as well. $AC5^{(-/-)}$ mice have increased lifespan (~30%) and are protected against age-induced cardiac myopathy (which includes hypertrophy, apoptosis, fibrosis, and reduced cardiac function) [183]. AC5 disruption leads to a stimulation of the Raf/MEK/ERK pathway and an upregulation of superoxide dismutase, which may play roles in extending lifespan and resistance to oxidative stress [183].

The deletion of AC6 results in a somewhat different phenotype from that of AC5. Both isoforms are expressed equally at birth but in adult heart AC5 is dominant [184, 185]. Deletion of type 6 AC [186] resulted in no change in cAMP levels under basal conditions but cAMP levels were reduced by ~60% in left ventricular homogenates and by ~70% in cardiac myocytes under stimulated conditions. In addition, cardiac myocytes from $AC6^{(-/-)}$ show reductions in PKA activity (40%), Akt activity (60%), phospholamban phosphorylation (45%), and βAR-stimulated LV contractile function (~80%). The more severe decreases in AC activity and cAMP signaling compared

to AC5$^{(-/-)}$ are likely due to a dramatic decrease in AC5 protein levels by proteosomal degradation, although AC5 mRNA levels were unchanged. Thus these animals represent a functional double knockout of AC5 and AC6. Conclusions can still be made about roles for AC6 from these animals, particularly in regard to calcium handling. Deletion of AC6 decreased the Ca^{2+} affinity of SERCA2a by 3.5-fold and reduced caffeine-stimulated Ca^{2+} transients by 50%. These properties cannot be attributed to the decrease in AC5, since a small increase (not decrease) in Ca^{2+} uptake was observed in AC5$^{(-/-)}$ [178].

Overexpression of AC5, 6 and 8 has been examined in the heart with somewhat differing results. Overexpression of AC5 in wild-type mice led to increased basal cAMP, PKA activity, phosphorylated phospholamban, and baseline heart rates, without an enhancement of β-adrenergic receptor signaling or changes in global cardiac function [187, 188]. While in mice overexpressing AC6, basal heart rate and contractile function were unchanged, but cardiac responsiveness to β2-adrenergic receptor stimulation was increased [189]. In pigs, intracoronary delivery of an adenovirus expressing AC6 increases global LV contractile function with increased β_2-adrenergic receptor responsiveness and LV contractile function [190]. What is unclear in these overexpression models is whether the differences observed between AC5 and 6 point to abnormal coupling of these isoforms to various signaling pathways or an enhancement of their physiological roles in the heart.

Overexpression of AC8 enhanced AC activity 7- to 8-fold in cardiac membranes, increased basal PKA activity, and displayed Ca^{2+}-stimulation [191]. Ca^{2+}-stimulated AC8 is not normally expressed in the heart, yet AC8 overexpression had no deleterious effects on global cardiac function. Basal contractile rates and cardiac function (as measured by echocardiography) were unchanged despite elevated cAMP [188, 191]. However, recordings using cardiac catheterization or in isolated perfused hearts, measured a 2-fold increase in cardiac contractility under basal conditions [188, 191, 192], but no response to β_2-adrenergic receptor stimulation [191]. AC8 overexpression resulted in an increased Ca^{2+} sensitivity to cardiac contraction and faster SR uptake of Ca^{2+}, but no increase in L-type Ca^{2+} whole cell current [192]. These changes likely mediate the increased exercise capacity on treadmill testing by mice overexpressing AC8.

Paradoxically, overexpression studies paint a very different picture concerning the roles of cAMP in heart failure. Although AC5 deletion is protective against heart failure (in thoracic banding models), overexpression of AC5 or 6 improves survival rates in Gq-overexpression models. Overexpression of Gq is a cardiomyopathy model and leads to a decrease in cardiac responsiveness to catecholamines, reduced LV function, and decreased survival rates. Overexpression of AC5 or 6 improves these markers of heart failure, restoring basal cardiac AC activity, cardiac contractility, cardiac responsiveness to catecholamine stimulation, and survival rates [187, 193–195]. During congestive heart failure in pigs, AC6 overexpression increases LV function and attenuates deleterious LV modeling [196]. These studies suggest a beneficial role for AC5 and 6 in the pathogenesis of heart failure which is in contrast to the AC5 knockout.

In summary, AC5 and 6 are very closely related AC isoforms in terms of stimulation by Gsα and Gβγ and inhibition by Ca^{2+} and PKA. The differences in Giα regulation are subtle in that basal activity of AC5, but not AC6, is inhibited by Giα [34]. Both isoforms are more sensitive to Giα inhibition when weakly stimulated by Gsα; with increasing activation resulting in decreased Giα inhibition. The other major difference is in PKC regulation. AC5 is stimulated by PKC (α and ζ), while AC6 is inhibited by PKC (δ and ε). Thus both isoforms are highly regulated with numerous inhibitory inputs to carefully control cAMP levels. Cyclic AMP produced by endogenous AC5 may be harmful under stress conditions such as heart failure or aging; however, both AC5 and 6 overexpression can mitigate harmful effects of Gq overexpression. Clearly the heart has many ways to finely control the production and utility of cAMP.

Conclusions

Although AC expression patterns dictate much of the observed specificity in controlling physiological functions, clearly the regulation of individual AC isoforms is also an important factor. Another manner in which cAMP production may be fine-tuned for specific signaling pathways is by the creation of cAMP microdomains [197] or the formation of higher-order signaling complexes [111]. The latter strategy likely involves the use of A-kinase anchoring proteins to directly tether cAMP production to downstream effector molecules. This has recently been shown for AC5 and AKAP79, where complexes have been detected in rat brain tissue [110]. This complex facilitates phosphorylation of AC5 and sets up a negative feedback loop for cAMP production. Several strategies have been proposed for the creation of cAMP

microdomains or gradients, including diffusional barriers for cAMP, enzymatic barriers created by phosphodiesterases, or buffering of cAMP by PKA [57, 198]. Finally, the lipid composition of the plasma membrane itself may guide the formation of specific complexes either within or excluded from regions of high cholesterol and sphingolipids; all but AC2, 4, and 7 have been found within lipid rafts [for review see, 57]. Finally, the question of overlapping functions of ACs is still difficult to determine. The very nature of knockout and overexpression studies leads to the possibility of compensation at many levels. Without isoform-specific AC inhibitors and/or high quality specific AC antibodies, there are many open questions left to answer with regard to the physiological roles for distinct AC isoforms.

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