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Regulation and Role of Adenylyl Cyclase Isoforms

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■ **Abstract** At least nine closely related isoforms of adenylyl cyclases (ACs), the enzymes responsible for the synthesis of cyclic AMP (cAMP) from ATP, have been cloned and characterized in mammals. Depending on the properties and the relative levels of the isoforms expressed in a tissue or a cell type at a specific time, extracellular signals received through the G-protein-coupled receptors can be differentially integrated. The present review deals with various aspects of such regulations, emphasizing the role of calcium/calmodulin in activating AC1 and AC8 in the central nervous system, the potential inhibitory effect of calcium on AC5 and AC6, and the changes in the expression pattern of the isoforms during development. A particular emphasis is given to the role of cAMP during drug and ethanol dependency and to some experimental limitations (pitfalls in the interpretation of cellular transfection, scarcity of the invalidation models, existence of complex macromolecular structures, etc).

INTRODUCTION

Understanding of the now classical cyclic AMP (cAMP) signaling pathway, once considered simple and straightforward, has become very complex indeed. One reason is that cAMP acts not only by promoting protein phosphorylation via activation of a cAMP-dependent protein kinase (PKA) but also by inducing protein-protein interaction independently of any phosphorylation. Another reason for this complexity is the extreme variety of potential regulators of cAMP synthesis and degradation, caused by the multiplicity of phosphodiesterases ($\leq$40) and adenylyl cyclase (AC) isoforms.

This review deals with the latter enzymes, which convert ATP into cAMP. To date, at least nine closely related isoforms of AC (AC1–AC9) and two splice variants of AC8 have been cloned and characterized in mammals. They share a large sequence homology in the primary structure of their catalytic sites and the same predicted three-dimensional structure. Each AC isoform and variant consists of two hydrophobic domains (with six transmembrane spans) and two cytoplasmic

domains resulting in a pseudosymmetrical protein. The cytoplasmic domains (C1 and C2), which constitute the catalytic site, are subject to intracellular regulations specific for each subtype. In particular the catalytic activity, as well as the sites for interaction with forskolin (FSK) and Gαs, require both cytoplasmic moieties. Elucidation of the structure-function relationship of ACs has markedly progressed over the last 5 years thanks to a series of recent studies, including studies by crystallography and site-directed mutagenesis. The reader is referred to several more detailed recent reviews dealing with those aspects (1–6). General references up to 1997 can be found in Reference 7.

The present text, which does not try to be exhaustive, focuses on the potential physiological regulation of each AC isoform. The distinct properties of these ACs allow them to play an interpretative role in signal transduction instead of being just linear pathways for the activity of the G-protein-coupled receptors. Thus, depending on the properties and the relative levels of the isoforms expressed in a tissue or a cell type, extracellular signals received by the G-protein-coupled receptors can be differentially integrated. Many of the regulatory mechanisms reported here have yet to be rigorously assessed, and a few caveats should be kept in mind. As we discuss below, the lack of good specific antibodies precludes any attempt at identifying the subcellular localization of the isoforms or their relationship to the cytoskeleton or the scaffolding systems. Spurious stoichiometry often results from transfection experiments and may lead to erroneous interpretations. Furthermore, we still lack data from knockout experiments with all of the various isoforms of AC [only the knockout model for AC1 has been fully reported to date (8–10)]. Because cAMP plays a key role during development, conditional knockouts in various organs are probably required. This is all the more necessary because a compensatory increase of one isoform to supplement the loss of another cannot be excluded. From this point of view, it is striking that no human pathology linked to the alteration of a cyclase isoform has been reported so far, even though the pathology linked to other components of the cAMP signaling pathway (e.g. receptors and G proteins) is known.

TISSUE DISTRIBUTION AND PROPERTIES OF THE ADENYLYL CYCLASES

The mammalian ACs are a large family of enzymes encoded by at least nine independent genes, and the different subtypes are expressed as discrete patterns in only a limited number of tissues. The regulatory properties of the different ACs are listed in Table 1. In addition to their ability to respond to Gαs and to FSK, the different isoforms can receive signals from a variety of sources, including other G proteins, e.g. Gαi and G$\beta\gamma$, protein kinases (PKA, PKC, and calmodulin (CaM) kinase), phosphatases (calcineurin), calcium, and Ca^{2+}/CaM, and these isoforms are able to support and integrate differential regulatory pathways through cross-talk with other signal transduction systems.

TABLE 1 Regulatory properties of mammalian adenylyl cyclases

	Response to cAMP signaling pathway component[a]					
AC isoform	**Gαs**	**Gαi**	**G$\beta\gamma$**	**FSK**	**Calcium**	**Protein kinases**
AC1	↑	↓ (CaM- or FSK-stimulated activities)	↓	↑	↑ (CaM) ↓ (CaM kinase IV)	↑ PKC (weak) ↓ (CaM kinase IV)
AC2	↑	→	↑ (when stimulated by Gαs)	↑		↑ (PKC)
AC3	↑	↓		↑	↑ (CaM) (in vitro) ↓ (CaM kinase II)	↑ (PKC) (weak) ↓ (CaM kinase II)
AC4	↑		↑	↑		↑(PKC)
AC5	↑	↓	↓ ($\beta 1\gamma 2$)	↑	↓ (<1 μM)	↓ (PKA) ↑ (PKCα/ζ)
AC6	↑	↓	↓ ($\beta 1\gamma 2$)	↑	↓ (<1 μM)	↓ (PKA, PKC)
AC7	↑		↑	↑		↑ (PKC)
AC8	↑	↓ (Ca^{2+} rises)		↑	↑ (CaM)	→ (PKC)
AC9	↑	↓		↑ (weak)	↓ (calcineurin)	
sAC	→	→		→		

[a]↑, positive regulatory response; ↓, negative regulatory response; →, neutral response.

Determination of the precise expression pattern of each of the nine isoforms was difficult due to the low expression levels and the lack of specific high-affinity antibodies. Thus, tissue distribution has generally been determined by mRNA expression studies. All isoforms are expressed in brain cells, although the expression of any individual isoform is restricted to discrete structures of the central nervous system as demonstrated by in situ hybridization (11–17). In the peripheral tissues, the pattern of AC expression is more specific. Table 2 summarizes this distribution.

ADENYLYL CYCLASES AND BRAIN FUNCTION

Although all of the AC isoforms characterized so far are expressed in the central nervous system, the most abundant ones are the Ca^{2+}/CaM-stimulated AC1, the Ca^{2+}-insensitive AC2, and the Ca^{2+}/calcineurin-inhibited AC9 (12, 13, 18, 19).

Interestingly, research on the functional specificity of ACs in mammals has been driven by results obtained in *Drosophila melanogaster* concerning mutations that

TABLE 2 Tissue distribution of the mammalian adenylyl cyclases

AC isoform	Tissue distribution[a]	Potential associated function	Human chromosome
AC1	Brain (neuron), adrenal gland (medulla)	Circadian rhythm, synaptic plasticity, learning, memory, LTP, drug dependency	7p12
AC2	Brain, skeletal muscle, lung [heart]	Synaptic plasticity, arrest of cell proliferation	5p15
AC3	Brain, olfactory epithelium, male germ cells, pancreas, BAT [uterus, heart, lung]	Odorant stimulation	2p22–24
AC4	Brain [heart, kidney, liver, lung, BAT, uterus]		14q11.2
AC5	Heart, brain [kidney, liver, lung, uterus, adrenal, BAT]		3q13.2–q21
AC6	Widespread	Cell proliferation	12q12–13
AC7	Brain, platelets, widespread	Ethanol dependency	16q12–13
AC8	Brain, lung, [testis, adrenal, uterus, heart]	Synaptic plasticity, LTP, drug dependency	8q24
AC9	Brain, skeletal muscle, widespread		16p13.3
sAC	Testis [other tissues]		1q24

[a]Underlined tissue expressed this isoform at high level; [ ], low level of expression determined by PCR; BAT, brown adipose tissue.

affect memory. Mutant *rutabaga* flies fail to avoid a trained odor and appear to be deficient in a calcium-activated AC (20–22). This form of AC, characterized and cloned by Levin et al (23), appears most similar to the mammalian AC1, except for a long carboxy-terminal half, the function of which is unknown. A single-point mutation at position 1026 is sufficient to cause the complete loss of cyclase activity in vitro and to result in the biochemical and phenotypical defects seen in vivo. All of these observations point to a very important role for AC1 in learning and memory.

In the mammalian brain, both AC1 and AC2 have been implicated in synaptic plasticity. mRNA for these isoforms is highly expressed in regions associated with learning and memory, including the hippocampus, cerebral cortex, and cerebellum (9, 12, 13, 19, 24).

The various forms of long-term potentiation (LTP) in the hippocampus require entry of calcium through *N*-methyl-D-aspartate and voltage-gated Ca^{2+} channels. The enrichment of Ca^{2+}-sensitive ACs in areas exposed to high intracellular free calcium such as dendritic spines (15) leads to facilitation in the reception and

propagation of signals mediated by cAMP such as those that occur during the induction of the different forms of synaptic plasticity. The presence of Ca^{2+}-sensitive AC in the dendrites suggests that an increased level of postsynaptic Ca^{2+} concentration might upregulate the cyclase activity and control the propagation of the signal. Additionally, LTP and spatial learning are affected in AC1-mutant mice (9, 10, 25). Disruption of the *AC1* gene in mice results in a loss of calcium-sensitive AC activity in the cerebellum, cortex, and hippocampus equaling 62%, 38%, and 46%, respectively (10, 25, 26), with no obvious anatomical differences. The mutant mice exhibit a dampening of the long-term potentiation in hippocampus and a partial blockade in cerebellum tissue (9). A spontaneous loss-of-function mutation in the *AC1* gene has also been reported in *barrelless* mice (27, 28). This mutation is associated with a partial failure of patterning of the whisker-to-barrel pathway resulting in an incomplete formation of barreloids and an aberrant segregation of thalamocortical afferent arborization. It is therefore very likely that the AC1-signaling pathway plays an important role in pattern formation of the brain and in some forms of synaptic plasticity including learning and memory storage. Whether this might be related to the pattern of appearance of AC1 during development as we reported (24) merits further investigation. In fact, synaptogenesis and expression of LTP in rodents occur during the first 3 weeks after birth, concomitantly with an increased expression of AC1 mRNA in the cerebellum and hippocampus (24, 26).

The hypothesis of the implication of the Ca^{2+}/CaM-stimulated AC in the generation of signals necessary for late-LTP (L-LTP) and long-term memory (LTM) has been tested using mice lacking either AC1 or AC8. Both types of mice exhibit normal L-LTP and LTM. In contrast, double-knockout mice lacking both AC1 and AC8 have no more L-LTP or LTM. On the other hand, infusion of FSK in the hippocampus of these mice restores normal LTM by increasing the cAMP level through other AC isoforms (29).

In a very recent paper, Laurent-Demir et al (30) analyzed extensively the distribution of AC1 and AC2 in the different regions of the hippocampus, particularly in the dentate gyrus at the subcellular level. They showed that AC1 mRNA is preferentially expressed in both cellular and molecular layers of the dentate gyrus, suggesting that it could be regulated by the presence of the afferent innervation, whereas AC2 mRNA is preferentially localized over the dentate granule cell bodies. Denervation of the cellular and molecular fields of the dentate gyrus by destruction of the entorhinal cortex fiber resulted in a dramatic reduction of the AC1 mRNA (30), without any effect on AC2 mRNA. This effect is transient, being maximal at 5 days and returning to control values within 30 days, when new synapse connections have been formed.

AC9 is the only isoform that is potentially inhibited by the calcium-dependent phosphatase calcineurin (cf 31). Invalidation of this isoform in *Caenorhabditis elegans* prevents the Gαs-induced neuronal-cell death (32, 33). AC9 might therefore be an important regulator, especially related to signaling in motoneurons. It is tempting to speculate that AC9 may also play such a role in mammals,

although there is no evidence for it at present. This isoform is expressed in various regions of the brain, including the hippocampus, dentate gyrus fields CA1 and CA3, the hypothalamus (supraoptic nucleus), and several peripheral endocrine tissues such as those of the adrenal cortex, ovaries, and testis. In the hippocampus, its expression is at the same level as AC1 and AC2. At the ultrastructural level, AC9 appears localized in the postsynaptic dendritic profiles of the neocortex and hippocampus (18). Recently, Antoni et al demonstrated the expression of AC9 in the somatodendritic compartment of nerve cells and its colocalization with calcineurin (34), a molecule that has been implicated in both long-term depression (35) and LTP (36). Finally, the presence in the same subregion of the hippocampus of calcium/CaM-stimulated ACs (AC1 and AC8), CaM kinase II, calcineurin, and calcium/calcineurin-inhibited AC (AC9) makes this isoform potentially important for the homeostasis of brain function and particularly LTP.

CALCIUM-INHIBITABLE ADENYLYL CYCLASES 5 AND 6

All AC activities are inhibited by high, nonphysiological concentrations of calcium in the submillimolar range, possibly by competition with magnesium. In certain tissues, including pituitary gland tissue, platelets, and heart tissue, AC activity has been reported to be inhibited by lower concentrations of calcium, in the submicromolar range (37). The affinity of Ca^{2+} for this site on AC is $\sim 0.2\ \mu M$ in the presence of a 5000-fold excess of Mg^{2+} (38). This inhibition appears to be caused by the presence of either one of the two closely related cyclase isoforms AC5 and AC6, which were cloned from heart (39, 40), liver and kidney (41), striatum (42), Reuber hepatoma (43), or NCB-20 cells (44). When expressed in a variety of recipient cell lines, these isoforms are inhibited by submicromolar concentrations of calcium, and the inhibition is additive to that elicited by receptors acting via $G\alpha i$. The physiological relevance of such an inhibition has been confirmed in a few instances in intact cells (45). For example, sympathetic stimulation of cardiac tissue elevates cAMP, which in turn leads to calcium influx through L-type channels (46). It has been proposed that the wave of calcium could lead to a rhythmic dissipation of the cAMP signal (47, 48). The capacitative entry of calcium (secondary to the emptying of the intracellular Ca^{2+} pool, e.g. by the use of the Ca^{2+} ATPase inhibitor thapsigargin) has been proposed to play a major role in regulating, positively (AC1 or AC8) or negatively (AC5 or AC6), AC activity (47, 49). It is not the only mechanism by which a change in cytosolic Ca^{2+} concentration can influence AC activity because the capacitative entry of calcium plays a minor role, if any, in excitable tissues such as heart and striatum, in which inhibitable AC activities are the highest.

The proximity of the AC to the site of calcium increase is probably the critical factor, as is nicely shown by Gao et al (50), who demonstrated that the subunits of L-type calcium channels and AC were colocalized along T tubules in intact cardiac myocytes.

The two calcium-inhibitable ACs, AC5 and AC6, possess numerous common characteristics. Both are activated by Gαs and FSK and are inhibited by Gαi. The $\beta\gamma$ subunits of G proteins are thought to be inactive (41), but recent data obtained in transfected COS-7 cells showed that specific $\beta\gamma$ dimers could inhibit AC5 and AC6 (51). Both AC5 and AC6 can also be inhibited by phosphorylation through PKA (52–54), a phenomenon that could account for desensitization of the AC activity by a short negative-feedback loop, as demonstrated in chick hepatocytes (53). However, AC5 and AC6 differ in many other instances. Using purified PKC and AC, Kawabe et al have demonstrated that the PKCζ isoform can directly phosphorylate AC5, leading to a 20-fold increase in AC activity (55). In vitro, the α and ζ isoforms directly phosphorylate and activate AC5. Although PKCα is a less potent activator of AC5, the two PKCs are additive in their capacity to activate AC5. Phosphorylation of AC5 by the different PKCs is particularly important in heart tissue, where growth factors including insulin are able to regulate cAMP production and contractility. Whereas the ζ isozyme activates AC5 in a calcium-independent manner, the α isozyme requires calcium. This provides another mechanism for the calcium-mediated regulation of AC5 activity in heart tissue. In cells expressing AC5, insulin augments cAMP production through phosphatidyl-inositol-triphosphate activation of the PKCζ (55). In heart tissue, all hormones or growth factors that activate phosphatidyl-inositol 3-kinase leading to the formation of phosphatidyl-inositol-3,4,5 triphosphate (which activates PKCζ) would be able to control cAMP production through a direct activation of AC5. In contrast, phorbol esters have been shown not to affect AC6 activity in several systems (56–58) or even to decrease it (59, 60), possibly by an interaction on the N-terminal part of the protein. Finally, several reports have stressed a selectivity of AC5 vs AC6, for stimulation by purinergic agonists (61) or epidermal growth factor (62) or inhibition by dopamine via the D3 receptor (63).

In addition, it should be noted that both AC5 (64) and AC6 (41, 60) can exist as multiple forms or variants, which adds an obvious degree of complexity. AC5 and AC6 are not evenly distributed in the various tissues of the body. Although the AC6 isoform is found in all organs, the distribution of AC5 is more restricted because it is mainly found in the striatum and in the heart. We (65) and others (66) have demonstrated that, whereas the two major isoforms in rat heart tissue, AC5 and AC6 mRNAs, are equally present at birth, the AC5 mRNA becomes predominant in the adult rat heart. Since the two forms are clearly related and are similarly regulated by calcium, there is no obvious physiological correlation for this genetic switch. One could hypothesize that the shift from AC6 to AC5 is related to the state of cellular differentiation or to the ratio of cardiomyocytes to fibroblasts (67). During heart failure, the level of AC6 decreases while that of AC5 remains constant (68). Cardiac overexpression of AC6 in mice leads to an increased sensitivity to epinephrine and an enhanced cardiac function (69) and can improve heart function in murine cardiomyopathy due to Gαq overexpression (70). In contrast, cardiac overexpression of AC5 leads to increased AC activity, without further sensitivity to epinephrine, and an impaired heart function (J Hanoune &

N Defer, unpublished data). Among the various reasons for such discrepancies, one could cite the various specific characteristics of AC5 or AC6 described above, as well as the possibility of different subcellular localization (see conclusion).

In the kidney, distribution of AC6 in the medulla (71) is mainly localized to the collecting tubule and thick ascending limb, in which AC6 is highly expressed (72). In a very detailed series of publications (see review in 73), the group of Chabardes has assessed the relative levels of AC5 and AC6 in different segments of the rat renal tubule by qualitative reverse transcription-polymerase chain reaction. AC6 is present all along the nephron, whereas the expression of AC5 is restricted to the glomerulus and the cortical and outer-medullary portions of the collecting duct. Calcium is known to interfere with urine concentration, and the mechanisms implicated for its action are finely tuned through capacitative Ca^{2+} entry and/or intracellular calcium release. Furthermore, different agonists inducing similar increases in overall Ca^{2+} elicit a variable inhibition of AC activity (73). It is interesting that, in two animal models [invalidation of Gαs in mice (74) and the homozygous Brattleboro rat with hereditary diabetes insipidus from lack of antidiuretic hormone (71)], AC isoforms have been found depressed when one would have expected some compensatory increase.

CELL DIFFERENTIATION

In many cell types, the intracellular concentration of cAMP affects progression within the cell cycle. Although cAMP can have a growth-stimulatory effect (75), elevation of intracellular cAMP usually suppresses mitogenic signaling through PKA phosphorylation of Raf (76). The molecular identity of the isoform expressed in the cell and the ability of this isoform to integrate signals from the growth factor receptors are determinants for cell proliferation. In NIH3T3 cells, the ectopic expression of AC2, which can be stimulated by PKC, results in the inhibition of cell cycle progression and blocks the H-ras-induced transformation of the cells, whereas overexpression of AC6 has no effect on the cell cycle rate (77). In this context, it is noteworthy that AC2 and AC7 are expressed in postmitotic neuronal cells and in platelets, whereas most of the tissues that retain the ability to proliferate do not express significant amounts of these isoforms.

The importance of cAMP in cell differentiation has been further illustrated in various types of cells where differentiation is accompanied by the expression of specific AC isoforms. For example, mesodermal differentiation of pluripotent P19 cells is accompanied by the upregulation of AC2, AC5, and AC6, whereas neuronal differentiation is accompanied by the upregulation of AC2, AC5, and the Ca^{2+}/CaM-stimulated AC8 (78, 79). In both cases, cell contact and inhibition of cell proliferation are required before differentiation when AC2 is increased. Since this isoform has the highest specific activity and is activated by PKC, it is tempting to hypothesize that AC2 expression is a prerequisite for the arrest of cell proliferation allowing cell differentiation.

Many studies have established that motoneuron activity influences gene expression in developing and adult skeletal muscle fibers. Mouse gastrocnemius muscle is composed of ~100% type-II fast-twitch fibers. Denervation results in an increased amount of the slow-twitch fibers. In parallel, the levels of AC2 and AC9 mRNA decrease, whereas those of AC6 and AC7 increase (80). From our observation as well as those of Torgan & Kraus (81), it appears that AC2 and AC9 are restricted to fast-twitch fibers, whereas AC7 and AC6 are associated with reprogramming after destruction of the motoneuron. Those observations indicate that changes in the AC isoforms and probably their intracellular sublocalization may play a crucial role in muscle cell differentiation and muscle development.

Neural stimulation of brown adipose tissue (BAT) increases norepinephrine-stimulated AC activity and generates heat in response to cold stress. Immediately after birth, BAT undergoes an adaptive period, which involves the proliferation of the brown adipocyte precursors and their differentiation into mature cells (82). During this period, the ability of β-adrenergic ligands to stimulate AC activity is increased, peaking at 7 days. Among the different ACs expressed in BAT, only the mRNA level of AC3 is increased during the perinatal period, and the expression of this mRNA follows the changes in the AC activity (83, 84). Finally, the dramatic upregulation of AC3 mRNA by sympathetic nerve stimulation in the adult or by cold stress during the first postnatal weeks strongly suggests that this AC subtype plays an important role in BAT recruitment.

ADENYLYL CYCLASE AND THE MATURATION OF SPERMATOZOA

Many sperm functions, including maturation, motility and acrosome reaction, are thought to be mediated by cAMP, although the exact role of the cAMP-dependent pathway during spermiogenesis is largely unknown. cAMP-mediated mechanisms are involved in the regulation of gene expression in germ cells and a cAMP-responsive element modulator plays a crucial role in the regulation of transcription of genes necessary for spermatid maturation (85, 86). At least two AC isoforms are present in male germ cells, the olfactory AC3 isoform and a soluble AC form, but their respective roles are still unknown.

AC activity has been detected in cytosolic extracts from the early stages of spermatid cells. This activity is insensitive to any of the known activators of the membrane-bound AC activity and is dependent on the presence of the divalent cation Mn^{2+}. It has a low molecular mass (42 to 69 kDa). Based on these properties, the soluble form was predicted to be molecularly distinct from the other mammalian ACs (87). Very recently a soluble form (sAC) has been isolated from rat testis, the catalytic domains of which are closely related to those of cyanobacterial and myxobacterial ACs (88). This sAC is insensitive to FSK and to GTP, but its activity is highly dependent on the presence of Mn^{2+}. This sAC is encoded by a unique gene located on human chromosome 1. The full-length cDNA encoding sAC predicts

a protein of 187 kDa. When expressed in transfected cells, the Mn^{2+}-dependent activity is elevated in the particulate fraction. This sAC is widely expressed within the body at low levels, but a high level of expression occurs only in male germ cells. The expression of mRNA starts in pachytene spermatocytes and increases through spermiogenesis (89). This suggests that the protein is still present in mature spermatozoa, where it may have a specific function in male fertility.

AC3, initially identified as the specific isoform of the olfactory neuroepithelium (90, 91), was also found expressed in rat male germ cells, from pachytene spermatocytes to spermatids. A detailed study, performed by comparing the immunological staining in sections and in isolated germ cells, indicated that AC3 was localized on the acrosomal membranes of the round and the mature spermatids (92). Such a localization during spermiogenesis suggests a role of this AC in the biogenesis of acrosome and possibly in gamete production and fertilization. Several proteins of the olfactory signaling pathway, including the putative odorant receptor, Gαolf, AC3, and the olfactory nucleotide-gated channel (93, 94), as well as the olfactory neuron transcription factor Olf-1 (95), are expressed in rat testis in the same cell subpopulations (96–101). Taken together, these observations are consistent with the hypothesis that the signal transduction system used in olfaction may also be used in the function of the mature spermatozoa and may be implicated in sperm chemotaxis during fertilization.

ADENYLYL CYCLASES AND DRUG DEPENDENCY

Acute administration of morphine or opioids causes a decrease in AC activity via the Gi pathway. Chronic administration leads to the classical states of tolerance and dependence (102), which include behavioral and physical signs behind which is a complex array of biochemical phenomena. Among the various mechanisms underlying these phenomena, one of the most studied since the early work of Sharma et al on NG 108-15 cells (103) is an upregulation of the cAMP system, including AC, PKA, and the transcriptional factor cAMP response element-binding protein (CREB). Thus, after long-term in vivo morphine treatment followed by administration of the antagonist naloxone, one can observe an increase in AC activity in the cerebral cortex (104, 105), the striatum (106, 107), and the locus coeruleus, but not in the cerebellum, which is devoid of receptor. For example, after morphine withdrawal, there is a 30% increase in basal- and FSK-stimulated AC activity in the striatum, an increase which is no longer seen in receptor-deficient animals (106). It is also noteworthy that, in extensive studies of the critical role of cAMP in morphine dependence in the rat, Lane-Ladd et al found an increase in AC1, AC8, PKA, and CREB in the locus coeruleus, a major site responsible for the physical signs of dependence (17). These data confirm our earlier results on the involvement of AC8 in the locus coeruleus (108).

These changes have been reproduced in various cell culture systems, and one can more or less readily observe an upregulation of AC activity which has the

following characteristics: (*a*) it can be observed after treatment with a variety of inhibitory ligands, including endomorphins (109), κ receptor agonists (110, 111), adenosine (112), muscarinic agents, and somatostatin (113); (*b*) it is long-lived; (*c*) depending on the system studied, it may or may not involve a transcriptional step (114); (*d*) the $\beta\gamma$ subunits seem to play a specific, although not direct role (115–117); (*e*) the effect may be specific for certain isoforms of AC (118, 119). Along the same line, it is worth noting that opiates can have bimodal acute effects on cAMP production in the myenteric plexus, depending on the concentration used (120).

Most of the recent results originate from studies involving artificial, transient, or permanent transfections of various AC isoforms. The nature and stoichiometry of the components involved may provide spurious results, and therefore these data should be considered with caution until they can be directly confirmed in better models. However, the upregulation of the AC system is at present the best explanation available for the dependent state after opiate administration.

Interestingly, the recently described model of cannabis withdrawal confirms those data. The availability of a specific CB1 receptor antagonist, SR141716A, has resulted in the development of an in vivo model of cannabis abstinence. After 6 days of treatment with Δ-9 tetrahydrocannabinol, followed by the administration of the antagonist, the mice exhibit several somatic signs, which could be interpreted as being part of a withdrawal syndrome. Interestingly, the same animals exhibit a 100% increase in the basal, FSK, and calcium/CaM-stimulated AC activity in the cerebellum (rich in CB1 receptors) but not in the cortex or the striatum (121). More recently (122), we have shown that this increase in the activity of the cAMP pathway is directly associated with the expression of the physical signs of withdrawal. Indeed, microinjection of Rp-cAMP (an inhibitory cAMP analog) in the cerebellum markedly attenuates the expression of the withdrawal syndrome. In COS-7 cells cotransfected with CB1 receptors and individual AC isozymes, Rhee et al (123) reported a selective superactivation for AC1, AC3, AC5, AC6, and AC8, but not for AC2, AC4, and AC7. Interestingly, the superactivation of AC5 was abolished by treatment with pertussis toxin and by cotransfection with the C terminus of the β-adrenergic receptor kinase, which might imply a role for the $\beta\gamma$ subunits in the present system too.

CYCLIC AMP PATHWAY AND ALCOHOL ABUSE AND ADDICTION

Alcohol is the most widely abused drug in the world, and its effects as a psychoactive drug are unique since they are not initiated through specific receptors. Chronic exposure of cells in culture or whole animals to ethanol produces notable adaptive changes in the function of the AC signaling pathway (124–126). Since the first demonstration in liver (127), studies using membrane preparations from various organs or cells in culture have shown that ethanol significantly

enhanced Gs-stimulated AC activity (128–131). Brief exposure to ethanol increased basal and receptor-stimulated cAMP production in neuronal cells in culture as well as in isolated brain preparations (124, 132–135). By contrast, a decrease in β-adrenoceptor-stimulated AC activity was observed in cerebral cortical membranes of chronically ethanol-treated mice (136). Cells or tissues that have been exposed chronically to ethanol exhibit a reduced responsiveness of AC to agents that usually enhance its activity. For example, AC activity in platelets of alcoholics exhibits a decreased responsiveness to PGE1 (132). A selective effect of ethanol on cAMP synthesis by a specific AC isoform has been demonstrated using HEK293 cells transfected with different types of AC. Cells transfected with AC7 are the most sensitive to ethanol, whereas cells transfected with AC1 or AC3 are not, and only modest effects are observed in cells transfected with AC2, AC5, and AC6 (131, 137). Using assays on membrane preparations, no differential sensitivity to ethanol has been observed, suggesting no direct effect of ethanol on AC (126). The feature linking the different AC isoforms sensitive to ethanol is their sensitivity to phorbol ester and their possible phosphorylation by PKC, as has been proposed for the phosphorylation of AC7 through PKCδ (138).

A brief exposure to ethanol increases basal and receptor-stimulated cAMP production in neuronal cells in culture (132, 134) and causes an increase in both phosphorylation of CREB and cAMP-responsive element binding activity in rat cerebellum (139). In contrast, chronic exposure to ethanol results in an adaptative desensitization of cAMP production in cells in culture (140), and a decrease in the phosphorylation of CREB in granule cells of rat cerebellum (141). During the prolonged exposure to ethanol the catalytic subunit of PKA, Cα, is translocated to the nucleus where it remains for as long as ethanol is present (142). These observations and genetic data indicative of a cAMP signaling pathway in alcohol-related behavior (143, 144) favor a role for cAMP-dependent gene transcription in the molecular mechanism implicated in alcoholism.

Parsian et al (145) observed that basal and fluoride-stimulated platelet AC activity of alcoholics are lower than that in controls. Moreover, Ikeda et al (146) suggested that Gpp(NH)p and FSK-stimulated platelet AC activity may distinguish between subtypes of alcoholics (those who develop a negative mood in response to drinking, those who continue drinking despite health effects, and those who become violent while drinking). Recently, Rastma et al (147) reported that the FSK-stimulated AC activity is considerably lower in platelets of children of alcoholics (children that are at high risk of acquisition of alcoholism but not yet consuming alcohol). Furthermore, the reduced AC activity was observed only in platelets of children from multigenerational families prone to alcoholism. Thus, the platelet AC, most probably AC7, may represent a trait marker for predisposition to alcoholism.

The differential distribution in the neurons of brain can contribute to the characteristic spectrum of ethanol's action in an intoxicated individual. AC7 is highly concentrated in the cerebellar granule and Purkinje cell layers, suggesting a presynaptic localization (148). Such a localization for AC7 in brain favors the hypothesis

that this isoform could be implicated in the development of acute and chronic tolerance. Transgenic mice overexpressing AC7 specifically in the central nervous system under the control of the rat synapsyn I gene promoter show behavioral and electrophysiological changes in their sensitivity to ethanol (149). Moreover, the Purkinje cells of these transgenic mice rapidly become resistant to the depressant effects of ethanol on cell firing, which may explain their sensitivity to the uncoordinating effects of ethanol (150).

ADENYLYL CYCLASE AND GENETIC DISEASES

At present, several pathological states are known to be linked to mutations in various receptors or Gα subunits, which result in constitutive activation of cAMP production (151, 152). Whether such hyperfunction could be due to a modification of AC activity itself is an interesting possibility, although no example is known as yet. Conversely, could a pathological state be due to a decrease in cAMP production reflecting a mutation of AC itself? cAMP is probably too important for life for such a mutation not to be lethal if only one AC isoform existed. But, because at least nine different isoforms are known, each coded by a different gene on a different chromosome, it is likely that a mutation restricted to a specific isoform would be compatible with life and might give rise to a unique pathological phenotype. The detailed study that we and others have performed on the chromosomal localization of the various isoforms in humans has not identified, as yet, a potential link between the gene localization of a specific AC and a known disease (6, 153), although there was an unconfirmed claim that a specific form of pseudohypoparathyroïdism could be due to an abnormal AC (154). Interestingly, Leber's congenital amaurosis, the earliest and most severe form of inherited retinopathy, has been recently demonstrated to be linked to mutations of the gene of retinal guanylyl cyclase, leading to the synthesis of a membrane enzyme lacking the cytosolic catalytic moiety (155). Finally, Abdel-Halim et al (156) reported that a two-point mutation in the promoter region of the AC3 gene might be associated with a decrease in the glucose-induced insulin release in spontaneously diabetic rats, possibly through an alteration of AC mRNA transcription.

GENETICALLY MODIFIED MICE TO ASSESS THE FUNCTIONAL ROLE OF THE SPECIFIC ISOFORMS

The in vivo functional relevance of the different ACs can be examined in genetically modified mice. Invalidation of AC1 and AC8 affects long-term memory. Transgenic mice overexpressing AC7 in the brain (149) or AC5, AC6, or AC8 in the heart (69, 157, 158; J. Hanoune & N. Defer, unpublished data) have been created. Although AC5 and AC6 share most of their properties, the phenotypes of the transgenic mice are quite different. Mice overexpressing AC6 exhibit a normal

heart function under basal conditions and an increased cardiac responsiveness to adrenergic stimulation, without any histological abnormality even in older age ($\geq$19 months old). By contrast, mice overexpressing AC5 show no significant modification of the cardiac contractile functions under both basal and catecholamine-stimulated conditions, whatever the method used in young age (157; J. Hanoune & N. Defer, unpublished data). Over the life of the animal, AC5 overexpression results in myocardial damage characterized by cellular degeneration and fibrosis, while the remaining cells undergo compensatory hypertrophy. Moreover, homozygous AC5 mice develop, very early in their lives, a ventricular hypertrophy, which progresses towards a dilated cardiomyopathy. Another difference is the possibility for AC6 and not for AC5 to improve heart failure induced by Gq transgenesis (70, 159).

In contrast to AC5 and AC6, the neural isoform AC8 is stimulated by calcium. To modify the overall balance of activities in the heart, we have generated transgenic mice that express AC8 specifically in the cardiomyocytes. In the hearts of those transgenic animals, AC8 is able to drive cAMP synthesis and to stimulate PKA activity, without any detrimental consequences on global cardiac function. Basal heart rate and contractile function were unchanged. In contrast, upon release of parasympathetic tone, the intrinsic contractility is heightened and unresponsive to further β-adrenergic receptor stimulation (158).

Transgenic mice overexpressing AC7 specifically in brain tissue have also been produced. These mice have a modified sensitivity toward ethanol but also an increased sensitivity to morphine analgesia (149, 160).

PHARMACOLOGY OF ADENYLYL CYCLASES

Unlike phosphodiesterases, ACs never were important targets for drug development. Most of the available pharmacology is still limited to the activating natural product FSK and to the so-called P-site inhibitors. With the increased knowledge of the AC isoforms, one can expect some type-specific agents to become available in the future.

Forskolin

A diterpene extracted from the plant *Coleus forskohlii*, FSK activates all AC isoforms except AC9 and the newly described testis form. Despite a very hydrophobic nature, its action is not limited to the native membrane-bound form of the enzyme because it can readily stimulate some synthetic soluble ACs. FSK binds to the same cleft that contains the active site (161, 162). It glues together the two cytoplasmic domains by a combination of hydrophobic and hydrogen-binding interactions (163). AC9 is nonresponsive to FSK because of a Ser→Ala and a Leu→Tyr change in the binding pocket, a change which can be reversed by site-directed mutagenesis (164). The possibility that the FSK-binding site might accommodate an endogenous FSK-like small molecule is attractive (5).

Adenosine Derivatives

Adenosine and various analogs of the nucleoside known as P-site inhibitors (requiring an intact purine ring) inhibit all isoforms of AC (165). As shown by the group of Johnson (166–169), those derivatives, ordered by decreasing potency, include 2′,5′-dideoxyadenosine-3′-tetraphosphate > 2′,5′-dideoxy-3′-ATP > 2′,5′-dideoxy-3′-ADP > 2′,5′-dideoxy-3′-AMP > 2′-deoxy-3′-AMP > 3′-AMP > 2′-deoxyadenosine > adenosine. The best compounds can directly inhibit AC with K_i values of ~20–40 nM. Owing to the structural relationship between ATP and those P-site compounds, it is noteworthy that the inhibition is noncompetitive, with the apparent potency increasing when the enzyme is activated. The P-site inhibitors form a dead-end complex by binding to the active site in the presence of pyrophosphate, one of the products of the reaction (165). In the basal state the slow step of the reaction is the ATP cycling. Upon activation by Gαs or FSK, the release of PPi becomes partially rate limiting, permitting the binding of P-site inhibitors. There is some difference in the affinity of the various AC isoforms for the inhibitors, although the best inhibitor tested (2′,5′-dd-3′-ATP) was found to be equally efficacious for all isoforms tested (170). Some P-site ligands are cell permeable and have been utilized in a variety of mammalian tissues and isolated cells (171). A striking example is the differentiation of preadipocytes induced by 2′,5′-dd-Ado, which is more rapid and complete than that classically induced by insulin and T3 (171). Along the same line, it is interesting that a cyclic nucleoside phosphonate antiviral agent, derivative of 9-(2-phosphonyl methoxyethyl) adenine, may also act in part by inhibiting AC (172).

Other Potential Cyclase Inhibitors

AC activity has been shown to be inhibited by a variety of compounds: the shell fish toxin domain acid (173), the mycotoxin secalonic acid (174), ceramides (175, 176), derivatives of fatty acids such as 9-hydroxy 10-*trans*, 12 *cis*-octodeca dienoic acid (177), or 12(*S*)-hydroxyeicosatetraenoic acid (178), lithium (179), the antibiotic minocycline (180), the vitamin riboflavin (181), quinones (182), and glycerophosphoinositol (183). However most of the above mentioned effects are obtained at relatively high concentrations and might not be specific for AC.

CONCLUSION

Since the discovery of cAMP by Sutherland in the late 1950s, the amount and the quality of the data produced have confirmed its rank as the archetypal intracellular-signaling molecule. The various proteins involved in the pathway (receptors, G proteins, phosphodiesterases, effectors, regulators, etc) have been cloned and extensively studied, from both the structural and the regulatory points of view. Is the wealth of information that we possess now on the family of ACs the final stone, the harbinger of the end of the cAMP story? Or are we rather stepping into

another domain of biological complexity, requiring new experimental tools and approaches? We believe at this point that at least two major questions remain to be answered.

Is Adenylyl Cyclase Part of a Higher-Order Membrane Structure?

At the simplest level, this question refers to the stoichiometry of the AC moiety with receptors and G proteins. It has been directly assessed in a few systems such as in S49 cell lines (184) or rat ventricular myocytes (185). In the latter system, it appears that for one β-adrenergic receptor, there are 224 Gαs molecules and only 3 AC molecules. This demonstrates that Gαs is in great excess relative to either receptor or effector. Furthermore, the amplification of the signal between receptor and Gs is not sustained at the cyclase level. MacEwan et al (186) have transfected neuroblastoma-glioma hybrid NG 108–15 cells with various amounts of AC2, an isoform that normally does not exist in these cells. They showed that the maximal hormonal stimulation of cAMP production could be increased by raising total levels of AC, thus confirming that AC was the limiting component for receptor-mediated stimulation of AC activity. Along the same line, Gao et al (187) have used recombinant adenovirus to increase the AC6 isoform expression in neonatal rat cardiac myocytes, resulting in a marked increase in the response to isoproterenol. It has been further demonstrated that increasing the expression of AC6 in the heart improves cardiac performance in normal animal models and in transgenic animal models of heart failure (69, 70). Those results clearly confirm that AC is the limiting component for receptor-mediated stimulation of AC activity. More recently, the same group (188) further demonstrated that, while overexpression of AC6 enhanced responses to FSK and isoproterenol, responses to other agonists that increase cAMP in myocytes (PGE2, adenosine, glucagon, and histamine) were unchanged.

Among many possibilities, an attractive explanation would be that adrenergic receptors and ACs are not randomly distributed in the plasma membrane, interacting via a stochastic collision coupling, but they are colocalized. Indeed the authors showed that those two moieties could be found, perhaps together with muscarinic receptors, in caveolar microdomains of the cardiac sarcolemma (188). Interestingly, cardiac AC can be inhibited in vitro by the caveolin-3 scaffolding domain peptide (189). Such a specific subcellular localization could clearly have important physiological consequences. One could very well imagine that the two main AC isoforms found in the heart, AC5 and AC6, are indeed present in different membrane fractions. This could explain why a specific AC isoform seems to be associated with a specific hormone receptor in certain systems. For example, Chen et al (62) transfected HEK 293 cells with various AC isoforms and found that epidermal growth factor could increase AC activity only in cells expressing AC5. Using the same cells, Puceat et al have shown that AC5, but not AC4 or AC6, was responsive to purinergic stimulation and relatively insensitive to muscarinic inhibition (61). That purinergic activation of AC is additive to that by isoproterenol

in cardiomyocytes would fit with a model in which AC5 and AC6 are in different compartments of the same membrane. Furthermore, such a compartmentation would nicely explain why overexpression of AC6 in transgenic mice increases the response to adrenergic agents, whereas that of AC5 does not [157; J. Hanoune & N. Defer, unpublished data]. One obvious requirement for this hypothesis would be that AC5 and AC6 contain different addressing domains for the different compartments of the membrane. Considering the very high degree of sequence identity between the two isoforms, this is not evident. Furthermore, the above hypothesis is not compatible with the recent demonstration from Schwencke et al (190) that AC5 is also localized in caveolin-rich membrane nor with the report by Huang et al (191) that adrenergic receptors and AC are colocalized in a low-density membrane fraction from lymphoma S49 cells, which are known to lack caveolin. Furthermore, receptors have been shown to move in and out of caveolae (192). Only the use of specific and highly sensitive antibodies for AC5 and AC6 will definitively answer this question. These antibodies are not presently available.

Other levels of structural complexity exist as well. Very early target size analysis suggested that AC was part of a higher-order complex (193, 194), while more recent data are compatible with homo- (or even more interestingly hetero-) dimerization of AC (195). The interaction of AC with elements of the cytoskeleton has also been debated for a long time (196). The tubulin dimer has been shown to stimulate AC activity in rat cerebral cortex (197) and striatum (198). Those observations may have some bearing on the modifications of cAMP production in response to mechanical stimuli (199) or during neuronal disorders such as Alzheimer's disease, known to be associated with an altered cytoskeleton (197, 200).

To What Extent Is the Modulation of Adenylyl Cyclase Critical?

In the early paradigm put forward by Robison et al (200a), production of cAMP was the result of a linear chain of events. In liver, binding of epinephrine or glucagon stimulates AC, activates PKA, and eventually leads to glycogenolysis and hyperglycemia. The multiplicity of regulatory pathways, the existence of families of proteins at each step of these pathways, and the multiplicity of potential regulations for each of those isoforms coincide to further complicate the picture. For example, AC1 has been proposed to be an excellent coincidence detector for synergic stimulation via αs and calcium/CaM, and AC2 has been proposed as a coincidence detector for αs and $\beta\gamma$ or PKC; alternatively, AC5 or AC6 have been proposed as detectors for inhibition via calcium vs αs, (or NO vs αs for AC6) (201–204). That the various interfering signals originate from pathways that do not primarily involve cAMP (such as β subunits or calcium released by hormones not acting via Gαs) can lead to endless possibilities of "cross-talk." At a more accessible level, it is clear that we mostly confront combinatorial interplays where it is difficult to pinpoint the limiting steps involved. Two systems stand out as such potential regulatory models. The first is the pineal gland, in which

the circadian modulation of AC (205) is part of a regulatory system involving a variety of proteins (β-adrenergic receptors, kinase, cAMP, Ca^{2+}-dependent transcription factors, etc) (206). The final output, that is, melatonin production, is highly regulated, being increased during the night and nearly nil during the day. So many interacting components of the pineal machinery oscillate with a day-night rhythm, with positive or negative feedback interactions, that it is clearly impossible to identify a prime mover. The second model is the uterus, where all regulatory components cooperate at stimulating muscular relaxation during pregnancy via cAMP action and at triggering contraction at term by an opposite resetting. In this model, one can observe massive, synergistic modifications of all the individual steps of the cAMP pathways including the adrenergic receptor (201), AC (200), G protein (207–210), PKA (211), and A kinase anchoring protein AKAP-150 (212). Here again, it seems fruitless to try to weigh the role of each step in the signaling pathway.

However, on the bright side, the availability of the complete sequence of the human genome, as well as the sequences of a series of important animal models, should allow a very informative conclusion regarding the natural history (ontogeny and phylogeny) of the various AC isoforms, and hopefully also their physiological relevance across the various species.

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