Tissue specificity and physiological relevance of various isoforms of adenylyl cyclase

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Defer, Nicole, Martin Best-Belpomme, and Jacques Hanoune. Tissue specificity and physiological relevance of various isoforms of adenylyl cyclase. Am J Physiol Renal Physiol 279: F400–F416, 2000.—The present review focuses on the potential physiological regulations involving different isoforms of adenylyl cyclase (AC), the enzymatic activity responsible for the synthesis of cAMP from ATP. Depending on the properties and the relative level of the isoforms expressed in a tissue or a cell type at a specific time, extracellular signals received by the G protein-coupled receptors can be differently integrated. We report here on various aspects of such regulations, emphasizing the role of 

Ca\(^{2+}\)/calmodulin in activating AC1 and AC8 in the central nervous system, the potential inhibitory effect of 

Ca\(^{2+}\) 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 dependence. Present experimental limitations are also underlined (pitfalls in the interpretation of cellular transfection, scarcity of the invalidation models, and so on).

adenylyl cyclase; calcium; calmodulin; kidney; heart; brain; spermatozoa; opiates; cannabis

FORTY YEARS AFTER ITS DISCOVERY by Earl Sutherland, cAMP is still the archetypal “second messenger.” But the cAMP signaling pathway, once considered to be simple and straightforward, has become very complex indeed. One reason is the fact that cAMP is acting not only by promoting protein phosphorylation via activation of protein kinase [protein kinase A (PKA)] but also by inducing protein-protein interaction independently of any phosphorylation (38, 81). Another reason is the extreme variety of potential regulations of cAMP synthesis and degradation, due to the multiplicity of phosphodiesterases (up to 40) and adenylyl cyclase (AC) isoforms.

The present review deals with the latter enzymes that convert ATP into cAMP. Today, at least nine closely related isoforms of AC, AC1–AC9, and two splice variants of AC8, have been cloned and characterized in mammals (63, 75, 145, 152). All of them share a large sequence homology in the primary structure of their catalytic site and the same predicted three-dimensional structure. Each of them consists of two hydrophobic domains (with 6 transmembrane spans) and of cytoplasmic domains, resulting in a pseudosymmetrical protein. Only 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 and G\(_\alpha\), requires both cytoplasmic moieties. Elucidation of the structure-function relationship of ACs has markedly progressed over the last three years due to a series of recent studies, including crystallography and site-directed mutagenesis. The reader is referred to more detailed recent reviews dealing with those aspects (69, 158, 165).

The distinct properties of the individual isoforms allow them to play an interpretative role in signal
transduction instead of being a linear pathway for the activity of the G protein-coupled receptors. Thus, depending on the properties and the relative level of the isoforms expressed in a tissue or a cell type, extracellular signals received by the G protein-coupled receptors can be differently integrated.

The present text will focus on the potential physiological regulations involving the different isoforms. Ideally, one should one day be able to correlate the existence of a specific AC isoform in a given tissue or cell with a specific function or “raison d’être.” We are far from it at present and, if we want to avoid tedious phenomenological listings and descriptions, we can only grope for a few well-defined and physiologically relevant systems.

As is usual when commuting between analytic biochemical data and complex signaling networks, one is faced by many potential pitfalls. It is well known, for example, that 1) overexpressing a protein in a cell culture system can greatly alter the stoichiometry of the components of the network and lead to spurious results; 2) a well-defined regulation in vitro may be lost in vivo into a complex and redundant integrated system, (however, this specific regulation may show up in a pathological state); 3) some technical approaches may be too sensitive (e.g., the PCR reaction) or too unsatisfactory (we are still lacking good specific antibodies for all the AC isoforms) to provide unambiguous data; and 4) the probable marked differences in the specific activity of the various isoforms add a further degree of complexity when one analyzes the results of transfection experiments. This has been studied in the case of AC2 vs. AC6 (129), but most likely applies to most isoforms.

It is, therefore, with those caveats in mind that we present here a “progress report” on the cell- and isoform-specific regulations of cAMP synthesis.

**Tissue Specificity of mRNA Expression**

Because of the unavailability of satisfactory antibodies for most of the isoforms, the tissue distribution has generally been determined by mRNA studies. Table 1 shows that, among the large diversity of the AC isoforms, some are widely expressed, such as AC2, AC4, and AC6, whereas others are more specifically expressed, for example AC1 in tissues of neural origin and AC5 in heart and striatum. Although mRNA for the various AC isoforms was found in brain, their expression is restricted to discrete structures of the central nervous system as demonstrated by in situ hybridization (cf. Table 1). This is particularly clear for the Ca^{2+}/calmodulin-stimulated isoforms, AC1, AC3, and AC8. AC1 is abundant in the dentate gyrus of the hippocampus and the cerebral cortex (189), the highest expression of AC3 is exhibited in the olfactory neuroepithelium (188), whereas the important area for AC8 expression is the hypothalamus (16, 102), where it is the only Ca^{2+}/calmodulin-stimulated isoform (110, 187, 188). In chick heart, AC5 is essentially expressed in myocytes, whereas AC6 is expressed in nonmyocyte cells (198). Moreover, the colocalization of the l-type Ca^{2+} channels with different elements of the cAMP-mediated signaling pathway, including AC in cardiomyocytes along the T tubule membranes (56), will provide new insights for understanding of the regulation of this pathway.

The cellular heterogeneity of most of the tissues studied does not allow one to determine precisely in which type of cell from a given tissue a specific cyclase isoform was specifically regulated. Therefore, the relative expression levels of the AC isoforms in various brain structures have been determined by different groups (54, 66, 87, 102, 142).

Table 1. Tissue-specific distribution of the adenylyl cyclase isoforms

<table>
<thead>
<tr>
<th>Adenylyl Cyclase Isoform</th>
<th>Brain</th>
<th>Heart</th>
<th>Kidney</th>
<th>Liver</th>
<th>Lung</th>
<th>Testis</th>
<th>Skeletal Muscle</th>
<th>Adrenal</th>
<th>Brown Adipose Tissue</th>
<th>Uterus</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC1</td>
<td>++</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+a</td>
<td>-p</td>
<td>-p</td>
</tr>
<tr>
<td>AC2</td>
<td>++</td>
<td>+/-</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+h</td>
<td>0</td>
<td>+h</td>
</tr>
<tr>
<td>AC3</td>
<td>+</td>
<td>+/-</td>
<td>0</td>
<td>0</td>
<td>+/-</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AC4</td>
<td>+/-</td>
<td>+</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AC5</td>
<td>+/-</td>
<td>+</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AC6</td>
<td>+/-</td>
<td>+</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AC7</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AC8</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AC9</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>sAC</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

AC, adenylyl cyclase. *Expressed only in the medulla (142); *expressed at low level at E19 and decreased on denervation in gastrocnemius mouse muscle (154); *expressed at low level in the adult muscle and increased on denervation (154); *only in germinal cells, by immunohistochemistry (35); *AC5 and AC6 are essentially expressed in the adrenal medulla and to a lesser extent in the zona glomerulosa (142); *results obtained by PCR (115); *expressed in the 3 regions of the adrenal cortex (142); *increases during the perinatal period (20); *splice variant of type 4 in rat myometrium (51); *by RT-PCR (51); *expressed all along the nephron but more abundant in distal segments (in the collecting tubule and in the thick ascending limb) (18); *expression restricted to the glomerulus and to the initial portions of the collecting duct (18); *only in the glomerulus (18); *by Northern blot: expressed in myometrium in rat and human (109); *only in rat (109); *nondetectable by Northern blot (109); *in chick heart, AC5 is essentially expressed in cardiomyocytes and AC6 in noncardiomyocyte cells (198); *expressed at low level as determined by Northern blot (179); *the level of expression in cortex and medulla is approximately the same (142); *the expression of the different AC isoforms in various brain structures has been determined by different groups (54, 66, 87, 102, 110–113); *this soluble (s) isoform is insensitive to G protein or forskolin regulation and preferentially expressed in testis. The presumptive catalytic domains of sAC are closely related to cyanobacterial adenylyl cyclases (14).
isoform is expressed, with the notable exception of kidney (18). At least five AC isoforms are expressed in rat kidney, AC6, AC5, AC4, AC7, and AC9, as found out by Northern blot (143, 179). The expression of AC4, AC5, and AC6 has been determined along the nephron. The pattern of distribution of AC6 suggests a greater concentration in the medulla than in the cortex. At the cellular level, this distribution is characterized by a widespread presence along the whole renal tubule. AC6 is more abundant in the distal segments (in the collecting tubule and in the thick ascending limb), whereas AC5 expression is restricted to the glomerulus and to the initial portions of the collecting duct, and AC4 only in the glomerulus (18). These observations raise the important questions as to whether more than one isoform can be expressed in one cell type and how it can be targeted within the cell compartments. Through the assay of hormone-dependent cAMP levels and on the basis of the properties of Ca2+-inhibitable AC isoforms, Chabardes et al. (18) proposed that “AC5 is mainly, if not exclusively, expressed in the glucagon-sensitive cells and that AC6 is present in the vasopressin-sensitive cells of the outer medullary collecting duct of the rat kidney.” The functional relevance of AC localizations in the kidney will be described later.

TRANSCRIPTIONAL REGULATION OF AC GENES

Whereas chromosomal localization of each of the nine isoforms has been determined both in human and in mouse (49, 60, 61, 66, 134, 150, 173) (Table 2), little is known concerning the promoter and the structure of the genes. Part of the promoter regions for AC3 and AC8 has been described with potential sequences for binding specific factors (115, 178). Moreover, 215,441 bp of the human chromosome 16p13.3 have been sequenced (GenBank accession no. AC005736), which should cover the complete AC9 gene. The AC9 gene extends over >150 kb and 9 introns.

AC3 was initially identified as the specific isoform of the olfactory neuroepithelium (10, 188). Several proteins of the olfactory signaling pathway, including the putative odorant receptor, Goαolf, AC3, and the olfactory nucleotide-gated channel, have been identified and localized in the cilia by immunohistochemichal and electrophysiological methods (78, 107). Wang et al. (178) have identified binding sites for the olfactory neuron-specific transcription factor Olf-1 in the sequence surrounding the transcription initiation site of all these genes. This suggests that, in sensory neurons, the expression of these genes is coordinated and involves tissue-specific transcription factors. AC3 has also been found expressed in many other tissues, including bovine adipose tissue (20, 58), male germ cells (35, 57), and luteal cells from bovine ovaries (100). Expression of AC3 mRNA has also been detected in human islets isolated from nondiabetic individuals (193). Recently, two point mutations in the promoter region of the AC3 gene have been associated with a decrease in the glucose-induced insulin release in spontaneously diabetic rats, possibly through an alteration of AC mRNA transcription (1). It will be important to determine whether mutations in the AC3 gene promoter are also present in patients with type 2 diabetes.

The complete structure of the murine AC8 gene has been recently characterized (115). The AC8 gene extends over 18 exons, which encompass ~200 kb of the mouse genomic DNA. In the 5’ end, a very long untranslated sequence (~2 kb upstream from the translation initiation site) is highly conserved among the different species, i.e., mouse, rat, and human (16, 36, 115). This suggests that posttranscriptional regulations play an important role in the expression and/or localization of AC8. As for the promoter region of the AC3 gene, the AC8 promoter does not contain any canonical TATA box but does include a consensus cAMP response element (CRE). The presence of a putative CRE sequence in the AC8 gene promoter might have some relevance because the induction of AC8 expression in specific regions of the brain during chronic administration of morphine (87, 103) is attenuated by injection of a cAMP-responsive element binding protein (CREB) antisense oligonucleotide (87), and because CREB (~/-)mutant mice have reduced morphine abstinence syndrome (98).

All the sequence data available to date allow only limited conclusions. However, it is noteworthy that the structure of the different AC genes appears to be different: the first exon of AC3 is not translated (178), and <1 kb of the promoter region is sufficient to control the level of expression of this isoform (1). The mouse AC8 and human AC9 genes have a different exon organization with apparently no conservation of the splice donor sites, whereas some conservation exists between the mouse AC8 gene and the Drosophila melanogaster rutabaga gene (115). Finally, Muglia et al. (115) demonstrated that DNA sequences within the 10 kb preceding the first exon of the AC8 gene are critical for the establishment of region-specific pattern of expression of this isoform.

POTENTIAL REGULATIONS OF MAMMALIAN ACs

As with most of the proteins involved in signal transduction, the fact that ACs exist as multiple isoforms with different regulatory properties (Table 3) allows

<table>
<thead>
<tr>
<th>AC Isoform</th>
<th>Human Chromosome</th>
<th>Mouse Chromosome</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC1</td>
<td>7p12 (173)</td>
<td>11A2</td>
</tr>
<tr>
<td>AC2</td>
<td>5p15 (150)</td>
<td>13C1</td>
</tr>
<tr>
<td>AC3</td>
<td>2p22-24 (60)</td>
<td>12A-B</td>
</tr>
<tr>
<td>AC4</td>
<td>14q11.2 (49)</td>
<td>14D3</td>
</tr>
<tr>
<td>AC5</td>
<td>3q13.2-q21 (60)</td>
<td>16B5</td>
</tr>
<tr>
<td>AC6</td>
<td>12q12-13 (60)</td>
<td>15F</td>
</tr>
<tr>
<td>AC7</td>
<td>16q12-13 (66)</td>
<td>8C3-D (66)</td>
</tr>
<tr>
<td>AC8</td>
<td>8q24 (150)</td>
<td>15 (115)</td>
</tr>
<tr>
<td>AC9</td>
<td>16p13.3 (134)</td>
<td>16B1 (61)</td>
</tr>
<tr>
<td>AC1</td>
<td>1q24 (14)</td>
<td></td>
</tr>
</tbody>
</table>

Reference nos. are in parentheses.
Table 3. Regulations of mammalian adenylyl cyclases

<table>
<thead>
<tr>
<th>Regulatory Signal</th>
<th>Regulation(s)</th>
<th>Ref. No(s.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$G_{\alpha}$</td>
<td>All are stimulated</td>
<td>(145, 157, 165, 191)</td>
</tr>
<tr>
<td>$G_{\alpha}$</td>
<td>$G_{i}$, $G_{o}$ inhibit calmodulin- and FSK-stimulated AC1 activity</td>
<td>(85, 161, 163)</td>
</tr>
<tr>
<td></td>
<td>$G_{i}$ inhibits AC5 and AC6</td>
<td>(22, 163)</td>
</tr>
<tr>
<td></td>
<td>No effect on AC2</td>
<td>(12, 162)</td>
</tr>
<tr>
<td>$G_{\beta y}$</td>
<td>AC2, AC4, and AC7 are stimulated</td>
<td>(156)</td>
</tr>
<tr>
<td></td>
<td>AC1 is inhibited</td>
<td>(162)</td>
</tr>
<tr>
<td></td>
<td>AC5 and AC6 might be inhibited by the $\beta_{1}\gamma_{2}$ (?)</td>
<td>(12)</td>
</tr>
<tr>
<td>FSK</td>
<td>All except AC9 are stimulated</td>
<td>(166, 192, 200)</td>
</tr>
<tr>
<td>PKA</td>
<td>AC5 and AC6 are inhibited</td>
<td>(24, 74)</td>
</tr>
<tr>
<td>PKC</td>
<td>Stimulates AC1, AC2, AC3, and AC7</td>
<td>(28, 47, 76, 95, 145, 194, 201)</td>
</tr>
<tr>
<td>Calcium</td>
<td>At high concentration, inhibits all cyclases</td>
<td>(79)</td>
</tr>
<tr>
<td></td>
<td>At micromolar concentration, inhibits AC5 and AC6</td>
<td>(30, 33, 73, 111, 139, 195)</td>
</tr>
<tr>
<td></td>
<td>No effect on AC2, AC4, and AC7</td>
<td>(16, 59, 90, 158, 175, 185)</td>
</tr>
<tr>
<td></td>
<td>Stimulates AC1 and AC8 through calmodulin binding</td>
<td>(145, 157, 183)</td>
</tr>
<tr>
<td></td>
<td>Stimulates AC5 through PKC-$\alpha$ activity</td>
<td>(128)</td>
</tr>
<tr>
<td></td>
<td>Stimulates AC2 and AC7 through PKC</td>
<td>(180)</td>
</tr>
<tr>
<td></td>
<td>Inhibits AC1 through calmodulin kinase IV activity</td>
<td>(27, 181)</td>
</tr>
<tr>
<td></td>
<td>Inhibits AC3 through calmodulin kinase II activity</td>
<td>(128)</td>
</tr>
<tr>
<td></td>
<td>Inhibits AC9 through calcineurin activity (?)</td>
<td>(128)</td>
</tr>
</tbody>
</table>

FSK, forskolin; PKA, protein kinase A; PKC, protein kinase C.

complex signal integration, but may also lead to spurious conclusions. In most of the cases, regulatory properties of the cAMP-synthesizing machinery have been determined on purified membranes, and it is not clear by which combinatorial process the observed properties are integrated in the intact cells to produce a specific response to external stimuli. In some cases, the regulatory properties of individual isoforms have been determined in stably transfected, intact cells. Although these conditions permit the integration of informations from multiple signals, the transfection experiments are generally performed in cell types with low levels of cyclase activity or in cell types that do not express the transfected isoform. In those conditions, the physiological effectors needed for a given isoform may be absent, leading to nonreproducible data from cell type to cell type. Given the cell heterogeneity of most of the tissues, and the fact that most of the cells probably express multiple isoforms of AC, the analysis of the regulatory properties of one AC in its own context may be an unending challenge. The renal epithelial cells of the distal portion of the collecting tubule, where only one isoform, namely AC6, has been detected, may be an exception.

**Stimulation by $G_{\alpha}-$Subunit and by Forskolin**

Stimulation through $G_{\alpha}$ is the major mechanism by which ACs are activated and the cAMP level is elevated. By expressing AC1, AC2, and AC6 in insect cells, Harry et al. (65) demonstrated that different ACs have different affinities for $G_{\alpha}$, which may provide an explanation for the various responses of different cell types to hormones and neurotransmitters that elevate cAMP. These differences are abolished in the presence of 100 $\mu$M forskolin. This indicates a conserved mechanism by which forskolin regulates $G_{\alpha}$ coupling to the different ACs.

All ACs, with the possible exception of AC9, are activated by the diterpen forskolin. One major surprise in the elucidation of the structure of the ACs was the existence of a highly hydrophobic pocket at the interface of C1a/C2a, where forskolin acts. This pocket is different in AC9 compared with the other types of mammalian ACs. A single mutation transforming the Tyr$^{1082}$ → Leu of mouse AC9 can confer both binding and activation by forskolin (192). At this position the Drosophila AC9 protein, which is forskolin-sensitive, contains a Leu (72). Together, forskolin and $G_{\alpha}$ contribute for a higher cyclase activity. Gilman and colleagues (40, 157, 183) have shown that fragments of the two cytoplasmic domains of mammalian ACs can be synthesized independently as soluble proteins. On their mixture, both $G_{\alpha}$- and forskolin-stimulated activity can be restored. Using this system, Dessauer et al. (41) have characterized the interaction of AC with forskolin and ATP, although each one has its own binding site. The affinity of forskolin to AC is greatly reduced in the absence of $G_{\alpha}$ (41).

**Inhibition of AC Activity by $G_{i}$**

The complexity of the hormonal control of AC was first evidenced by Rodbell and co-workers (138), who discovered the dual stimulatory and inhibitory G protein ($G_{i}$)-regulatory pathways. The inhibitory action of G protein-coupled receptors on AC activity can be blocked by pertussis toxin. Whereas all isoforms of ACs are potentially activated by $G_{\alpha}$-coupled receptors, the inhibition by $G_{\alpha}$-coupled receptors appears to be isoyme specific; the $\alpha$-subunit of the $G_{i}$ protein, $G_{i}\alpha$, acts as a noncompetitive inhibitor of $G_{\alpha}$-stimulated AC5 and AC6 but has no effect on AC2 and AC8 (22, 85, 161, 163). Using a soluble enzyme system composed of the C1 and C2 domains of AC5 and AC2, the Gilman group (42) have dem-
onstrated that only the C1 domain of AC5 retains the ability to bind G\(\alpha\) within a site close to the active site of the enzyme.

**Regulations by the \(\beta\gamma\)-Subunits of the G Proteins**

Classically, G\(\beta\gamma\) was thought to inhibit AC activity by chelating and deactivating stimulatory G\(\alpha\). The possibility of specifically expressing individual AC isoforms in cultured cells has led to a complete reappraisal of this view. 1) G\(\beta\gamma\) has no direct effect on the activity of a few isoforms (AC3, AC8, AC9) (134, 156). 2) G\(\beta\gamma\) activates AC2, AC4, and presumably AC7, directly, but only in the presence of activated G\(\alpha\) (55, 95, 139, 156). This was a surprise and has the potential of explaining many aspects of cross-talk between different receptors (15, 53, 160, 199). For example, \(\alpha_1\)-adrenergic stimulation of the G\(\alpha\)-subunit can lead to an increase in cAMP through the \(\beta\gamma\)-complex, thus explaining the convergent action of Ca\(^{2+}\) and cAMP on the same target. Here again, the presence of specific isoforms of AC in a given cell will determine which regulatory pathways might be involved. Alternatively, a hormone or a neurotransmitter acting via a receptor normally coupled to G\(\alpha\) could produce a biphasic action on cAMP production depending on whether G\(\alpha\) or G\(\beta\gamma\) is predominantly influencing the enzyme (124, 125). 3) Finally, G\(\beta\gamma\) directly inhibits the calmodulin- or G\(\alpha\)-stimulated AC1 activity (156, 162).

In contradiction to previous data (132, 196), Bayewitch et al. (11, 12) have also shown, by transient cotransfection into COS-7 cells of AC isoforms and \(\beta\)-and \(\gamma\)-heterotrimeric G subunit, that AC5 and AC6 are markedly inhibited by G\(\beta\gamma\) (particularly \(\beta_1\gamma_2\)), in conditions where AC2 activity is stimulated. If this were true in a physiological context, we would have then a new kind of cross-talk between receptors, whereby a receptor, not coupled to G\(\alpha\) or to AC, could inhibit the cAMP formation in an unexpected manner if AC1, AC5, or AC6 was the predominant isoform in a given tissue. However, whether this observation is physiologically relevant is not demonstrated at present.

**Regulations by Protein Phosphorylation**

Modulation of the enzymatic activity by phosphorylation is a common signature of downstream and feedback regulations in the transduction cascades. In this context, phosphorylation of the ACs by PKA provides a means of desensitization at the effector level. The profiles of the regulatory sensitivity of ACs to protein kinases is different according to each subtype. Both AC5 and AC6 are directly phosphorylated, and inhibited, by PKA (24, 74). Phosphorylation by PKA directly inhibits AC5 activity by decreasing the maximal velocity of the enzyme (74). Phosphorylation of AC6 at the level of Ser\(^{874}\) would disrupt the functional G\(\alpha\) binding site, leading to the inhibition of AC activity (24). This mechanism could explain the cAMP-dependent desensitization of glucagon stimulation described several years ago in hepatocytes (133). This might be particularly important in the heart where AC5 and AC6 are the most abundant isoforms and where AC activity has to be strictly controlled. This suggests the presence of a negative feedback loop at the level of the cyclase itself as a potential mechanism of desensitization of the cAMP signaling pathway.

Phosphorylation by protein kinase C (PKC) often results from the activation of G\(\alpha\), and phospholipase C (PLC)-linked receptors, which in turn leads to mobilization of Ca\(^{2+}\), synthesis of diacylglycerol, and activation of PKC. On PKC activation, cAMP production within the cells is altered. Phorbol 12-myristate 13-acetate is able to increase AC activity in cells transfected with AC1, AC2, AC3, AC5, or AC7. Potentiation of AC1 activity by PKC can be observed only on Ca\(^{2+}\)/calmodulin stimulation (76), whereas inhibition of AC4 activity by PKC-\(\alpha\) is not observed on basal activity but after G\(\alpha\) stimulation (201). Whether PKC directly modulates AC activity has been controversial. In insect cells, AC2 activity is clearly activated by PKC-\(\alpha\), but this activity is lost on membrane solubilization or AC2 purification, although it retains the stimulation by G\(\alpha\) and forskolin (47). On the other hand, using purified PKC and AC, Kawabe et al. (80) have demonstrated that PKC-\(\zeta\) can directly phosphorylate AC5, leading to a 20-fold increase in AC activity. Although PKC-\(\alpha\) is less potent to activate AC5, the two PKCs are additive in their capacity to activate AC. Phosphorylation of AC5 by the different PKCs is particularly important in the heart, where growth factors including insulin are able to regulate cAMP production and contractility. In vitro, the \(\alpha\)- and \(\zeta\)-isoforms directly phosphorylate and activate AC5. Whereas the \(\zeta\)-isozyme activates AC5 in a Ca\(^{2+}\)-independent manner, the \(\alpha\)-isozyme requires Ca\(^{2+}\). This affords another mechanism for the Ca\(^{2+}\)-mediated regulation of AC5 activity in heart. In cells expressing AC5, insulin augments cAMP production through phosphatidylinositol-3,4,5 triphosphate (PIP\(_3\)) activation of the PKC-\(\zeta\) (79). In the heart, all hormones or growth factors that activate PI\(_3\)-kinase, leading to the formation of PIP\(_3\), which activates PKC-\(\zeta\), would be able to control cAMP production through a direct activation of AC5. All these observations demonstrate that PKC can alter the ability of the AC isoforms to integrate signals derived from multiple inputs. ACs therefore appear to be important targets for direct or PKC-mediated modulatory effects of Ca\(^{2+}\). The other very important regulations of AC by Ca\(^{2+}\), either negative or positive, are dealt with later.

The mechanism by which a cell can integrate multiple signals to modulate AC activity is well documented in a paper of Marjamaki et al. (101). AC2, AC3, and AC4 have been transfected in DDT1-MF2 cells which already expressed AC6, AC7, AC8, and AC9. Whereas AC2 and AC4 exhibit a high amino acid sequence homology, and share most of their in vitro regulatory properties, they can be submitted to different hor-
monal regulations in vivo: in cells transfected with AC2 or AC4, \( \alpha_2 \)-adrenergic receptor (AR) stimulation initiates both positive (through \( \beta \gamma \)) and negative (through \( G_i \)) effects on \( G_\alpha \)-stimulated activity; however, PKC blocks the negative input from the \( \alpha_2 \)-AR in AC2-transfected cells, whereas it blocks the positive input in AC4-transfected cells (101). These observations demonstrate the complexity of integration of multiple signals by ACs. The authors concluded that this dynamic process is dependent on the enzyme type and the state of phosphorylation. The ability of the AC to integrate multiple information certainly plays a key role in the signaling plasticity observed during a wide range of physiological or pathological processes and during development.

**Regulations by Ca\(^{2+}\)**

All AC activities are inhibited by high, nonphysiological concentrations of \( \text{Ca}^{2+} \) in the submillimolar range, possibly by competition with magnesium. In certain tissues, including the pituitary gland, platelets, and heart, AC activity has been reported to be inhibited by concentrations of \( \text{Ca}^{2+} \) in the micromolar range. This appears to be a feature of the two closely related cyclase isoforms, AC5 and AC6, cloned from heart, liver, kidney, striatum, Reuber hepatoma, or NCB-20 cells (30, 31, 111). When expressed in a variety of recipient cells lines, these isoforms are inhibited by micromolar concentrations of \( \text{Ca}^{2+} \), and the inhibition is additive to that elicited by receptors acting via \( G_\alpha \). Whether \( \text{Ca}^{2+} \) modulates AC5 and AC6 activities directly or via a \( \text{Ca}^{2+} \)-binding protein remains to be determined.

\( \text{Ca}^{2+} \)/calmodulin activates AC1 and AC8 by direct binding to a putative calmodulin binding site located in a C1b helical region of AC1 (90, 175) or in the C2 region of AC8 (59). The precise activation mechanism is unknown. It has been proposed, on the basis of other \( \text{Ca}^{2+} \)/calmodulin binding proteins, that calmodulin binding would disrupt an autoinhibitory interaction between the C1b or C2b region and the catalytic core.

**Conclusion**

It thus appears that 1) the different ACs have different potential regulatory properties, delineated by their primary structure and/or activity in vitro; 2) the same effector can exert positive or negative effects on the various isoforms; 3) according to the specific pattern of protein expression in the different cell types, the same isoform may be regulated differently; and 4) finally, through \( \text{Ca}^{2+} \) regulations, the different signaling pathways, using the various G proteins, can talk together to (hopefully) better regulate cell functions. It is clear that the integration of the multiple signals by AC is a dynamic process and that the ability of the different AC types to respond to activated \( G_\alpha \), \( G_\beta \gamma \), AC4, and phosphorylation places the enzyme at a central point for cross-talk between different signaling pathways.

**FUNCTIONAL RELEVANCE OF SPECIFIC ISOFORM EXPRESSION**

**Is the Inhibition of AC by Micromolar Concentration of \( \text{Ca}^{2+} \) Physiologically Relevant?**

Heart and kidney are among the major organs in which \( \text{Ca}^{2+} \)-inhibitable AC isoforms are predominant. However, it is difficult to clearly attribute a physiological role to this specific regulation. We and others have demonstrated that, although the two major isoforms in rat heart, AC5 and AC6, are equivalent at birth, the AC5 mRNA becomes predominant in the adult rat heart (52, 169). Sympathetic stimulation of cardiac tissue elevates cAMP, which in turn leads to an increase in intracellular \( \text{Ca}^{2+} \), and the wave of \( \text{Ca}^{2+} \) has been proposed to lead to a rhythmic dissipation of the cAMP signal (32). The capacitative entry of \( \text{Ca}^{2+} \), secondary to the emptying of intracellular \( \text{Ca}^{2+} \) pool (e.g., by the use of the \( \text{Ca}^{2+} \)-ATPase inhibitor thapsigargin), has been proposed to play a major role in positively (AC1 or AC8) or negatively (AC5 or AC6) regulating AC activity (26, 33). That it is the only mechanism by which a change in cytosolic \( \text{Ca}^{2+} \) concentration can influence AC activity is probably still open to question, especially in excitable tissues where the capacitative entry of \( \text{Ca}^{2+} \) plays a minor role if any.

In fact, the relative effects of \( \text{Ca}^{2+} \) and cAMP are much more complex. We have just demonstrated that overexpression of AC8, a neural, \( \text{Ca}^{2+} \)/calmodulin-stimulatable AC isoform in mice heart (91a) is not only compatible with normal heart function but even leads to enhanced function, with no cardiomegaly or fibrosis in 3-5 mo-old animals. If the rhythmic \( \text{Ca}^{2+} \) inhibition of cAMP formation were of major importance, we would have expected this transfection to be lethal.

In the kidney, the preferential distribution of AC6 in the medulla (143) is due to the presence of two segments, collecting tubule and thick ascending limb, in which AC6 is highly expressed (18). An important point is the subcellular location of the AC in the epithelial cells. It is generally accepted that AC is localized to the basolateral domain (144). However, a growing body of literature suggests that receptors are asymmetrically expressed in the renal epithelial cells: \( \alpha_1 \)-adenosine receptors and \( \beta_2 \)-ARs are thought to mediate the effects of agonist exposure at the apical membrane (17, 64, 96), whereas the \( \alpha_2 \beta \)-AR is known to be expressed at the basolateral membrane of the proximal tubule cells (68). In this context, it has been proposed that apical \( \beta_1 \)-AR requires endocytosis to activate a basolateral AC in proximal tubule epithelial cells of rat kidney (64), which essentially express the AC6 isoform. On the other hand, Okusa et al. (123) have concluded that, in LLC-PK1 cells stably transfected with two G protein-coupled receptors known to be targeted to the opposite domains in the renal epithelial cells, the apical \( \alpha_1 \)-adenosine receptor and the basolateral \( \alpha_2 \beta \)-AR, the AC activity is present at, or near, the apical and the basolateral domains of the cells and that the local AC activity can be regulated by \( G_\beta \gamma \)-coupled receptors.
therefore appears very important to determine the targeting of the various AC isoforms by using modern tools, such as flag labeling and confocal microscopy.

Depending on the cell type, the cytosolic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_e\)) can be increased by various mechanisms. Activation of PLC-coupled receptors, by substance K or bradykinin, causes an inhibition of the agonist-stimulated cAMP production: in C6–2B cells, which express mainly AC6, the inhibition of cAMP accumulation is temporally correlated with, and dependent on, initial [Ca\(^{2+}\)]\(_e\) rise evoked by Ca\(^{2+}\)-mobilizing agents (34). In parathyroid cells, where extracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_o\)) plays a crucial role, eliciting a negative feedback on parathyroid hormone secretion, increasing [Ca\(^{2+}\)]\(_o\) stimulates PLC activity and inhibits hormone-dependent cAMP accumulation (21, 82). In kidney, the cortical thick ascending limb ensures the cAMP-stimulated paracellular Ca\(^{2+}\) reabsorption from the lumen to the extracellular fluid compartment of the renal tubule; an increase in [Ca\(^{2+}\)]\(_e\) decreases the hormone-dependent cAMP accumulation by a mechanism that is independent of direct inhibition of AC activity, most probably AC6 (18, 155). In both bovine parathyroid cells and in rat kidney, a Ca\(^{2+}\)-sensing receptor has been described that is activated by [Ca\(^{2+}\)]\(_o\), and stimulates PLC activity. In the cortical thick ascending limb cells of rat kidney, this receptor is coexpressed with the Ca\(^{2+}\)-inhibitable AC, AC6 (37). An increase in extracellular Ca\(^{2+}\), coupled to PLC activation, induces a dose-dependent inhibition of the vasopressin-dependent cAMP increase (155). Experiments on microperfused rat cortical thick ascending limb have demonstrated that [Ca\(^{2+}\)]\(_e\) inhibits the transtubular electrolyte reabsorption (37), which supports a direct physiological role for an inhibition of AC6. The best hypothesis to explain the inhibitory effect of extracellular Ca\(^{2+}\) on AC activity in the thick ascending limb (37) is an inhibition elicited by an increase in intracellular Ca\(^{2+}\) (due to capacitative Ca\(^{2+}\) entry and/or Ca\(^{2+}\) release).

Invalidation of the G\(_{\alpha}\)-subunit in mice has shed additional light on those mechanisms. In the thick ascending limb, acute exposure to vasopressin increases NaCl transport probably through the apical Na-K-2Cl cotransporter. In the heterozygous G\(_{\alpha}\)-knockout mice, the Na-K-2Cl cotransporter protein is markedly reduced (48). In parallel, PLC production, on glucagon stimulation, and the abundance of AC6 are diminished in thick ascending limb. In this system the abundance of AC6 is probably regulated by a feedforward regulatory mechanism (48), the amount of AC6 being positively correlated with that of G\(_{\alpha}\) and not subject to compensatory overexpression. Along the same line, it is interesting to note that all the AC isoforms expressed in the kidney were found to be depressed in the homozygous Brattleboro rats, animals with an hereditary diabetes insipidus (DI) lacking antidiuretic hormone (143), when one would have expected some compensatory increase.

AC5 and AC6 are inhibitable by both G\(_i\)-coupled receptors and Ca\(^{2+}\). The characteristics of inhibitory regulation of AC activity by Ca\(^{2+}\) and G proteins were examined in dispersed gastric smooth muscle cells. These inhibitions can be mediated independently by G\(_i\) proteins and Ca\(^{2+}\) influx. When both mechanisms are triggered concurrently, inhibition is exclusively mediated by G\(_i\) proteins (116).

The Role of Ca\(^{2+}\)/Calmodulin-Activated ACs in Brain Function

The central nervous system possesses all the forms of ACs characterized so far. The presence of Ca\(^{2+}\)-stimulated cyclase activity has been known for many years and is now ascribed to the two specific isoforms, AC1 and AC8.

AC1 has been the first isoform to be cloned (86). It can be activated by G\(_i\)-coupled receptors as well as by the Ca\(^{2+}\)-calmodulin complex and, therefore, can function as a coincidence detector for the two signaling pathways. AC8 is also stimulated by Ca\(^{2+}\)/calmodulin, albeit at a 5–10 times higher Ca\(^{2+}\) concentration. Although there is not definite evidence for it, AC8 is supposed to rather act as a pure Ca\(^{2+}\) detector (190). AC1 is present in various areas of the brain, mainly the cortex, the hippocampus, the cerebellum, and the pineal gland. Interestingly, research in mammals has been driven by previous results obtained in Drosophila concerning mutations affecting memory. The flies can be trained to avoid a particular odor by coupling exposure to that odor with an electric shock. The rutabaga-mutant flies fail to avoid the “trained” odor and appear to be deficient in Ca\(^{2+}\)-activated AC (43, 44, 94). This form of AC has been characterized and cloned by Levin et al. (89) and appears most similar to the mammalian AC1, with the exception of a very long COOH terminal, the function of one-half 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 this points to a very important role of AC1 in learning and memory.

Disruption of the AC1 gene in mice results in a loss of Ca\(^{2+}\)-sensitive AC activity in cerebellum, cortex, and hippocampus by 62, 38, and 46%, respectively (151, 172, 186), with no obvious anatomic differences. The mutant mice exhibit a dampening of the long-term potentiation in the hippocampus and a near-blockade in the cerebellum (171). A spontaneous loss-of-function mutation in the AC1 gene has also been reported in mice (barrelless) (2, 182). 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 (104) merits further investigation.
At the same time, AC1 does function as a good coincidence detector, and this is well demonstrated in the pineal gland (170), where AC1 is activated by norepinephrine via both the β-AR (through G_α,β) and the α1-AR (through Ca^{2+} release), to increase cAMP formation and ultimately N-acetyl transferase and melatonin synthesis. AC1 synthesis undergoes a striking circadian variation that makes it a key regulating step in melatonin production and release.

AC8 is also a major isoform in the brain although it has also been found in testis (36, 63) and lung (115). In the brain, it is mainly present throughout (16, 102, 115), especially in the cortex, cerebellum, brain stem, hypothalamus, hippocampus, and olfactory bulb. The specific localization in hypothalamic nuclei suggests a role in neuroendocrine function whereas its specific increase in some regions of the brain, and especially in the locus ceruleus during morphine administration and withdrawal, points to a role in drug dependence (87, 103).

It is interesting to note that another type of cyclase, AC9, is expressed to a high level in the brain (5). This isoform is weakly sensitive to forskolin and is not directly regulated by Ca^{2+} or βγ. It has been proposed to be inhibited by the Ca^{2+}/calmodulin-activated protein phosphatase 2B (calcineurin), at least in mice (4, 6) but maybe not in humans (61). The kinase that potentially phosphorylates AC9 has not been identified. Interestingly, invalidation of this isoform in Caenorhabditis elegans prevents G_α-induced neuronal cell death (13, 84). 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.

Is a Specific Isoform Associated With Cell Differentiation?

In many cell types, the intracellular concentration of cAMP affects the progression within the cell cycle. In some of them, growth-stimulatory effects have been observed, whereas in others inhibitory effects have been reported (46, 97). In most of the cases, it appears that elevation of intracellular cAMP, through G_α or forskolin activation of AC, blocks the transfer of signal from the growth factor receptors to MAP kinases, through PKA-dependent phosphorylation (23, 62, 184). As with many other undifferentiated or dedifferentiated cell types in culture, NIH3T3 cells express AC6 at a high level, whose activity is inhibited by a variety of signals, including Ca^{2+}, PKA, and PKC (75, 146). To investigate the potential role of a specific AC isoform in regulating proliferative responses, Smit et al. (147) have transfected NIH3T3 cells with different AC isoforms. They observed that overexpression of AC6 has no effect on the rate of cell proliferation; by contrast, overexpression of AC2, an isoform that is stimulated by PKC, resulted in inhibition of cell cycle progression and increased doubling time, resulting from an inhibition of signal flow from Ras to mitogen-activated protein kinase. Moreover, the suppressive effect of the platelet-derived growth factor-induced DNA synthesis was completely reversed by coexpression of a dominant negative mutant of PKA. Thus expression of specific isoforms of AC might function as an homeostatic element of proliferation.

The importance of cAMP in cell differentiation has been reported in various organisms and cell types. However, the molecular mechanism involved is still poorly known. To investigate the role of specific isoforms of AC during cell differentiation, we have used the P19 embryonic carcinoma cells, which are pluripotent stem cells that can mimic in vitro the first stages of cellular differentiation occurring during mouse embryogenesis (106). Retinoic acid treatment of P19 cells leads to neuronal differentiation, whereas DMSO induces differentiation into mesodermal derivatives including cardiomyocytes. We have shown that neuronal differentiation of P19 cells, which is mediated by the cAMP/PKA cascade in vivo as well as in vitro (126, 176), exhibits a stage-specific upregulation of specific mRNA isoforms of AC, AC2, AC5, and AC8 (92). On the other hand, mesodermal differentiation of P19 cells is accompanied by an increase in mRNAs for AC2, AC5, and AC6 (93). In both cases, cell contacts and inhibition of cell proliferation are required before differentiation. In both cases, the total AC activity was increased at least by 10-fold. This increase is mainly related to an increase in AC2 level, because the specific activity of AC2 is much higher than that of the other ACs (129). Together with results obtained in transfected cells, these results favor the hypothesis that AC2 expression at a high level is a prerequisite for arrest of cell proliferation, then allowing cell differentiation. It is noteworthy that AC2 and AC7, both of which are stimulated by PKC, are expressed largely in postmitotic neural cells and platelets, whereas cells that retained proliferative capability do not express significant levels of isoforms that can be activated by growth factors.

Isoform-Specific Regulations During Development

The two best-studied systems to date are the heart and the brain. We (52) and others (169) have demonstrated that, although the two major isoforms in rat heart, AC5 and AC6 mRNAs, are equivalent at birth, AC5 mRNA becomes predominant in the adult rat heart. Because the two forms are clearly related and are similarly regulated by Ca^{2+}, there is no obvious physiological correlation for this genetic switch. One could hypothesize that the shift from AC6 to AC5 could be related to the state of cellular differentiation. Interestingly, AC5 is absent in skeletal muscle, whereas cells that retained proliferative capability do not express significant levels of isoforms that can be activated by growth factors.

After denervation, the levels of AC9 and AC2 mRNAs decrease in skeletal muscle whereas those of AC6 and AC7 are increased, the latter pattern being identical to that observed in the fetus and the neonate. These results indicate that changes in AC activities as well as AC mRNAs play an important role in muscle development as well as during muscle atrophy (154).
In rat brain, we have studied the developmental pattern of AC1, AC2, and AC5 during the postnatal period by in situ hybridization (104). One of the very interesting features found is that during the early postnatal stage, AC1 transcripts are very high in the central cortex, the striatum, and several regions involved in the sensory relay nuclei (such as the superior and inferior colliculus). These AC transcripts subsequently decrease rapidly in these regions, to be replaced, for example, by the AC5 transcript in the striatum, whereas they dramatically increase in the cerebellum and the hippocampus. These results demonstrate that the various ACs are expressed in the developing rat brain in a region- and age-specific manner and that they may thus be important not only for synaptic transmission (e.g., for long-term potentiation and memory) but also in the differentiation and maturation of synapses between neuronal cells, especially in sensory pathways.

Is a Specific Isoform Associated With the Development and Function of Mature Spermatosa?

The cAMP-dependent pathway is known to play a critical role in the expression of genes involved in haploid germ cell differentiation, and several reports have indicated the existence of a unique soluble form of AC in mammalian germ cells with properties that differ from those of somatic cells: this AC activity is insensitive to G proteins, fluoride, and forskolin and is associated with a low-molecular-weight fraction, ranging from 42 to 69 kDa (3, 83, 121, 148, 149). The low-molecular-weight isoform has been described in the cytosol of the early stages of spermatide cells, whereas the AC activity is membrane-bound in mature spermatosa (3). That specialized isoforms are required for germ cell differentiation is suggested by observations in lower organisms, where an AC with a role of this AC in the biogenesis of acrosome and possibly in gamete production and fertilization. In olfactory epithelium, both AC3 and the olfactory Gα-subunit Gαolf have been localized to the same receptor cell compartment, the distal segments of the olfactory cilia (78, 107). Moreover, a selective localization of Gαolf putative odorant receptors, and associated desensitizing proteins have been shown in elongated spermatids and the midpiece of the sperm tail (142, and N. Defer, unpublished observations). 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 spermatosa and may be implicated in sperm chemotaxis during fertilization.

Is a Specific Isoform Associated with a Specific Hormone Action?

Studies from the laboratory of Patel and colleagues (118) have shown that epidermal growth factor (EGF) produces inotropic and chronotropic action in rat heart by increasing cAMP accumulation. This EGF-elicited stimulation of cellular cAMP accumulation in the heart is the result of stimulation of AC activity by a mechanism involving the participation of a Gα protein (119) and the tyrosine kinase activity of the EGF receptor (117). The EGF-receptor tyrosine kinase can phosphorylate Gα to tyrosine residues, and this phosphorylation increases its ability to stimulate AC activity. HEK-293 cells have been transfected with different isoforms of ACs, AC1, AC2, AC5, and AC6 (25). EGF increased AC activity and cAMP accumulation only in cells expressing AC5. Because all isoforms are potentially stimulated by Gα, these results suggest AC5 that activation reflects either the specific interaction between AC5 and the tyrosine-phosphorylated form of Gα or the presence of an additional regulatory element, potentially PKC-ζ, which could modify the sensitivity of the enzyme. In heart, growth factor-stimulated production of PIP3 through PI3K is able to stimulate PKC-ζ and activate AC5 by a mechanism independent of Ca2+. (91, 120).

Along the same line, another model is provided by the ATP stimulation of AC through purinergic receptors. For long time, it has been reported that extracellular purines act as intercellular messengers and exert a widespread influence on cellular function by acting through different types of cell surface receptors. Activation of P2Y purinoceptors has been linked to changes in the cAMP level. According to the cell type, purinoceptor activation may result in an increase in basal and stimulated cAMP production as observed in microvascular endothelial cells from adrenal medulla and in heart. ATP, which is released from the terminal sympathetic nerve together with norepinephrine under physiological conditions, increases the contractility of isolated cardiac preparations (88) and induces chrono-
tropic and dromotropic effect on mammalian sinoatrial node, by binding to P2 purinoceptors. In ischemic hearts, ATP could also be a source of arrhythmia. In an attempt to demonstrate the mechanism by which purinergic stimulation of cardiomyocytes increases intracellular cAMP, Pucéat et al. (135) demonstrated that purinergic stimulation of cardiomyocytes increased intracellular cAMP content through a Gs-mediated activation of an AC (135). Using HEK-293-transfected cells, they demonstrated that AC5, but not AC4 or AC6, is responsive to the purinergic stimulation. Moreover, purinergic activation of AC is additive to that of isoproterenol in cardiomyocytes. It was thus suggested that purines might act as modulators of cell functions already regulated by other neuromediators released from the same nerve terminals.

During the course of pregnancy and at the onset of parturition, the contractile activity of the uterus is under the control of steroid hormones. Progesterone, which culminates at midpregnancy, enhances myometrium relaxations by increasing the Gs-coupled β2-AR cAMP cascade (29, 50, 99, 174). The regulation of myometrium contractility implicates the AC-stimulatory pathways as a key component modulating the intracellular cAMP concentration and thus the contractile state of the uterus. Northern blot analysis revealed the presence of numerous isoforms in both humans and rats, AC6 being the major ones (109, 153). The level of expression of the AC mRNAs increases 1.7- to 3.4-fold during the course of pregnancy and diminishes near term and after delivery. In agreement with these findings, both basal and forskolin-stimulated AC activities exhibited a two- to threefold increase during the course of pregnancy, followed by a slight decrease near term (153). These data indicate that changes in the level of AC mRNA (and presumably proteins) that occur during pregnancy and after delivery may contribute to the essential role of cAMP in maintaining uterus quiescence. In this respect, the papers by Mhaouty et al. (108, 109) are particularly relevant. They have identified two types of Gs-coupled α2-AR in rat myometrium: α2A-AR transcript is present at midpregnancy, whereas α2B-AR mRNA is detected at term (108). At midpregnancy, the activation of the α2A-AR/Gs signaling cascade by micromolar concentration of clonidine, results in a potentiation of the β2-AR stimulation of the AC activity in myometrial membranes (108); addition of α-transducin, a Gβγ scavenger, blocks this potentiation in a dose-dependent manner (109). At the time of delivery, [Ca2+]i dramatically increases in response to external stimuli and may inhibit AC6 activity, which is also expressed at a high level. Thus, during the early stages of pregnancy, when it is important to maintain a relaxed state of myometrium, the α2A-AR activation augments the effect of isoproterenol on the cellular cAMP concentration, promoting smooth muscle relaxation. This effect is probably mediated through Gβγ activation of AC2. At the later stage of pregnancy, when contraction is important, α2-AR inhibits the stimulation of AC by the β-AR agonists. Marjamaki et al. (101) have proposed that such a switch in the consequences of α2-AR stimulation could be explained by a change in the phosphorylation status of AC2.

CAMP and Drug Dependence

Acute administration of morphine or opioids causes a decrease in AC activity via the Gi pathway, and chronic administration leads to the classic states of tolerance and dependence (122). Dependence includes 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. (141) on NG 108–15 cells is an upregulation of the cAMP system, including AC, PKA, and the transcriptional factor CREB. Thus, after a long-term in vivo morphine treatment followed by administration of the antagonist naloxone, an increase in the AC activity in the cerebral cortex (45, 98) and in striatum (105, 164) but not in the cerebellum devoid of receptor can be observed. For example, after morphine withdrawal, there is a 30% increase in basal and forskolin-stimulated AC activity in the striatum, an increase that is no longer seen in μ-receptor-deficient animals (105). It is also noteworthy that, in an extensive study of the critical role of cAMP in morphine dependence in the rat, Lane-Ladd et al. (87) found an increase in AC1, AC8, PKA, and CREB in the locus coeruleus, a major site responsible for the physical signs of dependence. These data confirm our earlier results on the involvement of AC8 in the locus coeruleus (103).

These changes have been reproduced in various cell culture systems, and one can more or less readily observe an upregulation of AC activity that has the following characteristics: 1) it can be observed after treatment with a variety of inhibitory ligands, including muscarinic agents and somatostatin (167); 2) it is long lived; 3) depending on the system studied, it may or may not involve a transcriptional step (7); 4) the βγ-subunits seem to play a specific, although not direct, role (8, 19, 168); and 5) the effect may be specific for certain isoforms of AC (9, 196). 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 (177).

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 cannabinoid withdrawal confirms this model. The recent availability of a specific CB1 antagonist, SR-141716A, has allowed one to set up an in vivo model of cannabis abstinence. After 6 days of treatment with Δ9-tetrahydrocannabinol, followed by the administration of the
antagonist, mice exhibit several somatic signs (wet-dog shakes, facial rubbing, ataxia, hunched posture, mastication) that could be interpreted as being part of a withdrawal syndrome. Interestingly, the same animals exhibit a 100% increase in the basal, forskolin-, and Ca\(^{2+}\)/calmodulin-stimulated AC activity in the cerebellum (rich in CB1 receptors) but not in the cortex or the striatum (70).

Alcohol is one of the most widely abused drugs in the world. Although ethanol does not act through a specific receptor, there is increasing evidence that the observed effects result from specific alterations. Among the various biological markers associated with certain subtypes of chronic alcoholism, a low-platelet AC activity has been proposed to reflect a genetic predisposition to alcohol dependence. In most cell culture systems, acute exposure to ethanol treatment has been found to potentiate the receptor-mediated cAMP synthesis. In contrast, chronic exposure often causes a decrease in cAMP production. Parsian et al. (127) have observed that basal and fluoride-stimulated platelet AC activity of alcoholic patients have lower value than in control subjects (127). Moreover, Ikeda et al. (71) have reported that 5’-guanylyl imidodiphosphate- and forskolin-stimulated platelet AC activity may help to distinguish between subtypes of alcoholic patients (those who develop a negative mood in response to drinking, those who continue drinking despite health effects, those who become violent while drinking) (71). Recently, Ratsma et al. (137) have described that the forskolin-stimulated AC activity is considerably lower in platelets of children of alcoholic patients (children who are at high risk for alcoholism but not yet consuming alcohol). Furthermore, the reduced AC activity was only observed in platelets of children from multigenerational family of alcoholism. The platelet AC may therefore represent a trait marker for genetic predisposition to alcoholism.

In human platelets, the major AC isoform expressed is AC7 (67). A selective effect of ethanol on cAMP synthesis through a specific AC isoform has been demonstrated by using HEK-293 cells transfected with different types of ACs: the stimulation of cAMP generation by ethanol was found two- to threefold greater in AC7-transfected cells than in cells transfected with other ACs (197). Recently it has been proposed that ethanol may act by promoting phosphorylation of AC7 (136).

To better understand the mechanism of action of ethanol, animal models have been used. On exposure to ethanol, *Drosophila* displays behavior quite similar to that observed on ethanol intoxication in rodents and humans. More readily accessible to genetic analysis, *Drosophila* represents a very attractive model to investigate the molecular mechanisms underlying ethanol dependence. Moore et al. (114) have demonstrated that ethanol intoxication in *Drosophila* is modulated through the cAMP pathway and probably through AC1 activity (114). Indeed, loss-of-function mutations in rutabaga AC (the *Drosophila* AC1) increases the sensitivity to ethanol, whereas flies lacking both the cAMP phosphodiesterase (dunce) and the isoform AC1 (rutabaga) are not different from wild-type control flies.

**GENERAL CONCLUSIONS**

The existence of large families of proteins at each level of the cAMP signaling pathways (receptors, G proteins, cyclases, phosphodiesterases) has opened the Pandora’s box of combinatorial regulations. No longer can we safely assume that a given hormone will always increase, or decrease, the cAMP content of a cell. We are overwhelmed by the variety of potential regulations of AC activity. Yet, most recent progress in the field of AC has focused on the structural components of the enzymes involved in potential regulations more than on their physiological relevance. For some time, we will probably have to face the usual problem of sorting out important regulatory loops from spurious ones.

In particular, we are still lacking data from knockout experiments with the all various isoforms of AC (only the knockout of AC1 has been reported to date). As cAMP plays a key role during development, conditional knockouts in various organs will be probably necessary. This is all the more needed as a compensatory increase of an isoform to supplement the loss of another one cannot be excluded. From this point of view, it is striking that no pathology linked to the alteration of a cyclase isoform has been reported to far, with the exception of the altered sensory patterning of somatosensory cortex of barrelless mice, whereas the pathology linked to the other components of the cAMP signaling pathway (receptors, G protein) is well known.

Therefore, some of the questions that are likely to be crucial in the years to come might be the following. 1) To what extent are the various isoforms redundant? 2) To what extent are the various cross-talks, potentially regulating cAMP formation, really physiological? For example, if the pineal gland is a good model for AC1 being a potential coincidence detector (for G\(_{\alpha}\) and calmodulin/Ca\(^{2+}\)), we have to admit that we have no direct experimental evidence for it. Similarly, the potential inhibition of AC5 and AC6 by Ca\(^{2+}\) is still in need of a convincing demonstration. 3) In the cascade “receptor-G protein-effector,” AC is probably limiting, as demonstrated by Post et al. (131) in various systems. However, is the likely cellular compartmentalization of AC interfering with the stoichiometry of the enzyme with respect to the other components? 4) Are the overexpressed, extraneous isoforms localized in the correct compartments? 5) To what extent is the demonstration that the cAMP signaling cascade occurring within a restricted, caveolin-enriched, microdomain of the plasma membrane (140), a constant phenomenon? 6) Are there endogenous analogs of forskolin or adenosine nucleoside polyphosphate (39, 77) that could further regulate the activities of the different isoforms?

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