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International Union of Basic and Clinical Pharmacology. CI. Structures and Small Molecule Modulators of Mammalian Adenylyl Cyclases

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Journal

Pharmacological Reviews, 69(2)

ISSN

0031-6997

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Publication Date

2017-04-01

DOI

10.1124/pr.116.013078

Peer reviewed

ASSOCIATE EDITOR: ELIOT H. OHLSTEIN

International Union of Basic and Clinical Pharmacology. CI. Structures and Small Molecule Modulators of Mammalian Adenylyl Cyclases

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This work was supported by the National Institutes of Health National Institute of General Medical Sciences [Grants GM107094 (to R.S.O.), GM60419 (to C.W.D.)], National Institute of Neurological Disorders and Stroke [Grant NS091759 to C.W.D.], National Institute of Mental Health [Grants MH096927 and MH101673 to V.J.W.], the American Heart Association [AHA 005140Z, AHA 0450120Z, 14GRNT20380762], and the Deutsche Forschungsgemeinschaft [Se 529/5-1, Se 529/5-2].

We dedicate this review to the late Alfred G. Gilman, Nobel laureate for Physiology 1994, who made seminal contributions to the field of adenylyl cyclase research.

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dx.doi.org/10.1124/pr.116.013078.

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Abstract—Adenylyl cyclases (ACs) generate the second messenger cAMP from ATP. Mammalian cells express nine transmembrane AC (mAC) isoforms (AC1-9) and a soluble AC (sAC, also referred to as AC10). This review will largely focus on mACs. mACs are activated by the G-protein G_{α_s} and regulated by multiple mechanisms. mACs are differentially expressed in tissues and regulate numerous and diverse cell functions. mACs localize in distinct membrane compartments and form signaling complexes. sAC is activated by bicarbonate with physiologic roles first described in testis. Crystal structures of the catalytic core of a hybrid mAC and sAC are available. These structures provide detailed insights into the catalytic mechanism and constitute the basis for the development of isoform-selective activators and inhibitors. Although potent competitive and noncompetitive mAC inhibitors are

available, it is challenging to obtain compounds with high isoform selectivity due to the conservation of the catalytic core. Accordingly, caution must be exerted with the interpretation of intact-cell studies. The development of isoform-selective activators, the plant diterpene forskolin being the starting compound, has been equally challenging. There is no known endogenous ligand for the forskolin binding site. Recently, development of selective sAC inhibitors was reported. An emerging field is the association of AC gene polymorphisms with human diseases. For example, mutations in the AC5 gene (*ADCY5*) cause hyperkinetic extrapyramidal motor disorders. Overall, in contrast to the guanylyl cyclase field, our understanding of the (patho)physiology of AC isoforms and the development of clinically useful drugs targeting ACs is still in its infancy.

I. Introduction

A. Overview of Adenylyl Cyclase Signaling

Adenylyl cyclases (ACs) catalyze the conversion of ATP into the second messenger cAMP. ACs are broadly expressed in the kingdom of life (Linder and Schultz, 2003; Gancedo, 2013). This IUPHAR review exclusively covers mammalian ACs and focuses on the nine membrane AC isoforms, AC1–AC9 (Alexander et al., 2015; <http://www.guidetopharmacology.org/GRAC/FamilyDisplayForward?familyId=257>). We will discuss

in detail structural aspects because knowledge of the molecular architecture of ACs constitutes the rational basis for the development of potent and AC isoform-selective inhibitors and activators.

Certain bacterial AC toxins such as edema factor from *Bacillus anthracis* and CyaA from *Bordetella pertussis* constitute potential antibacterial drug targets and are discussed elsewhere (Seifert and Dove, 2012, 2013). ACs from parasites such as *Trypanosoma brucei* are also drug targets (Seebeck et al., 2004; Makin and Gluenz, 2015).

AC and cAMP were first described by Nobel laureate Earl W. Sutherland (Rall and Sutherland, 1958; Sutherland et al., 1962). The early history of mammalian ACs and cAMP is covered by excellent reviews (Sutherland and Robison, 1966; Hardman et al., 1971). G-protein activation of AC was first described by Pfeuffer (1977) and Gilman (1995). Later, mammalian ACs were found to be under the dual stimulatory and inhibitory control of G-proteins (Rodbell, 1984; Gilman, 1987; Birnbaumer et al., 1990). Moreover, the plant diterpene forskolin (FSK) from *Coleus forskohlii* (an ancient Indian folk medicine) was identified as an AC activator and has become a very important experimental tool for probing the involvement of ACs in (patho)physiologic processes (Seamon et al., 1981; Daly, 1984; Insel and Ostrom, 2003; Alasbahi and Melzig, 2012). After purification of a mammalian AC and peptide sequencing (Smigel, 1986), subsequent cloning studies revealed a complex transporter-like topology of membrane ACs (mACs) (Krupinski et al., 1989) and the existence of nine isoforms (ACs 1–9) (Sunahara et al., 1996).

In the 1970s, when the field of signal transduction at the membrane was still in its infancy, an AC with unique biochemical properties was described in the soluble fraction of rat testis homogenate (Braun and Dods, 1975; Neer, 1978; Braun, 1991). The initial observation was confirmed by several laboratories (Gordeladze and Hansson, 1981; Gordeladze et al., 1982; Stengel and Hanoune, 1984), but the nature of the enzyme catalyzing this reaction remained elusive for almost three decades. The unique properties of sAC were conclusively elucidated with the cloning of cDNAs derived from mouse testis and human embryonic kidney (HEK) 293 cells (Buck et al., 1999; Geng et al., 2005).

Numerous reviews on the cloning, structure, function, regulation, and pharmacological modulation of mammalian ACs are available (Cooper, 1998; Tang and Hurley, 1998; Defer et al., 2000; Sunahara and Taussig, 2002; Sinha and Sprang, 2006; Willoughby and Cooper, 2007; Sadana and Dessauer, 2009; Pavan et al., 2009;

Pierre et al., 2009; Seifert et al., 2012; Steegborn, 2014; Schmid et al., 2014; Levin and Buck, 2015). Methods for the analysis of mammalian ACs have been reviewed in volume 345 of the book series *Methods in Enzymology* (Iyengar and Hildebrandt, 2002).

The purpose of this review is to bring together leading experts from the AC field and provide a state-of-the-art of the field with a strong focus on mACs 1–9. We discuss (patho)physiologic aspects and implications for drug development. Moreover, we try to integrate a historical perspective and cite representative papers from leading groups over the past six decades. However, we apologize to all colleagues whose works we could not cite due to the focus of this review and limitations in space.

We are fully aware of the current discussion about data reproducibility (Kannt and Wieland, 2016) and positive publication bias (Carvalho et al., 2016). Many “negative” studies trying to confirm published original data were shelved and never published. Confirmatory “positive” papers are similarly discouraged by most scientific journals (Kannt and Wieland, 2016). Therefore and whenever possible, we strived to draw conclusions on the basis of data from independent research groups to obtain a balanced picture, highlight gaps in our knowledge, and point to controversial data. A major problem in the field of pharmacological AC modulation is that the available experimental tools have not always been used critically (Seifert, 2014, 2016; Michel and Seifert, 2015) so that misinterpretations of data are likely to have occurred. Unfortunately, catalogs of chemical companies contribute to the misinformation of researchers because compounds are being advertised as being selective for a specific nucleotidyl cyclase without actually having this property. Thus, researchers should exert great caution when selecting pharmacological tools for their studies, particularly in intact-cell systems.

B. Nomenclature

The enzyme that catalyzes the production of cAMP from ATP was originally named *adenyl cyclase* [EC 4.6.1.1] (Sutherland et al., 1962). The name *adenylate*

ABBREVIATIONS: AC, adenylyl cyclase; AKAP, A-kinase-anchoring protein; β AR, β -adrenoceptor; 2C2, C2 catalytic domain of AC2; 5C1, C1 catalytic domain of AC5; CaM, calmodulin; 2CE, 2-hydroxyestradiol; cNMP, nucleoside 3,5-monophosphate; CNS, central nervous system; FDFM, familial dyskinesia with facial myokymia; FSK, forskolin; $G\alpha_i$, α -subunit of the inhibitory G-protein of adenylyl cyclase G_i ; $G\alpha_o$, α -subunit of the G-protein G_o that is widely expressed in the CNS and neuroendocrine cells and mediates inhibition of calcium channels; $G\alpha_{olf}$, α -subunit of the olfactory G-protein that stimulates adenylyl cyclase; $G\alpha_q$, α -subunit of a G-protein that stimulates phospholipase C; $G\alpha_s$, α -subunit of the stimulatory G-protein of adenylyl cyclase G_s ; $G\beta\gamma$, $\beta\gamma$ -subunits of G-proteins; GPCR, G-protein-coupled receptor; HEK, human embryonic kidney; IBMX, isobutylmethylxanthine; KH7, (\pm)-2-(1H-benzimidazol-2-ylthio)propanoic acid 2-[(5-bromo-2-hydroxyphenyl)methylene]hydrazide; mAC, membrane adenylyl cyclase; MANT, 2',(3')-O-(N-methylanthraniloyl); MRP, multidrug resistance associated protein; NB001, 5-[[2-(6-amino-9H-purin-9-yl)ethyl]amino]-1-pentanol; NKY80, 2-amino-7-(2-furanyl)-7,8-dihydro-5(6H)-quinazolinone; NO, nitric oxide; PDE, phosphodiesterase; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; sAC, soluble adenylyl cyclase; sGC, soluble guanylyl cyclase; pGC, particulate guanylyl cyclase; RGS proteins, regulator of G-protein signaling proteins; RU-0204277, 6-chloro-N⁴-cyclopropyl-N⁴-[(thiophen-3-yl)methyl]pyrimidine-2,4-diamine; SKF-83566, 8-bromo-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1H-3-benzazepin-7-ol hydrobromide; SQ22,536, 9-(tetrahydro-2-furanyl)-9H-purin-6-amine.

cyclase first appeared in print in 1970 (Stansfield and Franks, 1970), and it did not take long for this alteration in the name to take hold. As the story goes according to Louis Ignarro, over dinner sometime in the late 1970s, many of the leaders in the field all agreed that *guanylate* was far easier to pronounce than *guanylyl*. The same logic was true for *adenylate*, and thus a mixture of names appeared throughout the 1970s and 1980s. However, the name *adenylate cyclase* does not correctly describe the chemistry of the enzymatic reaction. Because the α,β oxygen leaves ATP upon attack of the O3 oxygen of the ribose ring (Walseth et al., 1981), technically it is an adenylyl moiety that is cyclized, not adenylate (adenylate is equivalent to AMP; if adenylate were cyclized, the reaction would end up with one excess oxygen). The decision by several prominent laboratories to switch to *adenylyl cyclase* took place slowly in the late 1980s and mid 1990s. Alfred G. Gilman relayed his recollections of events at the 1988 Cold Spring Harbor meeting shortly before his untimely death (and later confirmed by Lutz Birnbaumer). More senior scientists may remember the push for the term *N proteins* as opposed to *G proteins*. According to Gilman and in response to a call for clarification of G protein nomenclature,

Lutz proclaimed that he would accept G if we accepted adenylyl. This was a complete surprise to me. I was not familiar with the chemistry, did not realize that there was an argument, did not really give a damn, and quickly said “sold”. For some reason it all happened quickly and painlessly after that.

Thus, despite issues with pronunciation, the name *adenylyl cyclase* is here to stay. Technically, this should not be a problem because the abbreviation *AC* for *adenylyl cyclase* is broadly used and easy to pronounce.

The IUPHAR consortium on ACs discussed nucleotidyl cyclase nomenclature with the IUPHAR consortium on guanylyl cyclases (GCs). The consortia agreed that for chemical reasons, the terms *guanylyl cyclase* and *adenylyl cyclase* should be used to reflect the actual biochemistry of NTP cyclization. The International Union of Biochemistry and Molecular Biology still lists *adenylate cyclase* as the accepted name of AC, mentions a number of alternative names, but the entry into the database is from the year 1972, many years before the nomenclature discussion began. However, it should be kept in mind that specifically in the older literature, the term *adenylate cyclase* was used commonly. Hence for database searches, both the terms *adenylate cyclase* and *adenylyl cyclase* have to be used because with the latter search term, substantially fewer items will be retrieved than with the former.

ACs are encoded by specific genes and divided into five classes, specified with Roman numbers I–V (Linder

and Schultz, 2003). All mammalian ACs belong into the class III (Linder and Schultz, 2003; Sinha and Sprang, 2006; Steegborn, 2014). The mammalian AC genes are designated *ADCY1-10*. These terms are used in genetic studies and are of particular relevance for studies linking specific polymorphisms in AC isoforms to human diseases, an emerging scientific field. In biochemical and pharmacological studies on the actual proteins, the term AC is used. ACs 1–9 are membrane-delimited ACs (mACs) and also referred to as *mACs 1–9*. They belong to the class IIIa (Linder and Schultz, 2003). The term *transmembrane ACs* (tmACs) is used less frequently. *AC10* corresponds to the *soluble AC*, *sAC*. The terms AC10 and sAC are both used in the literature. sAC is a class IIIb AC (Linder and Schultz, 2003). The IUPHAR committee on ACs agreed that the term sAC should be preferred to reflect the initial description of the enzyme as soluble AC. This nomenclature provides an analogy to the GC field, where membrane and soluble GCs (sGCs) are distinguished from each other. In the GC field, the membrane GCs are referred to as *particulate GCs* (*pGCs*). Although the terms *membrane* in the AC field and *particulate* in the GC field describe identical enzyme anchoring modes, it was decided not to change these terms because they are very well established, specifically in light of the fact that the GC committee introduced a number systems for sGCs (GC1 and GC2) and a capital letter system for pGCs (GC-A–GC-G). With respect to numbering of AC isoforms, both Roman and Arabic numbers are used. The AC committee agreed that for better readability, Arabic numbers should be used.

The term AC implies that the enzyme is capable of producing only cAMP. This assumption is correct for mACs 1-9 (Sunahara et al., 1998; Hasan et al., 2014). However, sAC is more promiscuous in terms of substrate specificity and also synthesizes cGMP and the cyclic pyrimidine nucleotides cCMP and cUMP (Hasan et al., 2014; Kleinboelting et al., 2014b). Accordingly, from a biochemical perspective, sAC is an ATP-preferring nucleotidyl cyclase (Seifert, 2015; Seifert et al., 2015). pGCs are very ineffective at producing cAMP (Beste et al., 2013), whereas sGC is also capable of synthesizing cAMP, cCMP, and cUMP and constitutes a GTP-preferring nucleotidyl cyclase (Beste et al., 2012). The AC committee decided that these additional enzymatic activities of nucleotidyl cyclases will not change the historically established distinction into ACs and GCs. This is also justified in view of the fact that neither sAC nor sGC prefer CTP or UTP relative to ATP or GTP as substrates (Seifert, 2015; Seifert et al., 2015).

With regard to the mAC activator forskolin, both the abbreviations FS or FSK (Fsk) are being used. For literature searches in databases, the term FS is not sufficiently specific. Therefore, we recommend that the term FSK be used.

C. Model Systems for Adenylyl Cyclase Analysis

1. *From Organs to Purified Adenylyl Cyclases.* Research on ACs started with crude organ membrane fractions (Rall and Sutherland, 1958; Sutherland et al., 1962) and soluble organ fractions in case of sAC (Braun and Dods, 1975; Neer, 1978; Braun, 1991). Although organ membranes contain numerous AC isoforms, this system is relatively close to the physiologic situation. By combining physiologic and pharmacological activators, inhibitors, RT-PCR analysis of expressed mRNA and, if available, antibodies, analysis of ACs in organ membranes can be quite informative and yield valuable information on functionally dominant AC isoforms (Göttle et al., 2009; Erdorf and Seifert, 2011). Experiments with native organs have been successfully complemented with gene knockout studies and transgene overexpression studies in mice (Ferguson and Storm, 2004; Sadana and Dessauer, 2009).

Studies with membranes from native cells such as platelets (e.g., Wieland et al., 1992) or cultured cells such as neuroblastoma cells (e.g., Sharma et al., 1975a) also yielded most valuable insights into the mechanisms of AC regulation. Of particular importance in this regard are the S49 lymphoma wild-type cells and the $G\alpha_s$ -deficient cyc^- cells (named according to the observation that they do not respond to hormonal stimulation in terms of increased cAMP production and erroneously assumed to be AC deficient). These cell lines played a fundamental role in resolving and reconstituting protein components of the G-protein-coupled receptor (GPCR)–G-protein-AC signaling cascade (Shear et al., 1976; Ross et al., 1978; Hildebrandt et al., 1982; Jakobs et al., 1983a,b). Alterations in platelet membrane AC activity have been proposed as biomarkers for depression and alcoholism (Menninger et al., 2000; Hines and Tabakoff, 2005), fostering current studies analyzing polymorphisms in individual *ADCY* genes (see section II.E).

Although purification of the G-proteins was rather straightforward and the purified G_i - and G_s -proteins were stable (Gilman, 1987; Birnbaumer et al., 1990), purification of functionally and structurally intact GPCRs (Gether et al., 1997) and particularly mACs turned out to be much more difficult because of their very low abundance and conformational and thermal instability under the conditions used for purification (Ross, 1981; Pfeuffer et al., 1985, 1989; Smigel, 1986). However, crude detergent-dispersed AC from rat brain is quite stable and has been used for a number of studies (Johnson and Sutherland, 1973; Desaubry et al., 1996; Desaubry and Johnson, 1998; Gille et al., 2004).

The advent of mAC cloning (Krupinski et al., 1989) enabled studies with recombinantly expressed ACs. ACs can be overexpressed in mammalian cells, HEK293 cells being a very popular model system (Jacobowitz et al., 1994). One should keep in mind that HEK293 cells endogenously express several mACs,

providing background activity (Atwood et al., 2011; Yu et al., 2014). ACs can also be effectively overexpressed in *Spodoptera frugiperda* Sf9 cells (Taussig et al., 1994b). Although few studies have been performed with ACs purified from Sf9 cells (Taussig et al., 1993a,b), most studies are conducted with crude Sf9 membranes overexpressing a given AC isoform, increasing stability of the enzymes and facilitating the execution of extensive pharmacological studies (Pinto et al., 2008, 2011; Brand et al., 2013). Again, the background activity of the endogenous membrane AC of Sf9 cells needs to be properly considered in overexpression studies (Pinto et al., 2008). Like mammalian mACs, the membrane AC of Sf9 cells is potently inhibited by MANT [2',(3')-O-(N-methylanthraniloyl)] nucleotides (Gille et al., 2004) and effectively activated by FSK (Pinto et al., 2008).

In the case of GPCRs (Kobilka et al., 1988) and G-protein α -subunits (Woon et al., 1989; Conklin et al., 1993), the approach of constructing and expressing chimeras between various protein isoforms has been successful and provided valuable insights into functional domains. In contrast, in the case of ACs, there are relatively few studies on chimeras of full-length AC isoforms (Levin and Reed, 1995; Crossthwaite et al., 2005; Yoshimura et al., 2006; Diel et al., 2008). A major reason for this situation is the fact that the chimeras are unstable and, hence, functionally inactive (Seebacher et al., 2001). As a result, our current knowledge on the functional relevance of the transmembrane domains is rather poor. It has been proposed that the mammalian AC transmembrane domains may serve in solute transport (Krupinski et al., 1989), as a voltage sensor (Reddy et al., 1995) or dimerization domain (Cooper and Crossthwaite, 2006). Experimental validation of these proposals is still in the very beginning (Beltz et al., 2016).

2. *Studies with Purified C1 and C2 Subunits.* Although the transmembrane domains of mACs constitute a major reason for their extremely difficult biochemical tractability, the cytosolic domains C1 and C2 are much more readily accessible (Fig. 1). Expression in *Escherichia coli* of a fusion protein of parts of the C1 and C2 domains of ACs 1 and 2, respectively, with complete omission of the transmembrane domains, resulted in a FSK- and $G\alpha_s$ -stimulated enzyme (Tang and Gilman, 1995). This landmark study showed that for the essential aspects of catalysis and regulation, the transmembrane domains are dispensable, raising also the question whether these domains have just a membrane anchor function for the cytosolic domains. The C1:C2 approach has been successfully applied to a number of AC isoforms, expressing either fusion proteins or separate C1 and C2 subunits that form a catalytically active enzyme in solution (Yan et al., 1996, 1997; Whisnant et al., 1996; Scholich et al., 1997; Sunahara et al., 1997; Dessauer et al., 1998; Haunso et al., 2003). In contrast to the C1:C2 heterodimer (Whisnant et al., 1996; Sunahara et al., 1997), the C1:C1 homodimer and the

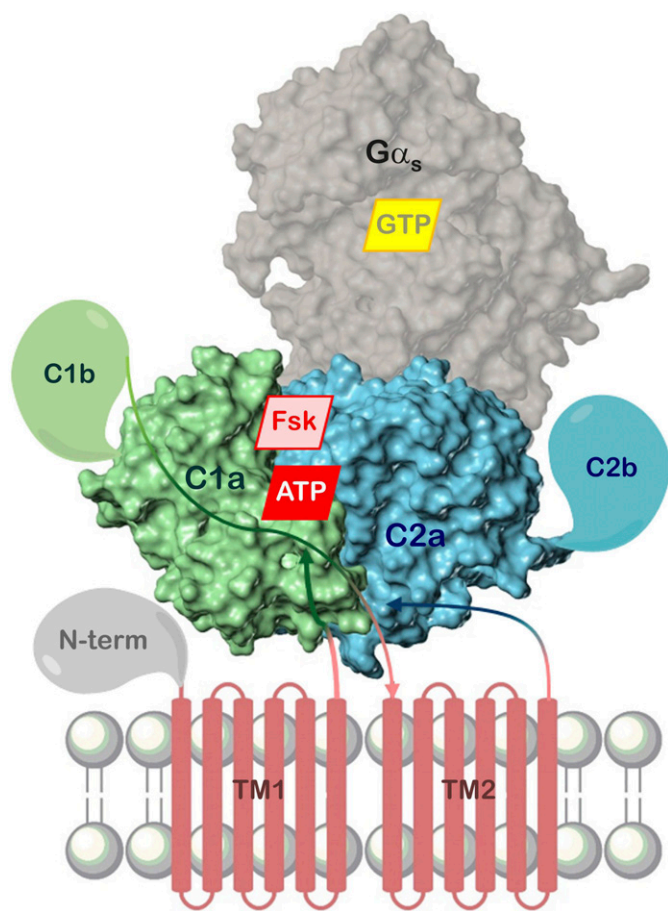


Fig. 1. Common domain structure of mACs. Components resolved in crystal structures are presented as surface model. Shown is the “ventral” surface of C1a:C2a for the sake of consistency with Figs. 2–4, 6–11, and 14 depicting crystal structures. The real position of the membrane is probably behind the hidden “dorsal” surface. FSK, forskolin site (AC1–8), N-term, N-terminal domain; C2b, C-terminal domain; TM, transmembrane domains; arrow lines, schematic course of the polypeptide chain.

C2:C2 homodimer are catalytically inactive (Whisnant et al., 1996; Zhang et al., 1997; Suryanarayana et al., 2009b).

The various C1:C2 systems have yielded valuable insights into the regulatory properties of mACs. The most widely used system in this regard has become the system with the C1 domain from AC5 (5C1) and the C2 domain from AC2 (2C2), referred to as 5C1:2C2 system. Historically, the term VC1:IIC2 has been used frequently. For the sake of consistency with the proposed AC isoform nomenclature, we will use the term 5C1:2C2. The heterodimer allowed molecular insights into the regulation of, and conformational changes in, mACs (Whisnant et al., 1996; Sunahara et al., 1997; Dessauer and Gilman, 1997; Dessauer et al., 1997, 1998, 2002; Mou et al., 2005, 2006, 2009; Suryanarayana et al., 2009a; Pinto et al., 2009, 2011) and the resolution of 17 crystal structures with different ligands in the catalytic site (Table 1). Moreover, this system has been used for extensive

pharmacological studies assessing the catalytic site and the allosteric regulatory site (Dessauer, et al., 1997; Gille et al., 2004; Mou et al., 2005, 2006; Suryanarayana et al., 2009a; Pinto et al., 2009, 2011). However, it should be kept in mind that this system constitutes a hybrid with subunits from different AC isoforms so that it can serve just as a model for mACs in general. There are some examples of successful expression of C1:C2 systems from a particular AC isoform, such as ACs 2, 5, 7, and 9 (Scholich et al., 1997; Dessauer et al., 1998; Yan et al., 2001; Haunso et al., 2003; Dokphrom et al., 2011). However, functionality of these systems seems to be very dependent on the specific length of the expressed subunit (Dessauer et al., 1998; Mou et al., 2005). The most widely used purified mAC system is 5C1(dog):2C2(rat) (Table 1). The high conservation of the catalytic domains between dog and rat tempers concerns regarding the use of proteins from different species, but regulation and interactions with the N terminus is missing in this system (Gao et al., 2007).

3. Methodological Aspects. For the analysis of AC activity in cell-free systems with purified proteins or membranes, reliable and sensitive one- and two-column methods for separation of the substrate [α - 32 P]ATP and the product [32 P]cAMP (eluted from the column) are available (Johnson et al., 1994; Post et al., 2000). For inhibition of cAMP-degrading phosphodiesterases (PDEs), cell-free AC assays often contain the PDE inhibitor isobutylmethylxanthine (IBMX). However, limited water solubility of the inhibitor (unless aqueous solutions are preheated or organic solvents are used) and IBMX resistance of certain PDEs (Bender and Beavo, 2006; Francis et al., 2011) could influence assay results. Other studies included cAMP (see, e.g., Gille et al., 2004), but cAMP at high concentrations can inhibit ACs (Dessauer and Gilman, 1997). A highly important variable in AC assays with cell-free preparations is the choice of the particular divalent cation, Mg^{2+} or Mn^{2+} . In general, with Mn^{2+} , higher catalytic activities than with Mg^{2+} are obtained (Gille et al., 2004; Mou et al., 2005), the affinities of inhibitors increase (Gille et al., 2004; Mou et al., 2005) and the pharmacological profile of diterpenes is affected (Erdorf et al., 2011). Moreover, Mn^{2+} can functionally uncouple AC from G_i -proteins (Hildebrandt et al., 1982; Jakobs et al., 1983a,b; Dessauer et al., 2002; Gille and Seifert, 2003) and alter basal AC activities (Pieroni et al., 1995). Although Mg^{2+} is usually assumed to be the physiologic cation of catalysis (Ross et al., 1978), indirect evidence points to a role of Mn^{2+} as cation for catalysis, at least for sGC (Bähre et al., 2014).

In many studies, AC activity is assessed in intact cells. For intact cells, various reliable immuno-based cAMP detection methods are available (Post et al., 2000), and high-throughput screening methods (with

TABLE 1
Crystal structures of the catalytic domains of mACs

PDB ID	C1a sequence	C2a sequence	Metal A	Metal B ^a	G α_s ^b	ATP-site	FSK-site	Res. ^c	Reference
1AB8 ^d	—	2C2 (rat) 871-1090	—	—	—	—	FSK	2.20	Zhang et al., 1997
1AZS	5C1 (dog) 361-575	2C2 (rat) 870-1081	—	—	+	—	Methylpiperazino-FSK	2.30	Tesmer et al., 1997
1CJK	5C1 (dog) 364-580	2C2 (rat) 870-1081	Mg ²⁺	+	GTP γ S	Adenosine-5'- <i>RP</i> -(α -thio- <i>PPP</i>)	FSK	3.00	Tesmer et al., 1999
1CJT	5C1 (dog) 364-580	2C2 (rat) 870-1081	Mg ²⁺	+	GTP γ S	2',3'-Dideoxy-ATP	FSK	2.80	Tesmer et al., 1999
1CJU	5C1 (dog) 364-580	2C2 (rat) 870-1081	Mg ²⁺	+	GTP γ S	2',3'-Dideoxy-ATP	FSK	2.80	Tesmer et al., 1999
1CJV	5C1 (dog) 364-580	2C2 (rat) 870-1081	Zn ²⁺	+	GTP γ S	2',3'-Dideoxy-ATP	FSK	3.00	Tesmer et al., 1999
1CS4	5C1 (dog) 361-580	2C2 (rat) 870-1081	—	+	GTP γ S	2'-Deoxyadenosine-3'- <i>P</i> + <i>PP</i>	FSK	2.50	Tesmer et al., 2000
1CUL	5C1 (dog) 364-580	2C2 (rat) 874-1081	Mg ²⁺	+	GTP γ S	2'-Deoxyadenosine-3'- <i>P</i>	FSK	2.40	Tesmer et al., 2000
1TL7	5C1 (dog) 364-580	2C2 (rat) 870-1081	Mn ²⁺	+	GTP γ S	3'-MANT-GTP	FSK	2.80	Mou et al., 2005
1UOH	5C1 (dog) 361-580	2C2 (rat) 870-1081	Mg ²⁺	+	GTP γ S	3'-MANT-GTP	FSK	2.90	Mou et al., 2005
2GVD	5C1 (dog) 363-580	2C2 (rat) 870-1081	Mn ²⁺	+	GTP γ S	TNP-ATP	Methylpiperazino-FSK	2.90	Mou et al., 2006
2GVZ	5C1 (dog) 363-580	2C2 (rat) 870-1081	Mn ²⁺	+	GTP γ S	3'-MANT-ATP	Methylpiperazino-FSK	3.27	Mou et al., 2006
3C14	5C1 (dog) 363-580	2C2 (rat) 870-1081	—	+	GTP γ S	<i>PP</i>	FSK	2.68	Mou et al., 2009
3C15	5C1 (dog) 363-580	2C2 (rat) 870-1081	—	+	GTP γ S	<i>PP</i>	FSK	2.78	Mou et al., 2009
3C16	5C1 (dog) 363-580	2C2 (rat) 870-1081	Mg ²⁺	+	GTP γ S	ATP	FSK	2.87	Mou et al., 2009
3G82	5C1 (dog) 363-580	2C2 (rat) 870-1081	Mn ²⁺	+	GTP γ S	3'-MANT-ITP	FSK	3.11	Hübner et al., 2011a
3MAA	5C1 (dog) 363-580	2C2 (rat) 870-1081	—	+	GTP γ S	Adenosine-5'- <i>RP</i> -(α -thio- <i>PPP</i>)	Methylpiperazino-FSK	3.00	Mou et al., 2009

P, monophosphate; PP, pyrophosphate; PPP, triphosphate; 3'-MANT, 3'-*O*-(*N*-methylanthraniloyl)-; TNP, 2',3'-*O*-(2,4,6-trinitrophenyl)-.

^aMetal ions A and B located in the nucleotide binding site.

^bBovine G α_s not present (—) or present (+) as chain C in the complex together with GTP γ S and mostly metal and Cl⁻ ions (not shown).

^cResolution [Å].

^dHomodimer of 2C2.

varying reliability) have been developed as well (Williams, 2004; Conley et al., 2013). However, formally, intact cell assays do not reflect AC activity assays but constitute cAMP accumulation assays. In intact cell assays, the ATP substrate concentration for ACs cannot be readily controlled. And although PDEs are usually inhibited by IBMX (but see the cautionary note above), it has to be taken into account that cAMP can also be very effectively exported from cells via multidrug resistance associated protein (MRP) isoforms 4 and 5 (Keppler, 2011; Copsel et al., 2011). These transporters are inhibited by numerous compounds such as probenecid (Copsel et al., 2011). Unfortunately, the pharmacological tools used to block PDEs and MRPs are not totally specific. On one hand, methylxanthines can block adenosine receptors and MRPs (Chen et al., 2013b; Xie et al., 2011) and activate ryanodine receptors (Guerreiro et al., 2011). On the other hand, several MRP blockers can also inhibit PDEs (Xie et al., 2011). Thus, caution must be exerted when interpreting the results of intact-cell studies with PDE- and MRP inhibitors.

II. Structures, Expression, Compartmentation, Regulation, and (Patho)physiologic Function of Adenylyl Cyclases

A. Structures of Membrane Adenylyl Cyclases and Comparison with Soluble Adenylyl Cyclase

1. Overall and Crystal Structures of Nucleotidyl Cyclases. ACs 1–9 differ with respect to sequence and length (between 1080 and 1353 amino acids). ACs 1–9 are single peptide chains consisting of an N-terminal cytosolic domain of varying length, two membrane-spanning domains (TM1, TM2), each with six transmembrane α -helices, and two cytosolic domains C1 and C2 subdivided into catalytic (C1a, C2a) and regulatory (C1b, C2b) subdomains (Fig. 1). ACs 1–9 are activated by G α_s , and ACs 1–8 are also activated by FSK. The interface of C1a and C2a contains the ATP binding site and a pseudosymmetric FSK site. C1a and C2a, each with \sim 190 amino acids, are structurally homologous to each other, among mAC isoforms and with corresponding catalytic domains of sAC, sAC-C1 and sAC-C2, and of sGC, sGC α_{cat} and sGC β_{cat} .

Our present knowledge about structure, function, and regulation of the catalytic domains of mammalian

mACs is mainly based on X-ray structure analysis of recombinant C1a and C2a originating from dog AC5 and rat AC2, respectively. Until now, 17 crystal structures have been released in the Brookhaven Protein Data-bank (PDB). Table 1 presents an overview of the structures, varying in cocrystallized metal ions and ligands of the ATP binding site.

C1a and C2a were expressed and purified as 25-kDa peptide chains forming heterodimers with similar AC activity and regulatory properties as the holoenzymes, whereas homodimers of C1a or C2a are catalytically inactive. The heterodimeric construct was termed 5C1:2C2 and in all cases cocrystallized with FSK or its methylpiperazino derivative as well as with recombinant $G\alpha_s$ and GTP γ S as $G\alpha_s$ ligand. ATP-site ligands comprise ATP analogs, P-site inhibitors, and 2',3'-substituted purine nucleotide inhibitors.

sAC exhibits an amino acid sequence and domain organization related more closely to cyanobacteria and myxobacteria nucleotidyl cyclases than to mammalian mACs (Buck et al., 1999). In human sAC, the first 470 amino acids comprise a short N-terminal tail as well as C1 and C2 connected by a linker of about 70 residues (Kleinboelting et al., 2014a). The C-terminal domain of variable length (human sAC ~1140 amino acids) contains a putative autoinhibitory domain, a P-site, and a protein/protein interaction domain. A potential heme-binding domain (sAC-HD) shows some sequence similarities to Per-ARNT-Sim (PAS) domains known as interaction modules in signaling proteins, and more extensive parts of the C-terminal region slightly resemble STAND domains (Signal Transduction ATPases with Numerous Domains) (Middelhaufe et al., 2012; Steegborn, 2014). No clearly defined

transmembrane domain is present in the sAC amino acid sequence. A full-length and a short form of sAC were identified by Western blot of testis extracts with the short form encompassing the C1/C2 domain. Initially, the short form was thought to be generated by proteolysis (Buck et al., 1999), this processing being required for expression of catalytic activity. However, it is now established that alternate splicing generates the testis short form (Jaiswal and Conti, 2001), usually termed truncated sAC (tsAC), as well as isoforms reported in other tissues. Some of these variants lack the C1 domain, whereas others have shortened C termini (Geng et al., 2005; Schmid et al., 2007; Chen et al., 2014a).

The catalytic domain structure of human sAC was resolved by X-ray crystallography. At present, the PDB provides 28 sAC crystal structures (Kleinboelting et al., 2014a,b, 2016; Saalau-Bethell et al., 2014; Ramos-Espiritu et al., 2016), including complexes with ATP, α,β -methylene adenosine-5'-triphosphate, cAMP, pyrophosphate, bicarbonate, biselenite, bisulfite, bithionol, and various inhibitors. Comparing structures of 5C1:2C2, of sAC-C1-C2 and of an inactive human sGC α_{cat} :sGC β_{cat} heterodimer (Allerston et al., 2013; Seeger et al., 2014) indicates high structural homology not only of the individual catalytic domains, but also of the whole complexes, and points to a common, well conserved mechanism of cNMP generation facilitated by two metal ions.

2. General Folds and Alignments of Catalytic Domains. All crystal structures of 5C1:2C2, sGC α_{cat} :sGC β_{cat} , and sAC C1-C2 show a heterodimeric architecture with a pseudo-twofold axis. In Fig. 2, 5C1 and 2C2 are shown in side-by-side comparison denoting

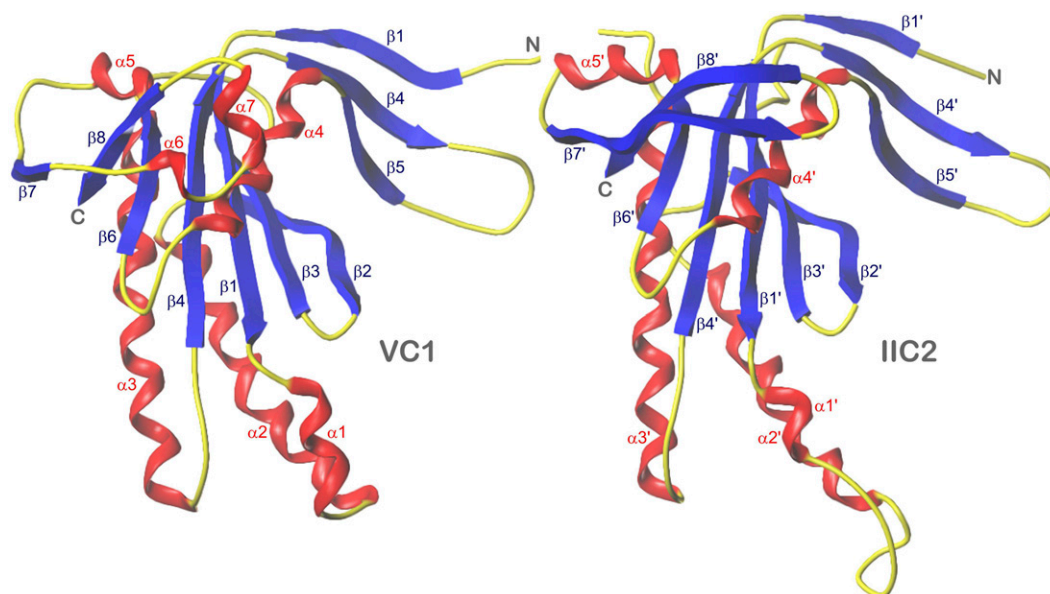


Fig. 2. 5C1 and 2C2 in side-by-side comparison of superimposed monomers. α -Helices, red; β -sheets, blue; loops and turns, yellow. Models are based on the crystal structure PDB 1CJU (Tesmer et al., 1997, 1999). Secondary structure elements as well as N and C termini are labeled.

secondary structure elements (Zhang et al., 1997), primed in the case of 2C2. A root mean square deviation (rmsd) of only 1.3 Å for 153 structurally equivalent C α atom pairs indicates the close similarity of 5C1 and 2C2 (Tesmer et al., 1997). More or less analogous fits result from pairwise superposition of 5C1, sGC α_{cat} , and sAC-C1 as well as 2C2, sGC β_{cat} , and sAC-C2, respectively, and of sGC α_{cat} with sGC β_{cat} as well as sAC-C1 with sAC-C2, in particular if only the sections between $\beta 1$ ($\beta 1'$) and $\alpha 5$ ($\alpha 5'$) are considered. In contrast, sequence alignments are less similar even if based on structure, but reflect the close evolutionary relatedness of mAC and sGC catalytic domains (amino acid identities: 5C1 versus sGC α_{cat} 29%, 2C2 versus sGC β_{cat} 27%), whereas sAC is quite distant from the other two enzymes (sAC-C1 versus 5C1 14%, versus sGC α_{cat} 17%, sAC-C2 versus 2C2 14%, versus sGC β_{cat} 15%). The amino acid identity between sGC α and sGC β is high (42%) in contrast to 5C1 and 2C2 (25%) and to the dissimilar sAC-C1 and sAC-C2 domains (12%).

Figure 3 presents 5C1:2C2 in complex with G α_s : GTP γ S, FSK, ATP, and Mg $^{2+}$ (Tesmer et al., 1997). The catalytic domains form a “head-to-tail, wreath-like dimer” (Tesmer et al., 1997) with a pseudo-twofold axis. Perpendicular to this axis and in front, the so-called “ventral” surface (Zhang et al., 1997) is visible (see also Fig. 1). Common of each domain are five α -helices surrounding an eight-stranded β -sheet core. Two strands ($\beta 1$, $\beta 4$ and $\beta 1'$, $\beta 4'$, respectively) are discontinuous. The 5C1-2C2 interface contains a spacious cavity with two pseudosymmetric binding sites for FSK and ATP + 2 Mg $^{2+}$ ions, respectively. The ligands probably approach from the ventral surface because of extensive 5C1-2C2 contacts at the dorsal side. G α_s interacts nearly exclusively with 2C2 ($\alpha 1'$ - $\alpha 2'$ loop, $\alpha 2'$, $\alpha 3'$, $\alpha 3'$ - $\beta 4'$ loop) and projects widely beyond the ventral surface (Fig. 1), suggesting that the dorsal side faces the membrane (Tesmer et al., 1997).

The structurally most conserved regions between $\beta 1$ and $\beta 4$ as well as $\beta 1'$ and $\beta 4'$, including the two metal-ion

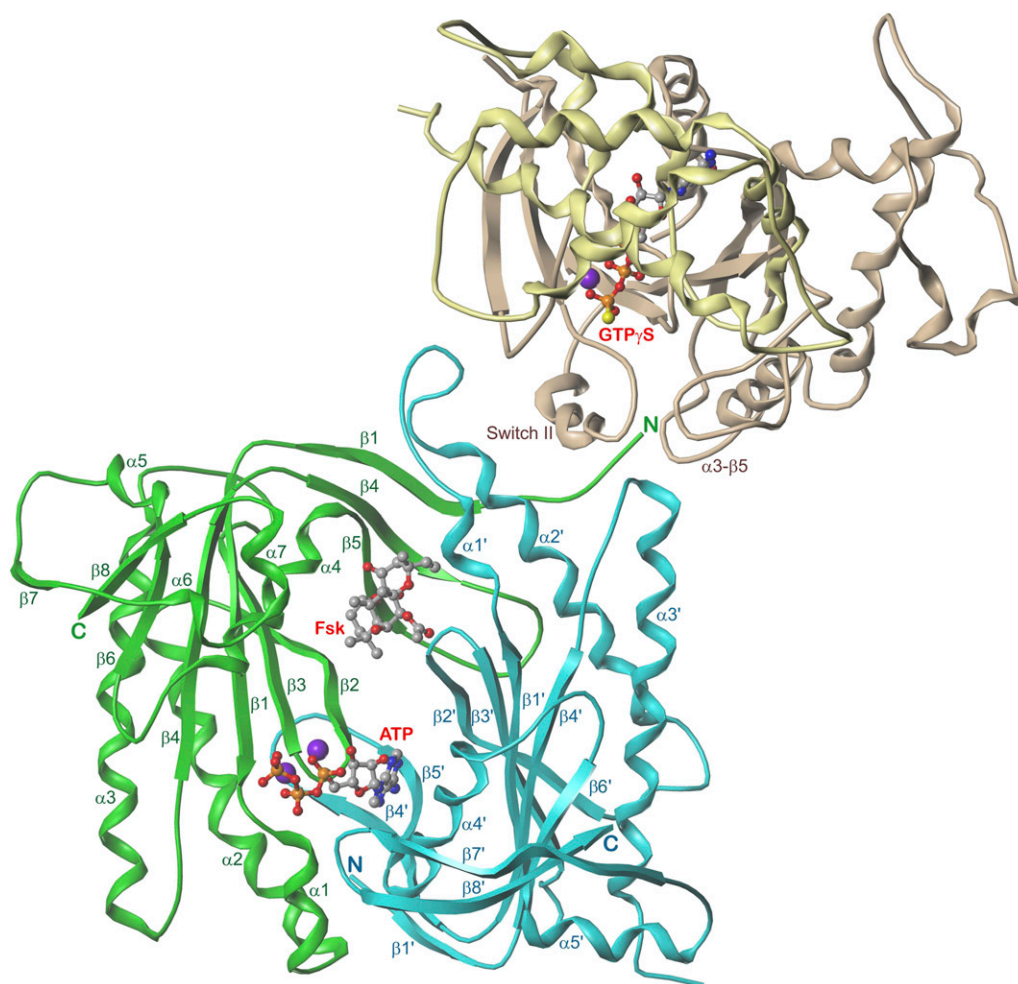


Fig. 3. 5C1:2C2 in complex with FSK, ATP, G α_s , and GTP γ S. View along the pseudo-twofold axis. 5C1, green; 2C2, cyan; G α_s helical domain, yellow; ras-like domain, mauve; atom colors: C, gray; N, blue; O, red; P, orange; Mg $^{2+}$, violet. Models are based on the crystal structure PDB 1CJU (Tesmer et al., 1997, 1999) with ATP docked instead of 2',3'-dideoxy-ATP. Secondary structure elements (G α_s only contact sites with 5C1:2C2) as well as N and C termini of 5C1:2C2 are labeled.

binding carboxylate residues in 5C1, constitute $\beta\alpha\beta\beta\alpha\beta$ catalytic cores present also in the Pol 1 family of nucleotide polymerases, which catalyze an attack of a 3'-hydroxyl upon a 5' phosphate, too (Artymiuk et al., 1997). It may be concluded that mammalian nucleotidyl cyclases have evolved from a very remote ancient nucleotide polymerase ancestor although evolutionary convergence cannot be ruled out.

The architecture of 5C1:2C2 (Fig. 3) is very similar to that of $sGC\alpha_{cat}:sGC\beta_{cat}$ (Allerston et al., 2013) and, to a lesser extent, sAC-C1-C2 (Kleinboelting et al., 2014a). If only the sequences between $\beta 1$ and $\alpha 4$ ($\beta 1'$ and $\alpha 4'$, respectively) are considered, more or less close superpositions are possible (Fig. 4). From these superpositions, a structure-based alignment of the catalytic domains of AC1, AC2, AC5, sAC, and sGC was derived (Fig. 5), being essentially in line with a previous comparison of mACs and sGC, published when only crystal structures of 5C1:2C2 were known (Tesmer et al., 1997), and with a recent alignment of 5C1:2C2, $sGC\alpha_{cat}:sGC\beta_{cat}$, and different prokaryotic and eukaryotic sAC-C1-C2 domains (Kleinboelting et al., 2014b). Exact mapping of secondary structure elements was based on 5C1:2C2, but α -helical and β -strand segments between $\alpha 2$ and $\alpha 4$ ($\alpha 2'$ and $\alpha 4'$) are nearly identical in size and position in all cases.

Differences are obvious between lengths of various loops and between secondary structure elements in the N- and C-terminal regions. The exceptionally long insert in 2C2 between $\beta 3'$ and $\alpha 3'$ is not fully resolved in 5C1:2C2 structures and probably involved in interactions with $G\beta\gamma$ (Chen et al., 1995), a characteristic modulator of AC2. The extended $\beta 2'-\beta 3'$ loop of sAC-C2 contributes to the conversion of the mAC-FSK site into a binding site for the sAC activator bicarbonate (Kleinboelting et al., 2014a). The $\alpha 1-\alpha 2$ and $\alpha 1'-\alpha 2'$ regions vary within and between the three structures. In 5C1:2C2, $\alpha 1$ is one helical turn longer than $\alpha 1'$ and connected by a hairpin turn with $\alpha 2$. The long, protruding $\alpha 1'-\alpha 2'$ loop is in keeping with its role as part of the $G\alpha_s$ binding site. By contrast, $\alpha 1$ and $\alpha 1'$ are of similar length (two turns) in $sGC\alpha_{cat}$ and $sGC\beta_{cat}$, respectively. Helix $\alpha 1'$ is not present in sAC-C2.

The most striking differences between the sequences and structures appear in the C-terminal regions (after $\alpha 5$ and $\alpha 5'$, respectively). In 5C1, a short $\beta 7$ -strand is connected with $\beta 8$ by a long Ω loop including two short α -helices which are not present in $sGC\alpha_{cat}$ and sAC-C1. Nevertheless, the course of $sGC\alpha_{cat}$ in this region roughly resembles that of 5C1. The $\beta 7$ -strand of sAC-C1 is longer, and the course between $\beta 7$ and $\beta 8$ differs compared with 5C1. The $\beta 7'-\beta 8'$ segments of the three structures are more similar. Two extended β -strands are connected by a reverse turn directed to the triphosphate group of the nucleotide.

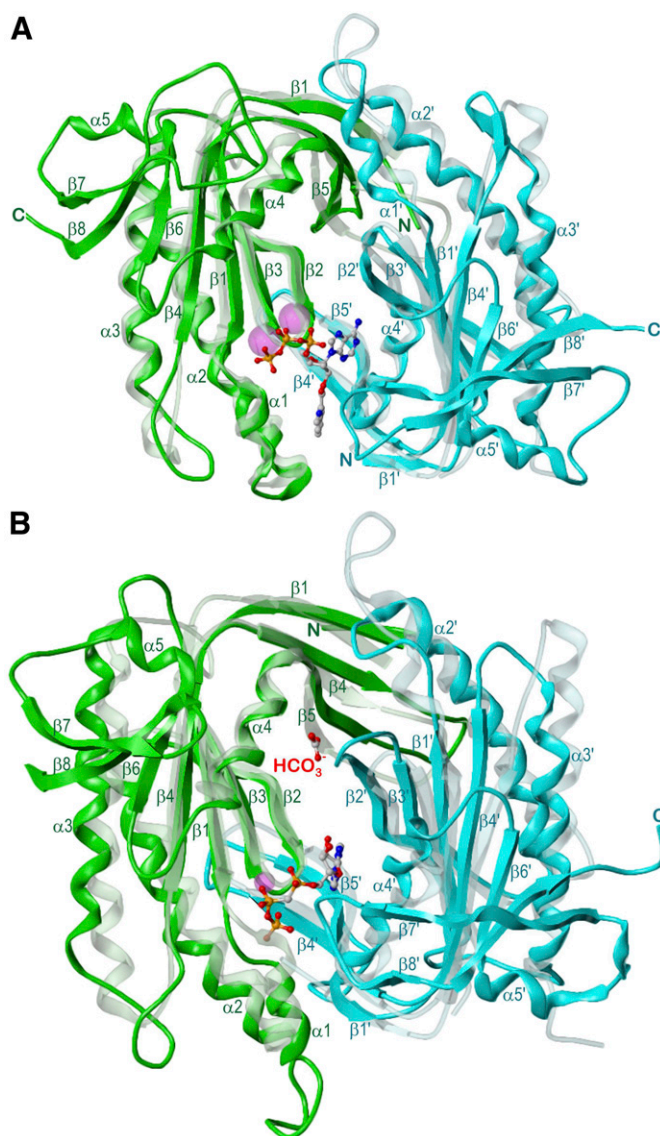


Fig. 4. Catalytic domains of sGC and sAC aligned with 5C1:2C2. Domains are drawn from $\beta 1$ to $\alpha 4$ and $\beta 1'$ to $\alpha 4'$, respectively. Views along the pseudo-twofold axes. $sdGC\alpha_{cat}$, sAC-C1; 5C1, green; $sGC\beta_{cat}$, sAC-C2; 2C2, cyan; atom colors: C, gray; N, blue; O, red; P, orange; Mn^{2+} and Ca^{2+} , magenta; secondary structure elements as well as N and C termini are labeled. (A) $sGC\alpha_{cat}:sGC\beta_{cat}$ in complex with 2'-MANT-3'-dATP and Mn^{2+} , model of the intermediate state based on the open state crystal structure PDB 3UVJ (Allerston et al., 2013) and on 5C1:2C2 PDB 1TL7 (Mou et al., 2005) as template (transparent model). (B) sAC in complex with α,β -methylene adenosine-5'-triphosphate, bicarbonate and Ca^{2+} , model based on the crystal structure PDB 4CLK (Kleinboelting et al., 2014a), 5C1:2C2 (transparent model) corresponding to Fig. 3.

3. Interaction of C1 Catalytic Domain of AC5:C2 Catalytic Domain of AC2 with α -Subunit of the Stimulatory G-protein of Adenylyl Cyclase G_s . The interface between 5C1:2C2 and $G\alpha_s$ is only about half as large as that between 5C1 and 2C2 (Tesmer et al., 1997), and only a few structural elements are involved in interactions (Figs. 3, 5, and 6). The vast majority of contacts are formed by 2C2. The switch II helix of $G\alpha_s$ is embedded into a groove enclosed by the $\alpha 1'-\alpha 2'$ loop, $\alpha 2'$, and the $\alpha 3'-\beta 4'$ loop, which, together with $\alpha 3'$, also contacts $\alpha 3$ and the $\alpha 3-\beta 5$ loop of $G\alpha_s$. 5C1 contributes

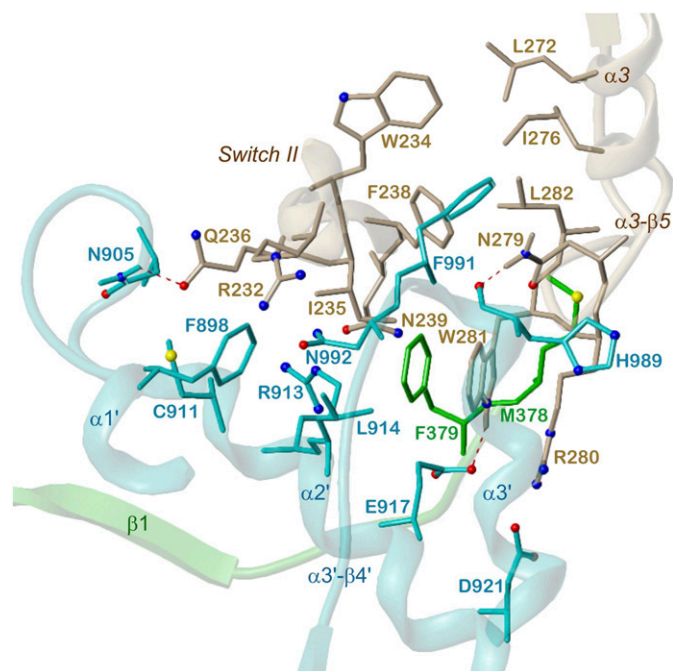


Fig. 6. Detailed 5C1:2C2- $G\alpha_s$ interactions. 5C1, green; 2C2, cyan; $G\alpha_s$, mauve; heteroatom colors: N, blue; O, red; S, yellow. Hydrogen bonds are drawn as red dashed lines. Model based on the crystal structure PDB 1CJU (Tesmer et al., 1997, 1999). Contacting amino acids (distance <3 Å) and secondary structure elements are labeled.

approaching W234 (switch II), L272, and I276 (α_3) of $G\alpha_s$. This pattern of AC- $G\alpha_s$ interactions is common for mAC isoforms, because all respective mAC amino acids are conserved (Fig. 5).

$G\alpha_i$ inhibits AC5 and AC6 noncompetitively with respect to $G\alpha_s$ (Taussig et al., 1994a), binding at a site that is opposite to that for $G\alpha_s$, i.e., between the α_1 - α_2 loop and the α_3 helix in the 5C1 domain (Tesmer et al., 1997; Dessauer et al., 1998).

4. C1 Catalytic Domain of AC5:C2 Catalytic Domain of AC2 Interface. The interface reflects the pseudo-symmetry of 5C1 and 2C2. Figure 7 shows the secondary structure segments and amino acids involved in polar and hydrophobic 5C1-2C2 interactions. Contacts (interatomic distances <4 Å) are formed by 28 amino acids of 5C1 and 33 of 2C2 (Tesmer et al., 1997), from which 17 are located at equivalent domain positions and 7 are identical (Fig. 5).

The rough symmetry of interacting secondary structure elements becomes obvious: contacts of α_1 - α_2 (α_1' - α_2') with β_1' , β_4' and the β_5' - α_4' turn (β_1 , β_4 , β_5 - α_4 turn) as well as of the C-terminal α_2 (α_2') section and β_2 (β_2') with the β_4' - β_5' (β_4 - β_5) loop. However, explicit interactions of amino acids are rather asymmetric. Only two charge-assisted hydrogen bonds between D424 (α_2) and the backbone NH moieties of A1012 and Q1013 (β_4' - β_5' loop) are reflected by positionally identical hydrogen bonds of D923 (α_2') with L499 and R500 (β_4 - β_5 loop). The asymmetry of the contacts correlates with the different shape of the FSK and the ATP binding

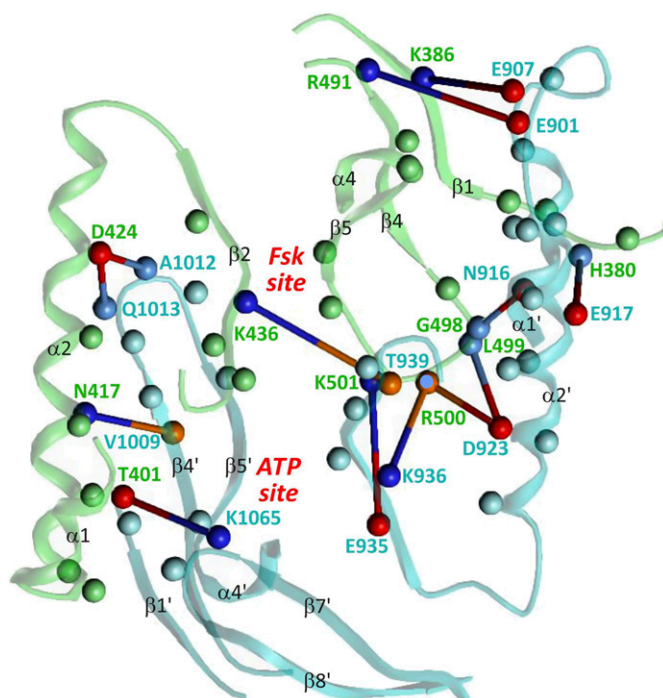


Fig. 7. 5C1-2C2 interface. α -atoms of all amino acids involved in 5C1-2C2 interactions (distance <4 Å, see Fig. 5) are drawn as balls, hydrogen bonds, and salt bridges of labeled residues as sticks. Colors: H-acceptor/acidic side chain, red; backbone O as H-acceptor, orange; H-donor/basic side chain, dark blue; backbone NH as H-donor, light blue; other contacting amino acids and framework of 5C1, green, of 2C2, cyan.

site. The “upper righthand” interface region is characterized by extensive contacts and additionally stabilized by FSK binding. A hydrogen bond between K436 (β_2) and the backbone oxygen of T939 (β_2') may be regarded as borderline between this region and the “lower left-hand” interface region with fewer contacts and a larger cavity involving the ATP and metal binding site. Characteristic of the lower region is a hydrogen bond between T401 and K1065, which keeps the β_7' - β_8' loop in an “open” position with respect to α_1 . This contact is broken on nucleotide binding. $G\alpha_s$ and FSK are suggested to activate mACs by changing the relative orientation of the catalytic domains while preserving nearly all interfacial contacts (Tesmer et al., 1997). Conformational switches are mainly based on a few alternate interactions of flexible segments (loops) of one domain with core components (helices, strands) of the other domain.

The interface in the structure of apo sAC-C1-C2 (Kleinboelting et al., 2014a) corresponds largely to that in 5C1:2C2 (Fig. 5). Thirty-two amino acids of sAC-C1 and thirty amino acids of sAC-C2 participate in contacts. Nineteen residues, five of which are identical, are located at equivalent domain positions. Interactions between secondary structure elements show the same rough symmetry as in case of 5C1:2C2 (see above and Figs. 4B and 7), and also the asymmetry of contacts is similar. The C1-C2 interface of sAC is stabilized by ten

hydrogen bonds. Among them, the following five hydrogen bonds overlap with hydrogen or ionic bonds between 5C1 and 2C2 (Fig. 7): Y38 ($\beta 1$)-E308 ($\alpha 2'$) with K386-E907, E84 ($\alpha 2$)-H398 ($\beta 4'$ - $\beta 5'$ loop) with D424-A1012, F96 ($\beta 2$)-K334 ($\beta 2'$) with K436-T939, G158 ($\beta 4$ - $\beta 5$ loop)-Q315 ($\alpha 2'$) with G498 - N916 and D159 ($\beta 4$ - $\beta 5$ loop)-Y318 ($\alpha 2'$) with L499-D923. The structure of apo sAC-C1-C2 resembles the “closed” state of 5C1:2C2 with no contact between $\alpha 1$ and the $\beta 7'$ - $\beta 8'$ loop.

5. Forskolin Binding Site. FSK binds in the “upper” pseudosymmetric region of the ventral cleft close to $G\alpha_s$ (Fig. 3). The binding site is located between $\beta 1$, $\beta 3$, and the $\beta 5$ - $\alpha 4$ loop of 5C1 on one hand and $\alpha 1'$ and the $\beta 2'$ - $\beta 3'$ loop of 2C2 on the other hand. Figure 8 presents the binding mode of FSK in more detail. The backbone oxygen of V506 ($\beta 5$), the side chain of T512 ($\alpha 4$), and the backbone NH moieties of S508 ($\beta 5$ - $\alpha 4$ loop) and S942 ($\beta 2'$ - $\beta 3'$ loop) form four direct hydrogen bonds with hydroxy and carboxy groups, respectively, of FSK. The side chain of S942 is further involved in a water-mediated H-bond network bridging the O1 and O4 atoms in FSK. Hydrophobic contacts with V511 ($\alpha 4$), K896 ($\alpha 1'$), I940, G941 ($\beta 2'$ - $\beta 3'$ loop) and a couple of aromatic amino acids, namely F394 ($\beta 1$), Y443 ($\beta 3$), W507 ($\beta 5$), F895 ($\alpha 1'$), and Y899 ($\alpha 1'$ - $\alpha 2'$ loop) complete the binding mode. Whereas FSK stabilizes 5C1-2C2 interactions by one order of magnitude (Sunahara et al., 1997), it does not affect the K_m value of ATP (Dessauer et al., 1997). However, V_{max} increases 60-fold (Sunahara

et al., 1997), indicating that FSK induces a conformational change of the ATP site leading to more efficient cAMP synthesis. Probably contacts between the $\beta 2$ - $\beta 3$ and the $\beta 2'$ - $\beta 3'$ loop link amino acids of the FSK site with residues contributing to catalysis (Tesmer et al., 1997).

The bicarbonate binding site of sAC overlaps with that of FSK at 5C1:2C2 (see Figs. 4B and 5). According to the structure of human sAC in complex with bicarbonate (Kleinboelting et al., 2014a), the bicarbonate ion forms charge-assisted hydrogen bonds with the backbone NH moieties of V167 ($\beta 5$) and M337 ($\beta 2'$ - $\beta 3'$ loop) as well as salt bridges with K95 ($\beta 2$) and R176 ($\alpha 4$). The bicarbonate binding site of sAC is too small to accommodate FSK (Kleinboelting et al., 2014a).

6. Nucleotide Binding Site. The nucleotide binding site in the “lower” pseudosymmetric region of the ventral cleft is more spacious than the FSK site, and interfacial contacts are weaker than in the “upper” region. The resulting high conformational flexibility facilitates ATP binding, catalysis, regulation, as well as cAMP and pyrophosphate dissociation. Moreover, not only $G\alpha_s$ but also ATP binding is necessary to transfer the inactive open into the active closed 5C1:2C2 state. In Fig. 9, an apo-AC 5C1:2C2 structure is aligned with an ATP bound structure (Tesmer et al., 1997). The closed state of the latter results from translocation and 7° -rotation of 5C1 around the center of the heterodimer, closing the catalytic site by moving $\alpha 1$ toward $\alpha 4'$ and the $\beta 7'$ - $\beta 8'$ loop of 2C2. Nevertheless, all interfacial

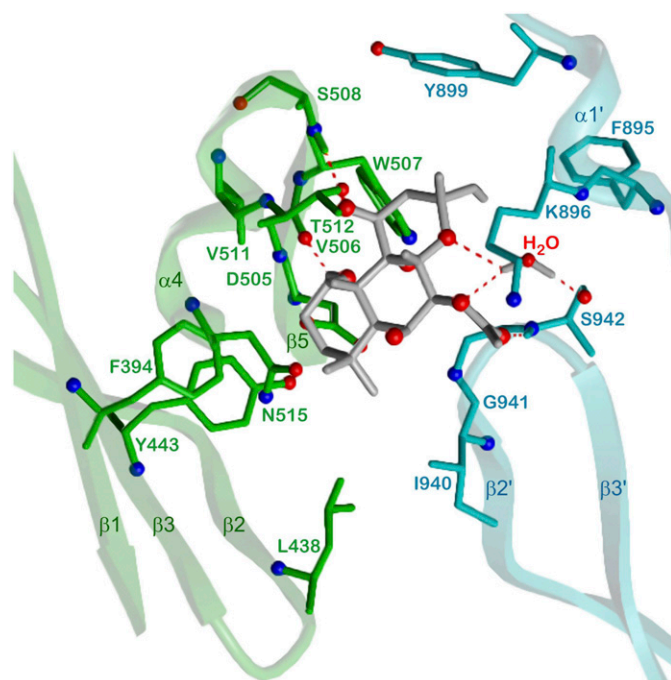


Fig. 8. Detailed 5C1:2C2-FSK interactions. 5C1, green; 2C2, cyan; FSK and water, gray; heteroatom colors: N, blue; O, red. Hydrogen bonds are drawn as red dashed lines. Model based on the crystal structure PDB 1CJU (Tesmer et al., 1997, 1999). Contacting amino acids (distance < 3.5 Å) and secondary structure elements are labeled.

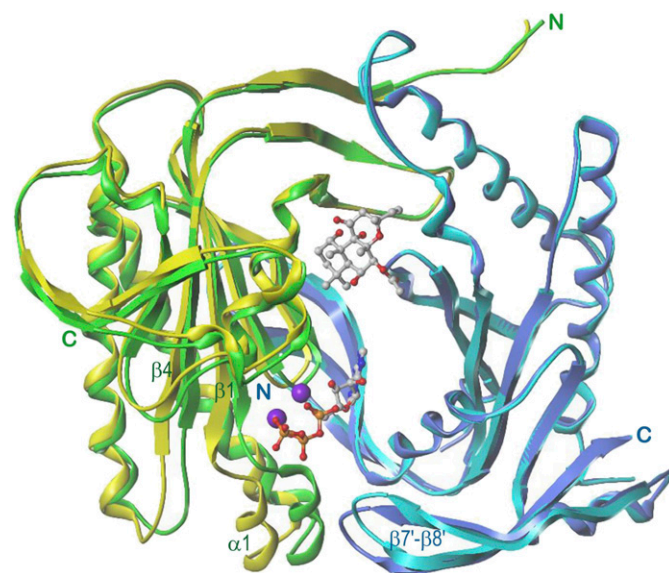


Fig. 9. Closed and open 5C1:2C2 conformations. Alignment of the complex with ATP, Mg^{2+} , and FSK with the open inactive state of apo-5C1:2C2. View along the pseudo-twofold axes. Closed state 5C1, green; 2C2, cyan; open state 5C1, yellow; 2C2, purple; atom colors: C, gray; N, blue; O, red; P, orange; Mg^{2+} , violet. Models are based on the crystal structures PDB 1CJU with ATP docked instead of 2',3'-dideoxy-ATP and 1AZS, respectively (Tesmer et al., 1997, 1999). Unfitting secondary structure elements as well as N and C termini are labeled.

contacts except two between $\alpha 1$ and the $\beta 7'$ - $\beta 8'$ loop are conserved in both states. With two inhibitory Ca^{2+} ions instead of Mg^{2+} , 5C1:2C2 binds ATP without switching into a closed state (Mou et al., 2009). The substituents of 2',3'-*O*-(*N*-methylanthraniloyl) nucleoside 5'-triphosphates and 2',3'-*O*-(2,4,6-trinitrophenyl)-NTPs bind between $\alpha 1$ and the $\beta 7'$ - $\beta 8'$ loop, preventing full closure of the catalytic site (Mou et al., 2005, 2006). As a consequence, the respective complexes represent intermediate (between closed and open) states of 5C1:2C2. In case of the sGC α_{cat} :sGC β_{cat} heterodimer, similar differences of an open and a GTP-bound closed conformation have been suggested (Allerston et al., 2013).

The structural similarity between mammalian mACs, sAC, and sGC in the nucleotide binding regions is exceptionally high. On one hand, common binding modes of ATP, GTP, and metal ions as well as common interactions and structural transformations indicate an evolutionarily well conserved catalytic mechanism of cNMP generation. On the other hand, differences observed in crystal structures result from substrate and inhibitor selectivity and from the flexibility of the ribosyl moiety and the triphosphate chain. The common nucleotide binding region of mACs, sAC and sGC and raw positions of aligned amino acids interacting with ATP and GTP, respectively, are shown in Fig. 10.

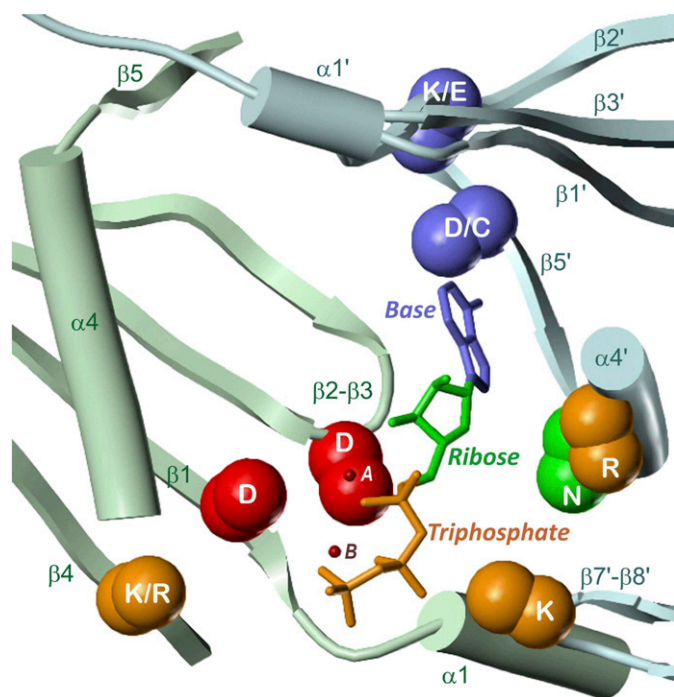


Fig. 10. Common nucleotide binding site of mAC, sGC and sAC. Essential amino acids are drawn as space fill models of α and β atoms; catalytic domain 1, green; catalytic domain 2, cyan; metals A and B, red balls; interactions of metal ions, red; triphosphate, orange; ribosyl moiety, green; nucleobase, blue. The model is based on the crystal structure PDB 1CJU (Tesmer et al., 1997, 1999) with ATP docked instead of 2',3'-dideoxy-ATP. Common secondary structure elements and amino acids of the binding site are labeled.

Metal-, triphosphate-, ribosyl-, and nucleobase-cyclase interactions are clearly distinguishable (Fig. 5). Two aspartates in $\beta 1$ and the $\beta 2$ - $\beta 3$ loop, respectively, coordinate metal ions A and B (Mn^{2+} or Mg^{2+} in native enzymes). The three phosphate moieties interact with conserved basic residues (Arg, Lys) in $\beta 4$, $\alpha 4'$ and the $\beta 7'$ - $\beta 8'$ loop. A relatively spacious cavity with the $\beta 2$ - $\beta 3$ loop as well as $\beta 5'$ and $\alpha 4'$ as lateral margins and with $\beta 2'$ and the $\beta 2'$ - $\beta 3'$ loop as roof accommodates the entire nucleoside moiety. Only two replacements between mACs and sGC, namely Lys by Glu ($\beta 2'$) and Asp by Cys ($\beta 5'$), are required to reverse adenine into guanine selectivity (Sunahara et al., 1998; Tucker et al., 1998).

Figure 11A shows detailed interactions between ATP and 5C1:2C2. Both Mg^{2+} ions are coordinated with D396 and D440, MgA additionally with the α -phosphate and the 3'-OH group of ATP, MgB with all phosphate moieties and with the backbone oxygen of I397. The three phosphates are integrated in a compact network of charge-assisted hydrogen bonds and salt bridges: the position and charge of the α -phosphate is stabilized by R1029 ($\alpha 4'$), the β -phosphate interacts with the backbone NH functions of F400 and T401 ($\alpha 1$), and the γ -phosphate with G399 (backbone NH, $\beta 1$ - $\alpha 1$ loop), R484 ($\beta 4$), and K1065 ($\beta 7'$ - $\beta 8'$ loop). The ribosyl moiety forms two hydrogen bonds between the ring oxygen and N1025 ($\alpha 4'$) and between the 2'-OH group and S1028 ($\alpha 4'$).

A two-metal-ion catalytic mechanism, previously derived from crystal structures in complex with ATP analogs and various divalent cations (Tesmer et al., 1999), was recently corroborated by density functional theory calculations of 5C1:2C2-ATP complexes (Hahn et al., 2015). Metal A acts as Lewis acid facilitating deprotonation of the 3'-OH group. Water-mediated transfer of the proton to the γ -phosphate is followed by changes in the coordination of both metal ions and in the conformation of ATP. The resulting shorter distance facilitates the nucleophilic attack of the 3'-oxygen on the α -phosphorus atom. The subsequent, concerted phosphoryl transfer step is rate-limiting and includes a transition state tightened by R1029. K1065 contributes to shifting the proton transfer equilibrium.

Binding of the nucleobase to mACs is relatively strong compared with sAC and sGC. The adenine moiety is involved in hydrogen bonds of the N1 nitrogen with K938 ($\beta 2'$) and of the 6- NH_2 group with D1018 and the backbone oxygen of I1019 ($\beta 5'$). Van der Waals contacts of the purine nucleus with L438 and G439 ($\beta 2$ - $\beta 3$ loop) and with F889 ($\beta 1'$) align the nucleobase between both domains.

The spacious nucleotide binding site of mACs is promiscuous for different nucleobases and accommodates various ligands, e.g., P-site inhibitors, 2'- and 3'-MANT as well as TNP [2',3'-*O*-(2,4,6-trinitrophenyl)]-substituted nucleotides, with often high affinity (Seifert

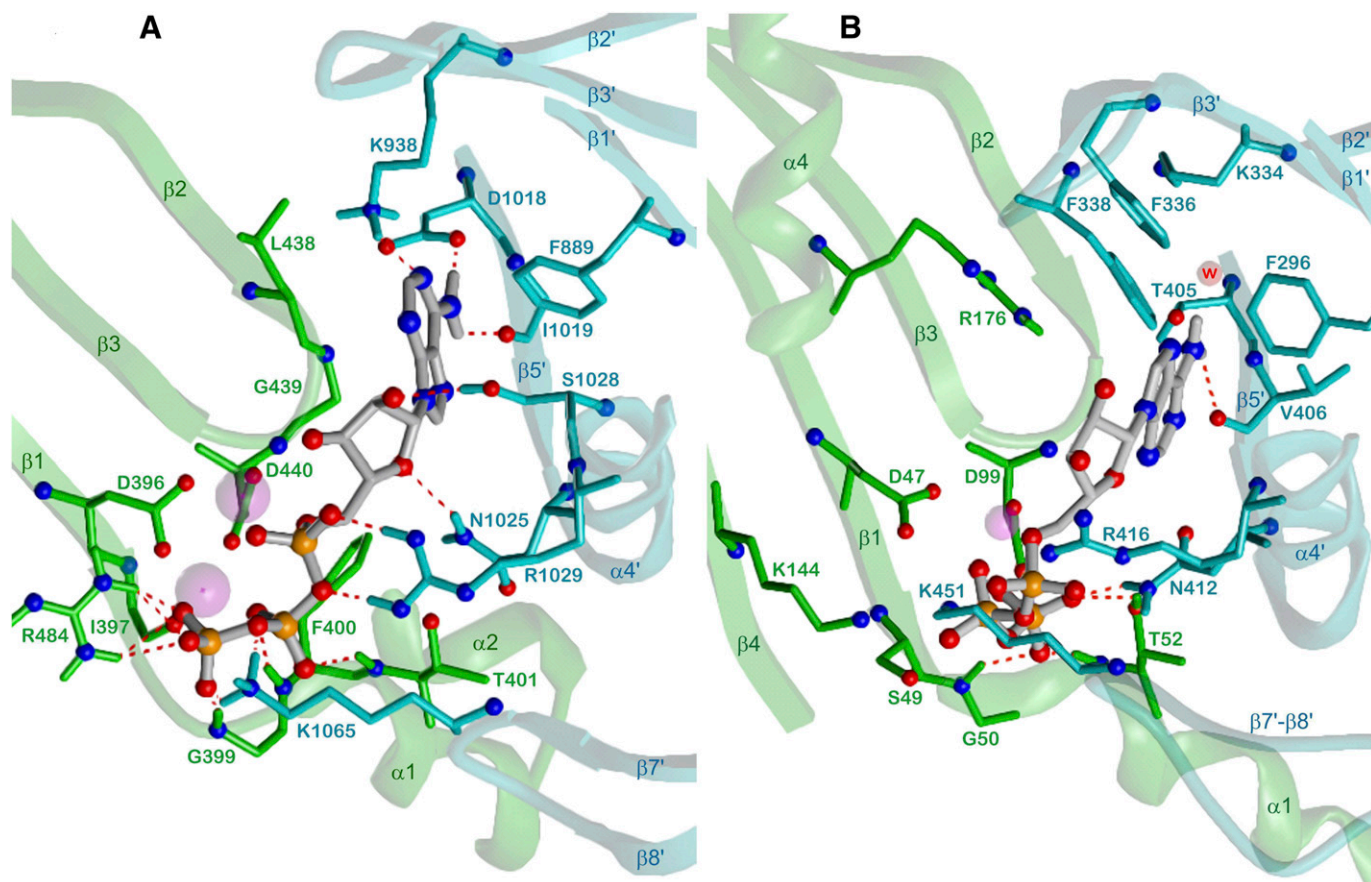


Fig. 11. Detailed interactions of mACs and sAC with ATP. C1, green; C2, cyan; ATP, gray; heteroatom colors: N, blue; O, red; P, orange; hydrogen bonds are drawn as red dashed lines, contacting amino acids (distance <3.5 Å) and secondary structure elements are labeled. (A) 5C1:2C2-ATP interactions, Mg²⁺, violet, model based on the crystal structure PDB 1CJU (Tesmer et al., 1997, 1999) with ATP docked instead of 2',3'-dideoxy-ATP. (B) sAC-ATP interactions, Na⁺, violet; w, water oxygen; model based on the crystal structure PDB 4USW (Kleinboelting et al., 2014b).

et al., 2012). The general binding mode of these inhibitors corresponds to that of ATP, but flexible fit of the phosphate and ribosyl moieties (ring pucker) leads to slightly different and specific interactions. However, NTPs other than ATP do not act as mAC substrates (Sunahara et al., 1998; Hasan et al., 2014), because coherent dynamic transformations rather than tight interactions account for kinetic processes. The high substrate selectivity of mACs with respect to variation of the nucleobase and the triphosphate moiety depends on specific stable and/or transient hydrogen bonds and on distinct flexibility compatible with a concerted catalytic mechanism.

Figure 11B demonstrates similarities and differences of the binding mode of ATP at sAC compared with that at mACs. The structure of the complex (Kleinboelting et al., 2014b) contains only one Na⁺ (or Ca²⁺) ion located somewhat between the reference positions of metals A and B in 5C1:2C2. The triphosphate moiety adopts a specific conformation with partially different interactions than in the case of 5C1:2C2. The α -phosphate interacts with R416 ($\alpha4'$) and K451 ($\beta7'$ - $\beta8'$ loop); the β -phosphate with S49 ($\beta1$), K144 ($\beta4$), and Na⁺; and the γ -phosphate forms three hydrogen

bonds with G50 (backbone NH, $\alpha1$), T52 ($\alpha1$), and N412 ($\alpha4'$) and contacts also Na⁺. R176 ($\alpha4$), in fact pointing to the regulatory bicarbonate site, was modeled as in the sAC complex with α,β -methylene-ATP (Kleinboelting et al., 2014a), because this amino acid together with R416 ($\alpha4'$) probably takes part in catalysis. The adenine base adopts a *syn* conformation in contrast to the *anti* conformation in most other mammalian nucleotidyl cyclase structures resolved so far. The 6-NH₂ group is involved in a hydrogen bond with the backbone oxygen of V406 ($\beta5'$) and in a water-mediated interaction with K334 ($\beta2'$). During catalysis, a transient direct contact between K334 and the 6-NH₂ substituent may be formed (Kleinboelting et al., 2014a). The catalytic constants of ATP and GTP as sAC substrates indicate that base discrimination takes place during turnover rather than by substrate binding (Kleinboelting et al., 2014b). K_m values for ATP for sAC are ~ 20 times higher than those for mACs (Kleinboelting et al., 2014a). Figure 11 provides possible reasons: lower affinity of ATP at sAC may be due to fewer explicit hydrogen bonds compared with 5C1:2C2. Specifically, the ribosyl moiety forms no hydrogen bonds

corresponding to those with N1025 (sAC N412) and S1028 (sAC A415) in the case of 5C1:2C2.

B. Expression of Adenylyl Cyclases

Understanding the specific role of AC isoform in cellular signaling has been hampered by the complex and heterogeneous expression pattern of ACs in nearly all cell types (Defer et al., 2000; Willoughby and Cooper, 2007; Sadana and Dessauer, 2009; Sanabra and Mengod, 2011). The specific signaling roles of each AC isoform are further complicated by their unique, but often overlapping, regulatory properties. Much of our understanding on the specific roles of individual AC isoforms in signaling pathways has been derived from studies with gene knockout and transgenic mice.

Description of the cellular expression patterns for each AC provides important information in delineating their signaling roles. However, identifying AC isoform distribution remains a challenge (Antoni et al., 2006). First and foremost, there is a dearth of well-characterized isoform-specific antibodies (Iwamoto et al., 2003; Antoni et al., 2006; Sadana and Dessauer, 2009; Göttle et al., 2009). In some cases, antibodies have been used along with other molecular (mRNA measures) or biochemical (functional assays) approaches revealing partially consistent results (Ostrom et al., 2003; Johnston et al., 2004; Liu et al., 2004; Göttle et al., 2009). A second factor concerning quantitative expression studies is the generally low levels of AC protein expression (Alousi et al., 1991; Seifert et al., 1998). For the reasons discussed above, most studies have used mRNA quantification methods to gain insight into the potential expression pattern of ACs. These approaches include RT-PCR analysis, Northern analysis, and in situ hybridization (Defer et al., 2000; Willoughby and Cooper, 2007; Sadana and Dessauer, 2009; Sanabra and Mengod, 2011). There is also an emerging body of information on mRNA expression using microarray analysis and next generation sequencing technology (Atwood et al., 2011). Despite the number of available data sets, the generally low correlation between mRNA and protein expression highlights the need for caution in using mRNA as a surrogate for protein expression (Maier et al., 2009; Ostlund and Sonnhammer, 2012; Werner et al., 2016). The reasons for the lack of correlation presumably reflect the complex processes ultimately contributing to protein expression including transcription, translation, and protein stability/turnover. Although meaningful comparisons of the mRNA expression levels between individual isoforms are discouraged, comparisons of a single isoform across tissues or after perturbation may be informative given that appropriate experimental controls are met.

The general expression patterns for AC mRNA have been expertly described in several previous reviews, and the major sites of robust mRNA expression for each isoform have been specified (Defer et al., 2000;

Willoughby and Cooper, 2007; Sadana and Dessauer, 2009). These reports used information from a variety of species including human, rat, mouse, and chicken. Much of the expression work has been supported by studies with transgenic or knockout mice. Perhaps the most thoroughly studied examples involve AC1 and AC8. Both isoforms are highly expressed in brain regions associated with learning and memory, synaptic plasticity, and opioid withdrawal, and experiments with single and double knockout animals support these implications (Ferguson and Storm, 2004; Sadana and Dessauer, 2009).

Rieg and Kohan (2014) described the expression pattern of AC isoforms within the nephron. This carefully written review focused primarily on mRNA studies and generally used antibody studies as supporting data. There was a notable absence of AC1 and AC8, consistent expression for AC6 and AC9, and a more mixed expression pattern of the other AC isoforms throughout the nephron (Rieg and Kohan, 2014). The distribution of AC isoforms in the digestive system was also reviewed (Sabbatini et al., 2014). Similar to the other organs/cells reported, mRNA for multiple ACs were found in hepatocytes and cholangiocytes (Strazzabosco et al., 2009), as well as in the pancreatic acini and duct (Sabbatini et al., 2013). Another paper describing the cAMP signaling elements in lung reported the AC protein/mRNA expression patterns in several tissues and cells including bronchial epithelium, vascular endothelium, airway smooth muscle cells, vascular smooth muscle cells, pulmonary fibroblasts (WI-38 cells), and inflammatory cells (Oldenburger et al., 2012). Although multiple AC isoforms were found in all cells, the number of AC isoforms expressed in the vascular endothelium appears to be more limited (e.g., four isoforms by antibodies) when compared with other cells including airway or vascular smooth muscle cells that appear to express mRNA for seven or all nine ACs, respectively (Xu et al., 2001; Jourdan et al., 2001; El-Haroun et al., 2004). It is likely that RT-PCR approaches would have identified more ACs in the vascular endothelium because of its enhanced sensitivity. The expression of multiple ACs is common in most if not all tissues/cells, including myometrium (Price and Bernal, 2001), adipocytes (Serazin-Leroy et al., 2001), skeletal muscle (Suzuki et al., 1998), and adrenal gland (Shen et al., 1997).

The heart also expresses multiple AC isoforms (Manolopoulos et al., 1995; Göttle et al., 2009). Differential expression of AC4–AC7 mRNA in various regions of the heart and vascular system of humans has been described (Wang and Brown, 2004). There are also cell-type expression differences with AC5 and AC6, proposed to be the most abundant AC isoforms in cardiomyocytes (Yu et al., 1995; Li et al., 2012). However, there is controversy with regard to the relative expression of AC5 versus AC6 in the heart as

well as the changes in expression that occur during development or stress (Tobise et al., 1994; Espinasse et al., 1995, 1999; Scarpace et al., 1996; Wang and Brown, 2004; Hu et al., 2009; Braeunig et al., 2013). AC1 mRNA is found only in the sinoatrial node (Mattick et al., 2007). Cardiac fibroblasts express ACs 2–8 with differential patterns in caveolar versus non-caveolar regions (Ostrom et al., 2003).

A number of studies examined AC isoform expression across the central nervous system (Matsuoka et al., 1997; Antoni et al., 1998; Nicol et al., 2005). In situ hybridization was used to examine the AC expression patterns in the rodent brain (Visel et al., 2006; Sanabra and Mengod, 2011). Differences in AC isoform expression between glutamatergic, GABAergic, and cholinergic neurons were revealed (Visel et al., 2006; Sanabra and Mengod, 2011). High levels of mRNA for AC1 and AC8 are found in areas important for learning and memory and high levels of AC5 mRNA in the striatum (Sanabra and Mengod, 2011). AC2 was found in limbic as well as in other areas. AC3 and AC6 were found widespread throughout the brain as was AC9 with enhanced expression in the neocortex and hippocampus (Sanabra and Mengod, 2011). There was limited expression of AC7 in thalamic and hypothalamic nuclei and AC4 was found only in blood vessels (Visel et al., 2006; Sanabra and Mengod, 2011). The reviews and reports highlighted above support a very broad distribution of ACs with multiple isoforms being expressed in any given tissue as well as cell type.

Studies with human tissues and cells are somewhat limited but also suggest that the mRNAs of many AC isoforms are broadly expressed across multiple tissues (Xu et al., 2001; Ludwig and Seuwen, 2002; Wang and Brown, 2004; Pinto et al., 2012). Of particular note is the study of the expression of mRNA for ACs 1–9 in 16 human tissues (Ludwig and Seuwen, 2002). These investigators attempted to visually quantify the RT-PCR products' band intensity and compare expression levels of individual isoforms across tissues (Table 2). Multiple

AC isoforms ranging from three isoforms in skeletal muscle to all nine mAC isoforms in the testis were found. The data also point to differences between human and mouse expression patterns in certain tissues including skeletal muscle (Suzuki et al., 1998; Ludwig and Seuwen, 2002). In addition to the brain, there is significant AC1 expression in heart, skeletal muscle, kidney, pancreas, spleen, testis, and ovary (Ludwig and Seuwen, 2002). AC8 showed the most restricted expression, being detected in the brain and testis. The generally broad AC expression patterns in humans as well as other species often contrast with the initial cloning studies where mRNA expression was examined at the whole tissue level. The discrepancies may be explained when one considers potential differences in methodology (Northern analysis versus RT-PCR) as well as when comparing whole tissue extracts to cell preparations where AC mRNAs may show selective expression in certain cell types.

In addition to knowing tissue expression patterns, investigators are interested in the AC isoform expression in relevant cell lines or primary cells. The majority of studies employed a RT-PCR approach to assess mRNA levels (Varga et al., 1998; Johnston et al., 2004; Liu et al., 2004), but microarray approaches have been used as well (Atwood et al., 2011; Zhu and Oxford, 2011). HEK293 cells are often used for examining both endogenous and exogenous AC signaling. Reports on AC mRNA expression in HEK293 cells indicate the presence of three to seven AC isoforms (Hellevuo et al., 1993; Premont, 1994; Ludwig and Seuwen, 2002; Lefkimmatis et al., 2009). Consensus analysis of these studies suggest that HEK293 cells express mRNA for AC1, AC3, AC5 (weak), AC6, AC7 (weak), and AC9. A similar pattern in HEK293 cells was found using microarray, with the exception that AC5 and AC7 were reported at similar levels to AC1, AC3, AC6, and AC9 (Atwood et al., 2011). The reported discrepancies in expression patterns of the same cell model could reflect any of the numerous experimental or methodological

TABLE 2
Expression of mAC isoforms at the mRNA level

Expression pattern of AC isoforms in rodent brain and human tissue. Sanabra and Mengod (2011) used in situ hybridization to examine and compare the AC isoform expression in rodent brain tissues. Semiquantitative RT-PCR was used to examine and compare the AC isoform expressions in 16 human tissues (Ludwig and Seuwen, 2002).

AC Isoform	Rodent CNS Expression (Sanabra and Mengod, 2011)	Relative Human Tissue Abundance (Ludwig and Seuwen, 2002)
AC1	Hippocampus, cerebellum, cortex, and thalamus	Peripheral blood leukocytes > heart ~ skeletal muscle ~ kidney ~ pancreas ~ spleen ~ testis ~ ovary
AC2	Limbic and widespread	Skeletal muscle ~ testis > heart ~ prostate ~ ovary ~ colon
AC3	Widespread	Placenta ~ testis ~ ovary ~ colon > lung ~ spleen ~ thymus
AC4	Not detected	Heart ~ placenta ~ lung ~ spleen ~ testis ~ ovary ~ small intestine
AC5	Caudate putamen and olfactory tubercle	Testis ~ heart ~ prostate ~ ovary ~ small intestine ~ colon
AC6	Widespread	Heart ~ kidney ~ prostate ~ testis ~ ovary ~ small intestine ~ colon
AC7	Thalamus and hypothalamus	Peripheral blood leukocytes > placenta ~ lung ~ spleen ~ thymus > heart ~ liver ~ ovary ~ colon
AC8	Hippocampus, cerebellum, cortex, thalamus and hypothalamus	Testis (limited detection)
AC9	Cortex and hippocampus	Heart ~ placenta ~ lung ~ liver ~ skeletal muscle ~ kidney ~ pancreas ~ testis ~ ovary ~ peripheral blood leukocytes

variations including differences in cell harvesting/preparation, cell growth/maintenance conditions, RT-PCR approach/conditions, or primer design.

Much remains unknown regarding the actual expression patterns of individual AC isoforms. The use of mRNA as a surrogate for protein expression has its limitations, but provides a starting point into understanding signaling pathways. The astute scientist is encouraged to gather converging pieces of evidence to establish a link between mRNA abundance with AC function. One example for such an approach is the elaboration of the FSK-insensitive AC9 as the functionally predominant AC isoform in human myeloid cells (Mahadeo et al., 2007; Brunskole Hummel et al., 2013; Werner et al., 2014, 2016). Combining mRNA studies with additional data from the use of well-characterized antibodies (when available) and gene silencing approaches like shRNA and siRNA (Fan et al., 2009; Emery et al., 2015) can further connect AC isoform expression to cellular signaling.

It is firmly established that sAC is expressed at high levels in the testis of all mammalian species studied so far including human, and its expression is confined to postmeiotic germ cells (Schmid et al., 2014; Levin and Buck, 2015). Two major protein variants are retrieved from testis and spermatozoa; i.e., a protein of 187 kDa corresponds to the full-length enzyme and a 48–50 kDa splicing tsAC variant containing only the C1/C2 domains. Both molecular species are catalytically active. Lower level expression has been reported in a wide range of additional tissues and cells (Schmid et al., 2014; Levin and Buck, 2015).

C. Compartmentation of Adenylyl Cyclases

1. Cellular Localization. Although many signaling processes can differently alter activity of each AC isoform, there is a single, primary signal output by ACs 1–10, i.e., production of the second messenger cAMP. This simple fact belies both the intuitive notion and the experimental observation that not all AC isoforms regulate identical cellular functions. To explain this complication, one must evoke the concept of cAMP signaling compartmentation (Ostrom et al., 2012; Cooper and Tabbasum, 2014). Indeed, specific AC isoforms localize in different cellular compartments where they associate with a subset of GPCRs and form distinct complexes with downstream effectors. ACs 3, 5, 6, and 8 associate with caveolar and lipid raft microdomains, whereas the other mACs are localized in nonraft plasma membrane domains (Schwencke et al., 1999; Fagan et al., 2000; Ostrom et al., 2000, 2002; Smith et al., 2002). This isoform-specific localization is remarkably stable across different cell types, species, and activation states (Ostrom and Insel, 2004). ACs partner with GPCRs and downstream effectors that are similarly localized, creating discrete signaling complexes. It is attractive to imagine that these complexes allow the

common second messenger, cAMP, to generate a wide array of specific responses, even in a single cell. This is especially true when one considers the large number of different GPCR expressed in a single cell that use cAMP as their primary intracellular signal.

GPCRs display some AC-specific coupling that reflects the colocalization of receptors and AC isoforms in lipid raft or nonraft plasma membranes. By using overexpression or knockdown of an AC isoform, specific GPCR-AC coupling was unmasked. AC6 overexpression in a number of cells, including airway smooth muscle, lung fibroblasts, and neonatal myocytes, enhances signaling by β -adrenoceptors (β ARs) without affecting signaling by a number of other $G\alpha_s$ -coupled receptors (Ostrom et al., 2000, 2002; Liu et al., 2008; Bogard et al., 2011). In each case, receptor coupling could be explained by the colocalization or exclusion of the receptor in lipid rafts (where AC6 resides). This concept of colocalization can be even more complex in cardiac myocytes. Because of specialized T-tubule and sarcolemmal membrane environments in these specialized cells, AC and GPCR localization is even more specific. AC5 is localized primarily in T-tubules, whereas AC6 resides in sarcolemmal membranes (Timofeyev et al., 2013). The β_1 -adrenoceptor (β_1 AR), but not the β_2 -adrenoceptor (β_2 AR), couples effectively to AC5 (Tsunematsu et al., 2015). This mirrors the differential distribution of the β_1 AR and β_2 AR in T-tubules (Cros and Brette, 2013).

In airway smooth muscle cells, β_2 AR signaling is selectively increased when AC6 is overexpressed and these components colocalize in lipid rafts (Bogard et al., 2011). In contrast, prostanoid EP receptors couple only to AC2 in nonraft domains. Unique cellular responses can be mapped to individual AC isoforms in these cells. By examining gene expression after FSK stimulation in cells overexpressing either AC2 or AC6, interleukin 6 expression is increased by AC2-produced cAMP, whereas somatostatin expression was regulated by cAMP emanating from AC6 (Bogard et al., 2014). Studies using Förster resonance energy transfer-based biosensors to detect cAMP in different subcellular domains have provided direct evidence of these signaling compartments (Agarwal et al., 2011, 2014). These findings show that cAMP is generated in discrete places by individual ACs and that the response machinery in these locations differs enough to yield contrasting regulation in the nucleus.

Specific examples of receptor regulation of AC activity depending on colocalization in membrane domains continue to emerge. Dopamine D1 receptors preferentially regulate AC3, AC5, and AC6 activity and this depends on interaction with lipid rafts, whereas dopamine D5 receptors are more nonraft localized and couple to just AC5 (Yu et al., 2014). Family B GPCRs also seem to depend upon colocalization of receptor and AC, as evidenced by the observation that the pituitary AC-activating polypeptide type 1 receptor was unable to

signal to downstream effectors when AC6 (but not AC7) expression was knocked down (Emery et al., 2015). Prostacyclin receptor inhibition of platelet activation depends upon intact lipid rafts (Raslan and Naseem, 2015), which is a known location of both prostacyclin receptor and AC5/6 expression (Liu et al., 2008).

Coupling of GPCRs to specific AC isoforms is highly regulated. In early pregnancy, α_2 -adrenoceptors inhibit uterine smooth muscle AC activity but then switch to being stimulatory during midterm pregnancy (Zhou et al., 2000). An upregulation of uterine AC2 expression at about the same time renders it likely that the switch in α_2 adrenoceptor signaling is due to the altered AC isoform expression (Zhou et al., 2007). Signaling by a coexpressed receptor can even dictate coupling between a specific GPCR and AC. Activation of prostaglandin EP1 receptors facilitates dopamine D1 receptor activation of cAMP production (Ehrlich et al., 2013). This effect depends on $G\beta\gamma$ signaling and expression of AC7, a $G\beta\gamma$ -sensitive isoform, and may explain how prostaglandin E2 can regulate neuronal dopamine signaling.

Lipid rafts exert a more complex function than just providing an assembly point for GPCR and ACs. AC regulation by capacitive calcium entry depends on intact lipid rafts and is specific to raft-localized AC isoforms (Cooper et al., 1995; Fagan et al., 1998, 2000; Smith et al., 2002). Disruption of lipid rafts causes a loss of coupling between capacitive calcium entry and calcium-regulated AC isoforms, AC6 and AC8. Thus, lipid rafts facilitate the juxtaposition of calcium-sensitive AC isoforms and capacitive calcium entry channels.

Given that AC isoforms are either lipid raft localized or not, a structural component must exist in all ACs to confer an affinity for one domain or the other. Intuition leads one to consider sequence-specific protein-protein or protein-lipid interactions and posttranslational modifications as likely mechanisms. Evidence of sequence-specific interactions come from studies making chimeras between raft and nonraft isoforms (Crossthwaite et al., 2005) as well as reductionist approaches examining fragments of ACs (Thangavel et al., 2009). Both of these approaches found evidence for roles of the intracellular C1 and C2 domains in targeting ACs to lipid rafts. These studies imply that AC isoforms have key protein-protein interactions that confer their lipid raft localization. However, protein-lipid interactions may be just as important. Mutation of three N-linked glycosylation sites on an extracellular loop of AC8 causes this lipid raft-localized isoform to appear in nonraft membranes (Pagano et al., 2009).

With intracellular protein-protein interactions facilitating lipid raft localization of AC, it is tempting to implicate a known scaffolding protein and resident of these domains, caveolin. However, caveolins are not responsible for anchoring ACs in lipid rafts. Specifically, AC isoforms have the same expression and localization

in cardiac fibroblasts isolated from both caveolin-1 knockout and wild-type mice (Thangavel et al., 2009). Additionally, cell lines that naturally lack caveolin expression still display lipid raft-localized AC isoforms (Thangavel et al., 2009).

sAC has been detected in a variety of organelles including nuclei, microtubules, cilia, and mitochondria (Zippin et al., 2003). Functional data supporting the expression of an active enzyme are available particularly for mitochondria and cilia of the airway epithelium. Intramitochondrial sAC activation is associated with an increase in cAMP measured by an immunodetection assay (Acin-Perez et al., 2009) or by monitoring Förster resonance energy transfer of mitochondria-targeted cAMP sensors (Di Benedetto et al., 2013). The increase in cAMP leads to PKA-mediated phosphorylation of respiratory chain enzymes (Acin-Perez et al., 2009; Di Benedetto et al., 2013) and an increase in ATP.

2. Organization of Adenylyl Cyclases with Downstream Effectors. A long-standing idea is that cAMP signaling is "tailored" in various cell types via the combinatorial expression of AC and PDE isoforms (Houslay and Milligan, 1997). However, a greater level of organization of signaling is achieved by A kinase anchoring proteins (AKAPs). Via their defining PKA binding motif, AKAPs organize cAMP-dependent protein kinase (PKA) signaling to a wide variety of other proteins (Colledge and Scott, 1999). They scaffold the kinase and its substrates and bring together a diverse array of other proteins involved in various signaling pathways. Several diverse members of the AKAP family interact with ACs in an isoform-specific manner and regulate cAMP signaling (Bauman et al., 2006). To date, the specific AKAP-AC interactions that have been defined are AKAP9/Yotiao with ACs 1, 2, 3, and 9 (Piggott et al., 2008), mAKAP with ACs 2 and 5 (Kapiloff et al., 2009) and AKAP79/150 with ACs 2, 3, 5, 6, 8, and 9 (Efendiev et al., 2010; Delint-Ramirez et al., 2011; Shen and Cooper, 2013).

The role of specific AC-AKAP associations in signal transduction was reviewed by Dessauer (2009). AKAP9/Yotiao has complex effects on AC regulation that depend on the AC isoform. AKAP9 has a pure scaffolding function, with no alteration of enzyme activity, when combined with AC1 and AC9 (Piggott et al., 2008). By contrast, this same AKAP inhibits the activity of AC2 and AC3 when these components interact. AC8 forms a complex with AKAP5 (AKAP79/150) and the effect appears to be a decrease in AC8 sensitivity to Ca^{2+} (Willoughby et al., 2010). But many more elements of the cAMP signaling cascade can be localized to a given site of signal generation. AKAP5 also interacts with the β_2AR and AC5 and AC6 (in addition, of course, to binding PKA) (Bauman et al., 2006). With this arrangement, PKA rapidly phosphorylates the β_2AR to initiate desensitization, receptor translocation and even G-protein switching

(Daaka et al., 1997), but the G-protein switch is discussed controversially (Friedman et al., 2002). PKA also inhibits AC5 and AC6 via phosphorylation (Beazely and Watts, 2006) and alters AC8 sensitivity to store-operated calcium entry; regulation by PKA is facilitated by AKAP5 (Delint-Ramirez et al., 2011). In each case, the AKAP-AC interaction establishes critical feedback regulation in the signaling cascade.

AKAPs can also regulate other ACs in an isoform-specific manner via targeting protein kinase C (PKC) phosphorylation. PKC increases activity of certain ACs (ACs 1, 2, 3, 5, 7) but inhibits the activity of others (AC6) (Sunahara et al., 1996; Lai et al., 1997). AKAP5 orchestrates muscarinic receptor activation of cAMP production by recruitment of PKC to a complex containing the PKC-stimulated AC2 (Shen and Cooper, 2013). PDE4 and PKA are also key elements in the complex. β AR regulation of postsynaptic AMPA-type glutamate receptors in mouse forebrain slices depends upon AKAP5 anchoring both PKA and AC (Zhang et al., 2013). Although the AC isoform in this model was not defined, these studies demonstrate that long-term potentiation is lost without this AKAP5-mediated organization.

AKAP-AC interactions have other pathophysiological implications (Efendiev and Dessauer, 2011). Mutations of the potassium channel KCNQ1 subunit interrupt its binding to Yotiao, causing a loss of PKA regulation of the channel, alteration in the action potential, and arrhythmias (Marx et al., 2002). Moreover, AKAP-AC complexes serve to sensitize bound PKA effectors to the effects of cAMP, shifting the concentration/response curves by two orders of

magnitude (Efendiev et al., 2010). Furthermore, PKA/PDE4D feedback contributes to local regulation of ACs (Mika and Conti, 2016).

D. Regulation of Adenylyl Cyclases

Table 3 summarizes regulation of mAC isoforms. Isoform-specific regulation of AC isoforms has been well described and extensively reviewed (Tang and Gilman, 1992; Sunahara et al., 1996; Hanoune and Defer, 2001; Patel et al., 2001; Cooper, 2003). All mACs are activated by $G\alpha_s$ (Tang and Gilman, 1992; Iyengar, 1993; Jones et al., 1990), although differences in AC activation may exist (Jones et al., 1990; Seifert, et al., 1998; Chan et al., 2011). Only ACs 1, 5, and 6 are directly inhibited by $G\alpha_i$ (Federman et al., 1992; Taussig et al., 1993a, 1994; Chen and Iyengar, 1993).

$G\beta\gamma$ subunits increase the activity of ACs 2 and 4 (Gao and Gilman, 1991; Tang and Gilman, 1991; Taussig et al., 1993b). The stimulatory effects of $G\beta\gamma$ typically require the presence of another activator such as $G\alpha_s$ or FSK (Tang and Gilman, 1991). When overexpressed, $G\beta\gamma$ subunits have been reported to inhibit ACs 5 and 6 (Bayewitch et al., 1998). However, cellular and in vitro assays using purified proteins display a conditional stimulation of AC5 and AC6 by $G\beta\gamma$ (Gao et al., 2007; Thomas and Hoffman, 1996) that involves different structural determinants in AC5 compared with AC6 (Brand et al., 2015).

Although all ACs are inhibited by high concentrations of Ca^{2+} , AC5 and AC6 are inhibited by submicromolar concentrations of Ca^{2+} ; AC1 and AC8, and to a lesser extent AC3, are stimulated by low concentrations of Ca^{2+} via CaM (Tang et al., 1991; Choi et al., 1992; Katsushika

TABLE 3
Regulation of mAC isoforms

This table focuses on known direct regulators of AC, although there are indirect routes to altering AC activity. Some of the effects listed have been clearly demonstrated as "direct" using purified, cell free components, whereas others have only been shown in intact cells. In the case of the latter, one must consider indirect mechanisms as well as the requirement for other factors for the effect to be realized. This table is adapted and updated from that published by Ostrom et al. (2012).

Regulator	Effect	AC Isoform	References
FSK (no known physiologic ligand for FSK site)	Stimulation	All isoforms (except AC9)	Premont et al. (1996); Yan et al. (1998); Onda et al. (2001); Cumbay and Watts (2004)
$G\alpha_s$	Stimulation	All isoforms	Iyengar (1993)
$G\alpha_i$	Inhibition	AC1, AC5, AC6; not AC2	Federman et al. (1992); Chen and Iyengar (1993); Taussig et al. (1993a, 1994a)
$G\beta\gamma$	Inhibition Stimulation (conditional)	AC1, AC3, AC8 AC2, AC4, AC5, AC6	Taussig et al., (1993b); Diel et al. (2006) Tang and Gilman (1991); Gao and Gilman (1991); Thomas and Hoffman (1996); Gao et al. (2007); Brand et al. (2015)
Ca^{2+}	Inhibition	AC5, AC6	Yoshimura and Cooper (1992); Katsushika et al. (1992)
Ca^{2+} /CaM	Stimulation	AC1, AC3 (weak), AC8	Tang et al. (1991); Choi et al. (1992); Cali et al. (1994)
CaM Kinase	Inhibition	AC1, AC3	Wayman et al. (1996); Wei et al. (1996)
PKC	Stimulation	AC1, AC2, AC3, AC5, AC7	Jacobowitz et al. (1993); Jacobowitz and Iyengar (1994); Kawabe et al. (1994); Watson et al. (1994); Bol et al. (1997)
PKC	Inhibition	AC6	Lai et al. (1997)
PKA	Inhibition	AC5, AC6, AC8	Iwami et al. (1995); Chen et al. (1997); Willoughby et al. (2012)
NO	Inhibition	AC5, AC6	McVey et al. (1999); Hill et al. (2000)
Raf kinase	Stimulation	AC2, AC5, AC6	Ding et al. (2004)
RGS proteins	Inhibition	AC3, AC5	Sinnarajah et al. (2001); Xie et al. (2012)

et al., 1992; Yoshimura and Cooper, 1992; Cali et al., 1994). Expression of AC5 and/or AC6 along with L-type Ca^{2+} channels allows an oscillatory regulation between Ca^{2+} and cAMP, because cAMP stimulates Ca^{2+} influx via the L type Ca^{2+} channel and the subsequent Ca^{2+} influx inhibits AC activity (Cooper et al., 1995). AC1 activation by Ca^{2+} /CaM is sensitive to oxidation of specific methionine residues in the N- and C-terminal domains of CaM (Lübker et al., 2015a,b). The oxidation-dependent CaM-regulation of AC1 may be relevant in ageing processes (Lübker et al., 2015a,b). The structural basis for AC inhibition by Ca^{2+} has been resolved (Mou et al., 2009). Transient receptor potential channels contribute to regulation of Ca^{2+} -sensitive ACs (Willoughby et al., 2014). In intact cell experiments, the Ca^{2+} ionophore A23187 is useful as an experimental tool to stimulate Ca^{2+} -dependent ACs (Vortherms et al., 2006). PKC can also stimulate many AC isoforms (AC1, AC2, AC3, AC5, and AC7) but reduces activity of AC6 (Jacobowitz et al., 1993; Kawabe et al., 1994; Lai et al., 1997). Phorbol esters are often used as experimental tools to modulate AC function via PKC activation (Olianas and Onali, 1986; Freyaldenhoven et al., 1992; Lin and Chen, 1997). Different PKC isoforms have different effects on AC activity, but there are no direct comparisons of multiple PKC isoforms on activity of individual AC isoforms. Regulator of G-protein signaling proteins (RGS proteins) modulate activity of some AC isoforms as well. RGS2 reduces AC3, AC5, and AC6 activity (Sinnarajah et al., 2001; Salim et al., 2003; Salim and Dessauer, 2004). RGS9-2 suppresses basal activity and modulates $\text{G}\beta\gamma$ activation of AC5 (Xie et al., 2012). Annexin A4 reduces AC activity and downstream cAMP signaling in HEK-293 cells and cardiac myocytes (Heinick et al., 2015). Coimmunoprecipitation of annexin A4 and AC5 was shown, but evidence that the observed effects are specific to this AC isoform is missing.

Several AC isoforms are also subject to a paradoxical increase in activity after persistent activation of G_i/G_o -linked receptors. This process is referred to as heterologous sensitization, supersensitization, superactivation, or cAMP overshoot (Sharma et al., 1975b). Persistent activation of opioid receptors on neuronal cells led to a marked increase in AC activity (Sharma et al., 1975b). This phenomenon has been demonstrated with a number of G_i/G_o -linked receptors and multiple AC isoforms (Watts and Neve, 2005). Moreover, this adaptive response has been observed in vivo after chronic administration of opioids or D2 dopamine receptor agonists (Brust et al., 2015).

The importance of the different regulatory properties of individual ACs cannot be overstated. Cells tailor their cAMP production characteristics in part by expressing different cohorts of AC isoforms. This was perhaps most clearly demonstrated by Henry Bourne's group (Federman et al., 1992) when exogenous expression of

AC2 (an isoform stimulated by $\text{G}\beta\gamma$) along with a constitutively active $\text{G}\alpha_s$, converted $\text{G}\alpha_i$ -coupled receptor inhibition of cAMP to a stimulation of cAMP production. Therefore, it is imperative to know the contribution of each AC isoform to a cell's total cAMP production capacity. Defining this is a challenge given that Mg^{2+} and Mn^{2+} levels cause sizable changes in AC activity and that isoforms may have varying sensitivities to both $\text{G}\alpha_s$ and FSK. AC isoform knockout serves as the most reliable tool for understanding the contribution of individual ACs to a given cell's signaling repertoire.

Regulation differences aside, mAC isoforms also differ in their basal and maximal activities. For example AC2 has a 10- to 15-fold higher basal activity compared with AC6, although FSK-stimulated activities only differ twofold (Pieroni et al., 1995). Differences in maximal catalytic activity (kcat or turnover number) are difficult to quantify and require purified AC isoforms or other means to accurately quantitate enzyme levels. Studies with purified AC1 and AC2 suggest FSK-stimulated AC1 activity is ~ 3.5 times higher than AC2, with a turnover number of ~ 15 per second (Taussig et al., 1993b). AC5 has even higher activity with a FSK/PKC-stimulated turnover number of 59 second^{-1} (Kawabe et al., 1994). This compares favorably with the $\text{G}\alpha_s$ - and $\text{G}\alpha_s$ /FSK-stimulated 5C1:2C2 turnover numbers of 30 and 100 second^{-1} , respectively (Dessauer et al., 1997). Thus in addition to regulatory control of ACs, relative intrinsic differences in basal and maximal AC activity may also influence their physiologic roles, as discussed below.

sAC is insensitive to G-protein regulation and FSK; it is instead activated by bicarbonate (Chen et al., 2000) and by Ca^{2+} (Jaiswal and Conti, 2003; Litvin et al., 2003). In contrast, mACs are not activated by bicarbonate (Xie et al., 2006). The crystal structure of human sAC indicates that binding of bicarbonate disrupts a salt bridge involving an aspartate residue that is involved in the substrate binding site (Kleinboelting et al., 2014a). Additional major allosteric changes induced by bicarbonate binding affecting the active site have been described in structural studies of a bicarbonate-regulated, cyanobacterial sAC (Kleinboelting et al., 2014b). Given the low affinity of this enzyme for ATP, sAC activity is sensitive to the ATP levels in the cell (Buck et al., 1999; Jaiswal and Conti, 2003). The removal of bicarbonate from the extracellular medium may be used to probe the involvement of sAC in a given physiologic process (Baudouin-Legros et al., 2008; Choi et al., 2012; Hasan et al., 2014).

Both the activities of sAC and mACs are pH dependent (Xie et al., 2006). At pH 7.4, basal activities of sAC and mACs are low and continuously increase until a plateau at pH 8.6–9.0 (Xie et al., 2006). The effect of pH on basal activity of mACs is more prominent than on sAC.

E. (Patho)physiologic Functions of Adenylyl Cyclase Isoforms

As described above, most cells and tissues express multiple AC isoforms. Therefore it has been difficult to tease apart the physiologic role for any given AC isoform. In view of the lack of isoform-specific inhibitors and isoform-specific antibodies, knockout and overexpression studies with mice have driven most of our current understanding (Table 4). Several reviews delve more deeply into the exact roles for different AC isoforms in a variety of systems (Sadana and Dessauer, 2009; Xia and Storm, 2012; Guellich et al., 2014; Nicol and Gaspar, 2014; Rieg and Kohan, 2014; Sabbatini et al., 2014; Lee, 2015). Overall, our knowledge on the role of specific AC isoforms in human pathophysiology is still very limited. For some isoforms such as ACs 1 and 5, quite a lot of information is available, whereas other isoforms, particularly AC4, are rather unexplored. Localization of AC isoforms in “interesting” or “important” locations such as brain and heart seems to play a role in the extent it has been studied.

1. Group I (Adenylyl Cyclases 1, 8, and 3).

a. Adenylyl cyclase 1. The first insights into roles for AC1 came from studies in *Drosophila melanogaster* and *Aplysia californica*. The *rutabaga* gene in flies is most closely related to AC1 and was named for its deficits in

learning and memory (Tempel et al., 1983; Levin et al., 1992). Deletion of AC1 in invertebrates or mice leads to a reduction of long-term potentiation (Levin et al., 1992; Wu et al., 1995) and impairments in numerous learning tasks, including spatial learning and learning to avoid a neutral odor (Sadana and Dessauer, 2009; Xia and Storm, 2012; Lee, 2015). Mice with AC1 deletion have deficits in neural wiring during brain development, including enhanced projections and/or branching patterns in the somatosensory system, the visual system, and in the peripheral nervous system (Abdel-Majid et al., 1998; Nicol et al., 2006; Dhande et al., 2012; Nicol and Gaspar, 2014; Suzuki et al., 2015). These developmental patterns are unique to AC1 knockout mice and are consistent with the higher levels of AC1 during development compared with AC8.

AC1 is also required for homeostatic plasticity during activity deprivation in mice (Gong et al., 2007) and sleep plasticity in flies (Donlea et al., 2009). Mice with overexpression of AC1 show improved recognition memory (Wang et al., 2004) but impaired spatial memory in older animals (Garelick et al., 2009) along with deficits in behavioral inhibition (Chen et al., 2015a). Finally, mice with deleted AC1 show decreased susceptibility to proteinuria (Xiao et al., 2011) and reductions in pain (Vadakkan et al., 2006). We are not aware of studies

TABLE 4
Knockout and transgenic (overexpression) studies with mice: (patho)physiologic functions of mAC isoforms
Because of the lack of selective mAC inhibitors and antibodies, advances in our knowledge on the functions of mACs isoforms largely depended on knockout and overexpression studies in mice. Table updated from Sadana and Dessauer (2009). With few exceptions, the table does not consider associations of *ADCY* gene polymorphisms with diseases in humans.

AC Isoform	Availability of		(Patho)physiologic Functions (Mice and Humans)
	Knockout (mice)	Overexpression (mice)	
AC1	Yes	Yes ^a	Mice: Learning, memory, synaptic plasticity, opiate withdrawal and cocaine sensitization, behavioral inhibition, brain development, pain
AC2	No	No	Mice: No data available Humans: AC2 impairment in Lesch-Nyhan syndrome (Kinast et al., 2012)?
AC3	Yes	No	Mice: Olfactory and pheromone responses, olfactory dependent and contextual memory, sperm function, diet-induced obesity, maternal behavior, inter-male aggressiveness
AC4	Yes ^b		Mice: No phenotype
AC5	Yes	Yes ^a	Mice: Cardiac contraction, motor coordination, striatum-dependent learning, opiate and ethanol dependency, pain responses, diet-induced obesity, renin secretion, stress responses. Humans: AC5 gain of function in human movement disorders associated with dystonia, myokymia and chorea (Chen et al., 2012c, 2014b)
AC6	Yes	Yes ^a	Mice: cardiac contraction and calcium sensitivity, polycystic kidney disease and renal function including mild Bartter syndrome, sympathetic tone, bone adaptations, pancreatic fluid secretion
AC7	Yes	Yes ^a	Mice: Ethanol dependency, depression, immune function
AC8	Yes	Yes ^a	Mice: Learning, memory, synaptic plasticity, opiate withdrawal, mood disorders, anxiety behavior, glucose homeostasis
AC9	Yes ^c	No	Mice: Immune response in monocytes, possible links to sepsis and cardiac repolarization

^aCardiac or brain directed overexpression.

^bTissue-specific expression in kidney collecting duct (Kittikulstuth et al., 2014a).

^cUnpublished results and International Mouse Phenotyping Consortium (<http://www.mousephenotype.org/>).

linking polymorphisms in the *ADCY1* gene with specific human diseases. An involvement of AC1 in the pathophysiology of alcoholism and Alzheimer's disease was proposed (Sohma et al., 1999; Yamamoto et al., 2000, 2001), but these studies hinged on the use of commercial antibodies with questionable specificity (Göttle et al., 2009).

b. Adenylyl cyclase 1 and adenylyl cyclase 8 redundancies. AC1 has many overlapping functions with AC8 that are often revealed in AC1/AC8 double knockouts. These include a reduction in neural responses to ethanol (Maas et al., 2005a,b), cocaine sensitization (DiRocco et al., 2009), and opiate withdrawal behaviors, including lowered morphine-induced hyperlocomotion and loss of cAMP-dependent transcription factor activation in the reward response circuitry of the brain (Li et al., 2006; Zachariou et al., 2008). In addition, double knockouts of AC1 and AC8 show deficits in memory acquisition, memory retention (Zhang et al., 2011), and fear memory (Sindreu et al., 2007; Wiczorek et al., 2010). Administration of FSK to double knockout mice can restore normal long-term memory (Wong et al., 1999), suggesting that stimulation of other AC isoforms is sufficient to overcome loss of AC1 and AC8.

Ca^{2+} -stimulated AC activity and AC1 and AC8 transcripts are also present in sinoatrial nodal cells of the heart. One or both of these AC isoforms appear to be important in sympathetic regulation of pacemaker current in guinea pig, mouse, and rat sinoatrial node cells (Mattick et al., 2007). The exact isoform involved in this process awaits confirmation at the whole animal level using knockouts of AC1 and/or AC8.

c. Adenylyl cyclase 8. AC8 is expressed at high levels in adult brain, and the AC8 knockout shows significant impairment in short-term plasticity and abnormal anxiety behavior under stress (Schaefer et al., 2000; Wang et al., 2003), in addition to the overlapping functions with AC1 discussed above. Lack of AC8 confers a hyperactive phenotype and an increased risk-taking behavior, similar to certain mood disorders (Razzoli et al., 2010). AC8 is also required for glucose homeostasis and hypothalamic adaptation to high-fat diet (Raoux et al., 2015). *ADCY8* gene polymorphisms may be linked to neuropsychiatric diseases, such as bipolar disorder, depression, and alcoholism (de Mooij-van Malsen et al., 2009; Zhang et al., 2010; Procopio et al., 2013; Wolf et al., 2014).

d. Adenylyl cyclase 3. AC3 is the predominant isoform in the olfactory epithelium and is largely responsible for odorant and pheromone detection in the olfactory bulb (reviewed in Wang et al., 2007). Odorants interact with GPCRs to stimulate AC via activation of the G-protein $\text{G}\alpha_{\text{olf}}$. All components of this signaling system, including AC3, are located within olfactory cilia (Wang and Storm, 2003). Deletion of AC3 leads to major defects in odorant-induced signaling, olfactory-

dependent learning, olfaction-based behavioral tests, olfactory axonal guidance processes, and mechanical force of airflow across olfactory cilia (Wong et al., 2000; Col et al., 2007; Chen et al., 2012b). Mice lacking AC3 are unable to detect mouse urine or pheromones and show a reduction in maternal behavior, inter-male aggressiveness, male sexual behavior, spermatozoa function, and male fertility (Livera et al., 2005; Wang et al., 2006; Wang and Storm, 2011). AC3 is also expressed in oocytes (Horner et al., 2003), and AC3 knockout mice show premature oocyte maturation (Horner et al., 2003).

Although the highest levels of expression of AC3 are present in olfactory tissue, AC3 is also found in the brain, hypothalamus, and at much lower levels in other tissues. Loss of AC3 in mice is associated with impaired short-term memory for novel objects and failure to exhibit extinction of contextual fear conditioning (Wang et al., 2011b), altered sleep patterns, and depression-like phenotypes (Chen et al., 2016). Key components of the olfactory signal transduction machinery (olfactory receptors, AC3, and $\text{G}\alpha_{\text{olf}}$) are expressed in the distal nephron (Pluznick et al., 2009). However, there is no effect on renal sodium or water excretion after selective deletion of AC3 in collecting ducts, suggesting compensation by other ACs (Kittikulsuth et al., 2014b). *ADCY3* gene polymorphisms have been associated with obesity (Nordman et al., 2008; Wang et al., 2009, 2010; Wen et al., 2012; Stergiakouli et al., 2014; Warrington et al., 2015), depression (Wray et al., 2012), and inflammatory bowel disease (Hulur et al., 2015).

1. Group II (Adenylyl Cyclases 2, 4, and 7).

a. Adenylyl cyclase 2. Reports of knockout phenotypes for AC2 are lacking. AC2 selectively couples to prostanoid EP2 receptors in smooth muscle cells, pointing to a potential role in regulating smooth muscle tone in response to prostaglandins (Bogard et al., 2012). AC2 and AC4 are the predominantly expressed AC isoforms in human airway smooth muscle cells, but AC2 expression is lacking in mouse bronchial smooth muscle cells, making confirmation of its function difficult (Bogard et al., 2011, 2012). Development of AC2-selective inhibitors may overcome the difficulties to elucidate the (patho)physiologic functions of AC2 (Pinto et al., 2008; Erdorf et al., 2011; Kinast et al., 2012; Conley et al., 2013). *ADCY2* polymorphisms may be related to neuropsychiatric (Mühleisen et al., 2014; Suarez-Rama et al., 2015) and pulmonary diseases (Hardin et al., 2012; Panasevich et al., 2013). Pharmacological studies point to a role of AC2 in the pathogenesis of Lesch-Nyhan disease, a neuropsychiatric disorder with severe self-injurious behavior (Kinast et al., 2012).

b. Adenylyl cyclase 4. A tissue-specific knockout of AC4 has been generated for loss of expression in kidney collecting duct principal cells. AC4 does not appear to play a role in water and sodium handling in kidney

(Kittikulsuth et al., 2014a). Phenotypes from other tissues have not yet been reported. We are not aware of studies linking *ADCY4* gene polymorphisms to specific human diseases.

c. Adenylyl cyclase 7. AC7 is a major contributor to cAMP synthesis in B and T lymphocytes. Mice with AC7 deletion produce fewer leukocytes and have a high mortality upon bacterial infections, producing less antigen-specific antibodies and displaying prolonged inflammation when challenged with zymosan (Duan et al., 2010; Jiang et al., 2013). Downregulation of AC7 is observed in monocytes of young men and is associated with significantly increased tumor necrosis factor- α release (Risoe et al., 2015).

AC7 also may play a role in pathways contributing to alcohol consumption. Starting from the early observation that ethanol at clinically relevant concentrations increases AC activity in mouse striatal membranes (Hoffman and Tabakoff, 1982), subsequent studies revealed that AC7 is particularly sensitive to stimulation by ethanol and that the effects of ethanol are mediated via the cytosolic domains (Yoshimura and Tabakoff, 1999; Yoshimura et al., 2006; Kou and Yoshimura, 2007; Dokphrom et al., 2011). Female mice with reduced AC7 levels show increased alcohol consumption, whereas increased AC7 levels lead to elevated ethanol-induced adrenocorticotropin and corticosterone levels (Pronko et al., 2010; Desrivieres et al., 2011). In humans, *ACDY7* polymorphisms have been linked to alcoholism (Hellevuo et al., 1997) and autoimmune diseases (Li et al., 2015). Human polymorphisms of *ACDY7* are associated with depression, supported by depressive phenotypes in AC7 knockout mice (Hines et al., 2006; Joeyen-Waldorf et al., 2012).

3. Group III (*Adenylyl Cyclases 5 and 6*).

a. Adenylyl cyclase 5. AC5 has received significant attention for its roles in heart and brain. Loss of AC5 results in improved basal left ventricular function but decreased isoproterenol-stimulated left ventricular ejection fraction, elimination of parasympathetic control of cAMP levels, and attenuation of baroreflexes that maintain blood pressure (Okumura et al., 2003a; Tang et al., 2006). Deletion of AC5 protects the heart against chronic β AR stimulation and chronic pressure overload by attenuating the decline in cardiac function and increased apoptosis (Okumura et al., 2003b, 2007). AC5 disruption is also protective against age-related cardiac myopathy and gives rise to an increased life span compared with wild-type animals (Yan et al., 2007). AC5 knockout mice show decreased body weight on standard and high-fat diet, with improved glucose tolerance and increased insulin sensitivity (Ho et al., 2015). However, loss of AC5 does not rescue the cardiomyopathy associated with overexpression of G_{α_q} (Timofeyev et al., 2010). AC5 gain-of-function mutations may be associated with heart failure (Chen et al., 2012c).

In contrast to the possible protection against cardiac and metabolic diseases, AC5 deletion has detrimental consequences for central nervous system (CNS) function. Specifically, AC5 knockout results in poor stress-coping mechanisms, presumably due to high AC5 expression levels in the reward and stress-related circuits of the dorsal striatum and nucleus accumbens (Kim and Han, 2009). Striatum-dependent learning and corticostriatal plasticity is also impaired upon loss of AC5 (Kheirbek et al., 2009). Most strikingly, AC5 knockout mice show a Parkinson-related motor disorder with impaired coordination and bradykinesia (Iwamoto et al., 2003). This phenotype can be partially rescued by dopamine D1- and D2-receptor stimulation (Iwamoto et al., 2003). Dopamine D1 receptor and D2 receptor signaling to AC is abolished in striatum upon knockout of AC5; the loss of D2 receptor function is consistent with a loss of motor activity responses to the antipsychotic drugs haloperidol and sulpiride (Lee et al., 2002). In addition, the dyskinesia induced by treatment with dopamine precursors, often observed in Parkinson's patients, is greatly reduced upon knockout of AC5 (Park et al., 2014). Finally, loss of AC5 leads to increased ethanol consumption and reduced sensitivity to high-dose ethanol (Kim et al., 2011a, 2012).

Initial reports suggested a role for AC5 in renin secretion (Ortiz-Capisano et al., 2007), but more recent studies show that these effects are largely due to AC6, with AC5 playing only a minor role in isoproterenol-stimulated plasma renin concentration (Aldehni et al., 2011).

Numerous studies have linked *ADCY5* gene polymorphisms to altered glucose metabolism, diabetes, and obesity (Barker et al., 2011; Rees et al., 2011; Wagner et al., 2011; Windholz et al., 2011; Holstein et al., 2013; Ng et al., 2013; Hodson et al., 2014; Knigge et al., 2015). *ADCY5* gene polymorphisms have also been linked to various neuropsychiatric diseases (Chen et al., 2012c; Procopio et al., 2013). Most notably, two nonconserved point mutations in *ADCY5* (R418W localized in the region between TM1 and C1a and A726T localized in the C1b region) have been associated with familial dyskinesia with facial myokymia (FDFM) (Chen et al., 2012c, 2014b). FDFM is a rare autosomal-dominant disease, characterized by paroxysmal and involuntary dystonic and choreiform movements of limb and facial muscles (Chen et al., 2012c, 2014b). Expression of the two AC5 mutants in intact HEK293 cells massively increased cAMP accumulation compared with wild-type AC5 upon stimulation with the β AR agonist isoproterenol (Chen et al., 2014b). These data indicate a gain-of-function in the nonconservative R418W and A726T mutants at the molecular level and are consistent with a gain-of-function on the extrapyramidal movement level (Chen et al., 2012c, 2014b; Chang et al., 2016). The AC regions in which the mutations are localized have not yet been resolved crystallographically. Other

nonconservative mutations in the *ADCY5* gene (R418G and R418Q) are associated with dystonia, myoclonus, and choreoathetosis, too (Chen et al., 2015b; Chang et al., 2016). In line with these data, the R418W mutation may cause benign hereditary chorea (Mencacci et al., 2015). The R418W and A726T mutants may also be associated with chronic heart failure (Chen et al., 2012c). Mutations of the *ADCY5* gene resulting in RNA instability can cause chorea and dystonia as well (Carapito et al., 2015). Finally, the nonconservative M1029K mutation in the *ADCY5* gene, localized in the C2a domain, has been linked to dystonia, hypotonia, and chorea (Chen et al., 2015c). Taken together, these data provide convincing evidence for an important role of AC5 in regulating the extrapyramidal motor system. It should be noted though, that presently, the characterization of the biochemical and pharmacological properties of disease-causing AC5 mutants is still very incomplete. It also remains to be determined whether loss-of-function mutations in the *ADCY5* gene cause Parkinson's disease in humans.

b. Adenylyl cyclase 6. AC6 is widely expressed with documented roles in heart, kidney, pancreas, bone, and brain. In heart, deletion of AC6 results in no change in basal cardiac cAMP levels but greatly reduced β AR-stimulated cAMP levels that may be due to a reduction in AC5 levels, in addition to loss of AC6 (Tang et al., 2008). AC6 knockout animals show greatly reduced β AR-stimulated left ventricular contractile function and reduced calcium transporter SERCA2a calcium affinity and calcium transients. Levels of AC6, but not AC5, appear to limit β AR signaling in heart (Gao et al., 1998; Tepe et al., 1999). Overexpression of AC6 in some heart failure models improves markers of heart failure, increasing cardiac contractility, cardiac responsiveness, and attenuating deleterious left ventricular remodeling (Roth et al., 1999, 2002; Lai et al., 2000, 2004). However, no improvement in heart function was observed in mouse chronic pressure overload models upon AC6 overexpression (Guellich et al., 2010). The differences between AC5 and AC6 roles in heart may be due to distinct compartmentalization of the two enzymes and the influence of local PDE pools in ventricular myocytes, with AC5 localized largely in the T-tubular region, whereas AC6 is found outside the T-tubules (Timofeyev et al., 2013).

In kidney, AC6 is localized in the tubules, collecting ducts, and juxtaglomerular cells, contributing to water homeostasis and renin secretion (Chien et al., 2010; Aldehni et al., 2011; Rieg and Kohan, 2014). Mice lacking AC6 have increased urine output, decreased urine osmolality, reduced responsiveness to arginine-vasopressin (AVP) receptor V_2 , and a mild Bartter syndrome-like phenotype (Rieg et al., 2010, 2013). Mice with a specific knockout of AC6 in collecting ducts also show a urinary concentrating defect and lack AVP-stimulated epithelial sodium channel activity

(Roos et al., 2012, 2013). The phenotypes associated with loss of AC6 are similar to that of nephrogenic diabetes insipidus and are consistent with a loss of vasopressin-induced cAMP in the inner medullary collecting ducts of the kidney. AVP-cAMP signaling within primary cilium directly regulates cyst growth in autosomal dominant polycystic kidney disease, the most common genetic cause of kidney failure (Rieg and Kohan, 2014). In a mouse model of polycystic kidney disease, loss of AC6 markedly reduced cyst formation and renal injury (Rees et al., 2014), consistent with AC6 association with an AKAP-scaffolded complex containing polycystin-2 in primary cilium (Choi et al., 2011). The localization of AC6 in osteocyte cilia likely contributes to its role in loading-induced bone adaptation (Lee et al., 2014). Other functions of AC6 include the regulation of basal sympathetic nerve discharge or sympathetic tone within the brain stem (Chien et al., 2013) and regulation of cAMP-dependent pancreatic amylase and fluid secretion (Sabbatini et al., 2013). In humans, the *ADCY6* S674 variant results in a hyperfunctional AC6 protein (Gros et al., 2007), but follow up studies are required. *ADCY6* gene polymorphisms have been linked to altered adhesion of red sickle cells (Eyler et al., 2008), but despite the importance of AC6 for heart function, no association of *ADCY6* gene polymorphisms with cardiovascular disease has been found in the study by Ikoma et al. (2003).

4. Group IV (Adenylyl Cyclase 9). Reports for AC9 knockout phenotypes are lacking. However, microRNAs targeting AC9 (miR-142-3p, miR-181a, and miR-181b) point to a decrease in AC9 expression in sepsis (Risoe et al., 2011) and potential roles in regulating cell proliferation in cervical cancer cells and differentiation of promyelocytic leukemia (Yang et al., 2014; Zhuang et al., 2014). Several reports suggest roles for AC9 in immune function. miR-142-3p restricts cAMP production in regulatory T cells (Huang et al., 2009), whereas knockdown or overexpression of AC9 in human neutrophils alters chemotaxis (Liu et al., 2010). In newly diagnosed patients with acute myelogenous leukemia and in those with a disease relapse, AC9 expression is lower than in cases of remission (Zhuang et al., 2014), suggesting that AC9 could be used as biomarker for diagnosis and clinical course of certain leukemia forms. In cervical carcinoma cells, miR-181b downregulates AC9 and promotes proliferation (Yang et al., 2014), pointing to a more general role of AC9 in cancer biology. AC9 is the functionally predominant AC isoform in neutrophils and monocytes (Mahadeo et al., 2007; Brunskole Hummel et al., 2013; Werner et al., 2014, 2016). Finally, association of AC9 with Yotiao and certain potassium channels in heart may point to a role in cardiac repolarization and certain forms of long-QT syndrome (Li et al., 2012). *ADCY9* gene polymorphisms are linked to different diseases including asthma (Small et al., 2003; Kim et al., 2011b), pancreatic cancer (Lee

and Gyu Song, 2015), mood disorders (Toyota et al., 2002), body weight (Berndt et al., 2013), and susceptibility to malaria (Manjurano et al., 2012; Maiga et al., 2013). *ADCY9* gene polymorphisms determine the efficacy of dalcetrapib, increasing high-density lipoprotein, on atherosclerosis (Tardif et al., 2015).

5. *Soluble Adenylyl Cyclase (Adenylyl Cyclase 10)*. Two mouse knockout lines for this gene have been generated [deletion of the first exon of the sAC gene, which prevents the expression of the C1 domain (C1KO)] (Esposito et al., 2004) and deletion of exons 12–14 that code for the C2 domain (C2KO) (Chen et al., 2013a). Both lines lead to similar phenotypes in the testis, including a complete loss of bicarbonate-sensitive AC activity in the testis and spermatozoa and undetectable cAMP levels in isolated spermatozoa, giving rise to infertility because of defective sperm motility (Esposito et al., 2004; Xie et al., 2006; Chen et al., 2013a). Additional phenotypes of these mouse lines have been reviewed elsewhere (Schmid et al., 2014; Levin and Buck, 2015). Reed et al. (2002) identified 18 base substitutions in the human sAC gene in a population of patients with absorptive hypercalciuria and increased propensity to kidney stones. Additional *ADCY10* gene polymorphisms suggest association with oligoazoospermia, asthenozoospermia, osteoporosis, and pancreatic cancer (Herrinton et al., 2011).

IV. Pharmacological Modulation of Adenylyl Cyclases

A. General Aspects

Given the highly differential expression, regulation and function of mAC isoforms discussed in the preceding text, ACs, in principle, constitute an excellent drug target. However, in marked contrast to the progress made in these basic science fields, to this end, translation of this knowledge into clinically useful drugs is still very limited. In contrast to ACs, the development of drugs targeting sGC and pGCs is much more advanced (Friebe et al., 2015). Specifically, the exploration of allosteric regulatory sites in sGC resulted in the development of potent NO-independent sGC activators (cinaciguat being the prototype) and NO-dependent sGC stimulators (riociguat being the prototype) with many clinical applications (Stasch et al., 2011, 2015).

Like GCs, ACs can be targeted at the catalytic site and allosteric regulatory sites, i.e., the FSK binding site in case of mACs and the bicarbonate binding site in case of sAC, and other sites distinct from these locations (Seifert and Beste, 2012). In case of mACs, it should also be possible to target the nonconserved transmembrane domains, but this field of pharmacology is still in its infancy (Beltz et al., 2016). The long-term goal in the field of AC pharmacology is to obtain stimulatory and inhibitory compounds with high affinity

and selectivity for a given AC isoform. So far, this goal has not been met.

Bacterial toxins constitute most valuable tools for the analysis of signal transduction processes in general and in many cases constitute pathogenic factors for disease development (Moss and Vaughan, 1989; Seifert and Dove, 2012, 2013; Simon et al., 2014; Seifert et al., 2015). Several bacterial toxins, specifically CyaA from *Bordetella pertussis*, edema factor from *Bacillus anthracis* and ExoY from *Pseudomonas aeruginosa* possess nucleotidyl cyclase activity and flood target cells with cAMP (and other cNMPs) (Seifert and Dove, 2012, 2013; Seifert et al., 2015). There is no known bacterial toxin that covalently modifies mACs. However, cholera toxin from *Vibrio cholera* ADP-ribosylates $G\alpha_s$ and, thereby, blocks its GTPase activity (Cassel and Selinger, 1977; Moss and Vaughan, 1979). As a result, $G\alpha_s$ is persistently activated and strongly activates mACs, resulting in massive cAMP production in enterocytes and, ultimately, profuse secretion with subsequent dehydration, the major clinical symptoms of cholera. Because ADP-ribosylation of $G\alpha_s$ is irreversible, symptomatic fluid and electrolyte substitution is crucial for successful recovery of patients suffering from cholera (Moss and Vaughan, 1989). Pertussis toxin from *Bordetella pertussis* ADP-ribosylates $G\alpha_i$ and, hence, uncouples GPCRs from inhibition of AC (Katada et al., 1982, 1984).

Currently, there is no drug on the market that exerts its main therapeutic effects via inhibition of mACs, although many patents have been filed (Seifert et al., 2012). However, as an off-target effect, certain antiviral compounds such as foscarnet and acyclic nucleoside phosphonates may inhibit mACs under clinical conditions (Shoshani et al., 1999; Kudlacek et al., 2001). It has been proposed that lithium and carbamazepine, mood stabilizers used for the treatment of bipolar disorder (Harwood and Agam, 2003), may act via preferential inhibition of AC5 (Mann et al., 2009). However, these data have not yet been confirmed independently, and mood stabilizers, most notably lithium, are known to act via multiple mechanisms (Harwood and Agam, 2003).

One important parameter when evaluating reports of AC-specific modulators is the system that was used. As noted in section I.C.3, a variety of methodologies are routinely used to assess AC activity, and two common approaches use either cellular membranes or intact cell preparations from cells overexpressing individual AC isoforms. The assays are biologically distinct (see section I.C.3) and have advantages and disadvantages that are important to consider when evaluating published reports. Studies with cell membranes can provide important mechanistic insight (i.e., direct versus indirect, competitive versus noncompetitive) and AC isoform specificity information (particularly when Sf9 membranes expressing a given AC isoform); however, the assays are generally of low throughput (Brand et al.,

2013). Intact cell studies are well suited for high-throughput studies but provide only limited information regarding modulator mechanism. For example, NB001 is reported to be a selective AC1 inhibitor; however, it is inactive in cell membrane preparations expressing AC1 (Brand et al., 2013). The use of intact cells for inhibitor studies also requires selective modulation of recombinant AC isoform of interest. For example, cells expressing AC1 and AC8 show robust cAMP responses to the Ca^{2+} ionophore A23187, and cells expressing AC2 are robustly activated by the phorbol ester PMA (Cumbay and Watts 2001). Although no selective activators of other AC isoform are available, overexpression allows for AC5 and others to be robustly activated by concentrations of FSK, showing little activity in wild-type cells (Cumbay and Watts 2001). Advantages of intact cell studies include a more native cellular environment, and presumably only cell permeable modulators will be active. However, counterscreening against other AC isoforms and studies examining the potential role of G proteins (e.g., pertussis toxin treatment and sequestering $G\beta\gamma$ subunits) are important considerations for intact cell studies. The results of intact cell studies can further be strengthened and supported by additional studies using cell membranes (Conley et al., 2013). Ultimately, one would like confirmation that pharmacological inhibition and genetic inhibition give rise to similar results. The considerations noted above should assist investigators when evaluating the strengths and weaknesses of reports examining AC isoform specific modulators.

B. Membrane Adenylyl Cyclase Activators

1. General Aspects. The FSK binding site in mACs constitutes the classic target for stimulatory drugs. Initially, FSK was characterized in membrane preparations containing a mixture of various mAC isoforms (Daly, 1984). ACs 1–8, but not AC9, are activated by FSK (Premont et al., 1996; Yan et al., 1998; Onda et al., 2001; Cumbay and Watts, 2004) (Table 3). There is marked synergism between $G\alpha_s$ and FSK with respect to increasing V_{max} of catalysis, characterized in detail with the 5C1:2C2 system (Sunahara et al., 1997; Mou et al., 2005). For identification of small molecule AC activators, low basal catalytic activity is acceptable.

The lack of FSK-activation of AC9 has been used to identify this isoform as functionally dominant AC in human myeloid cells (Mahadeo et al., 2007). Specifically, human monocytes and neutrophils express AC activity that is effectively stimulated by the $\beta_2\text{AR}$ and histamine H_2 receptor, whereas FSK is almost ineffective at increasing cAMP levels in these cell types (Brunskole Hummel et al., 2013; Werner et al., 2014, 2016). At the mRNA level, several AC isoforms including AC9 are expressed in myeloid cells (Mahadeo et al., 2007; Brunskole Hummel et al., 2013; Werner et al., 2014, 2016). The molecular basis for the FSK

resistance of AC9 is attributable to the presence of a tyrosine at position 1082, corresponding to a leucine in other AC isoforms (Yan et al., 1998). FSK can also be coupled to agarose and used as an affinity ligand for purification of mACs (Pfeuffer et al., 1985, 1989; Pfeuffer, 1991).

Despite intense efforts in the AC research community, no endogenous ligand for the FSK binding site has been identified so far (Laurenza et al., 1989; Seifert et al., 2012). Reports on the occurrence of an endogenous FSK-like molecule have not yet been confirmed (Putnam et al., 2007). Thus, the physiologic relevance of the FSK binding site is still elusive.

2. Structure-activity Relationships of Diterpenes. A limited set of systematic structure-activity relationship studies for FSK was performed at recombinant ACs 1, 2, and 5, representatives of the three major AC families, and the purified catalytic mAC subunits 5C1:2C2 (Table 5). Figure 12 shows the structures of the compounds examined. In the presence of Mn^{2+} , which is very often used in AC assays because it confers higher catalytic activity to these systems (Gille et al., 2004; Mou et al., 2005), FSK is the most potent and most efficacious diterpene at all ACs studied (Pinto et al., 2008). AC1 is the most sensitive isoform, but the difference in potency to the least sensitive AC isoform, AC2, is just 10-fold. In general, modifications such as the introduction of a hydrophilic *N*-methylpiperazonyl-butryloxy group, the removal of the 7-acetyl group or 7,6-isomerization of the acetyl group result in losses in potency and efficacy. Loss of the 9-hydroxyl group in FSK results in decreases in potency and efficacy, particularly for AC2. The removal of the 1-hydroxyl group in FSK is detrimental for AC activation, pointing to the crucial importance of a hydrogen bond between the 1-hydroxyl group and Val506 for activation (Pinto et al., 2008, 2009). Some degree of AC isoform selectivity of FSK and FSK derivatives was also reported in other studies (Sutkowski et al., 1994; Onda et al., 2001), but all available studies are incomplete to some extent, i.e., not all AC isoforms were studied, and complete concentration-response curves were not routinely generated.

The poor water solubility constitutes a serious problem in the pharmacological analysis of diterpenes, requiring dimethyl sulfoxide concentrations of up to 3% (w/v) to fully solubilize compounds (Pinto et al., 2008, 2009). Although such high solvent concentrations may be compatible with cell-free AC activity assays, experiments with intact cells generally do not tolerate dimethylsulfoxide concentrations higher than 1% (Brunskole Hummel et al., 2013). The high hydrophobicity of FSK also delays its dissociation from ACs and potentially prolongs drug action (Pinto et al., 2009). One strategy to circumvent these problems is the introduction of water-soluble FSK derivatives (Laurenza et al., 1987; Onda et al., 2001) (Fig. 12).

TABLE 5
Interaction of prototypical diterpenes with ACs 1, 2 and 5 and 5C1:2C2

AC activities were determined as described in the presence of Mn^{2+} . Reaction mixtures contained diterpenes at concentrations from 100 nM–300 μ M. Data were analyzed by nonlinear regression to determine the EC_{50} values. For recombinant holo-ACs, the efficacy for each analog was determined by dividing the maximal stimulation obtained for the analog by the maximum stimulation obtained by treatment with 300 μ M FSK expressed in percent. In case of 5C1:2C2, the efficacy for each analog was determined by dividing the maximal stimulation obtained for the analog by the maximum stimulation obtained by treatment with 100 μ M FSK expressed in percent. Data for holo-ACs were adapted from Erdorf et al. (2011), and data for 5C1:2C2 were taken from Pinto et al. (2009).

Diterpene	AC1		AC2		AC5		5C1:2C2	
	EC_{50}	Efficacy	EC_{50}	Efficacy	EC_{50}	Efficacy	EC_{50}	Efficacy
	μ M	%	μ M	%	μ M	%	μ M	%
FSK	3.3	100	38	100	18	100	7.4	100
DMB-FSK	2.8	65	50	46	11	81	28	41
6A7DA-FSK	3.0	110	17	70	13	70	2.6	83
7DA-FSK	9.3	109	81	62	38	124	3.1	86
9d-FSK	17	98	127	30	61	108	20	79
1d-FSK	N.D.	3	N.D.	0.4	N.D.	0.7	ine.	ine.
BODIPY-FSK	1.2	73	0.2	-19	2.7	69	N.D.	N.D.

N.D., not determined. Ine., ineffective. Structures of diterpenes are shown in Fig. 12. FSK, forskolin; DMB, 7-deacetyl-7-(*N*-methylpiperazino-butiryloxy); 6A7DA, 6-acetyl-7-deacetyl; 7DA, 7-deacetyl; 9d, 9-deoxy; 1d, 1-deoxy; BODIPY, 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene.

Not surprisingly, potencies and efficacies of ligands differ to some extent between 5C1:2C2 on one hand and ACs 1, 2, and 5 on the other, but the critical importance of the 1-hydroxyl group for activation was retained (Pinto et al., 2009). Interestingly, fluorescence

spectroscopy studies with MANT-nucleotides as sensors revealed that FSK derivatives without the 1-hydroxyl group can still bind to AC (Pinto et al., 2009), suggesting that formation of the hydrogen bond between the 1-hydroxyl group of FSK and Val506 is important for

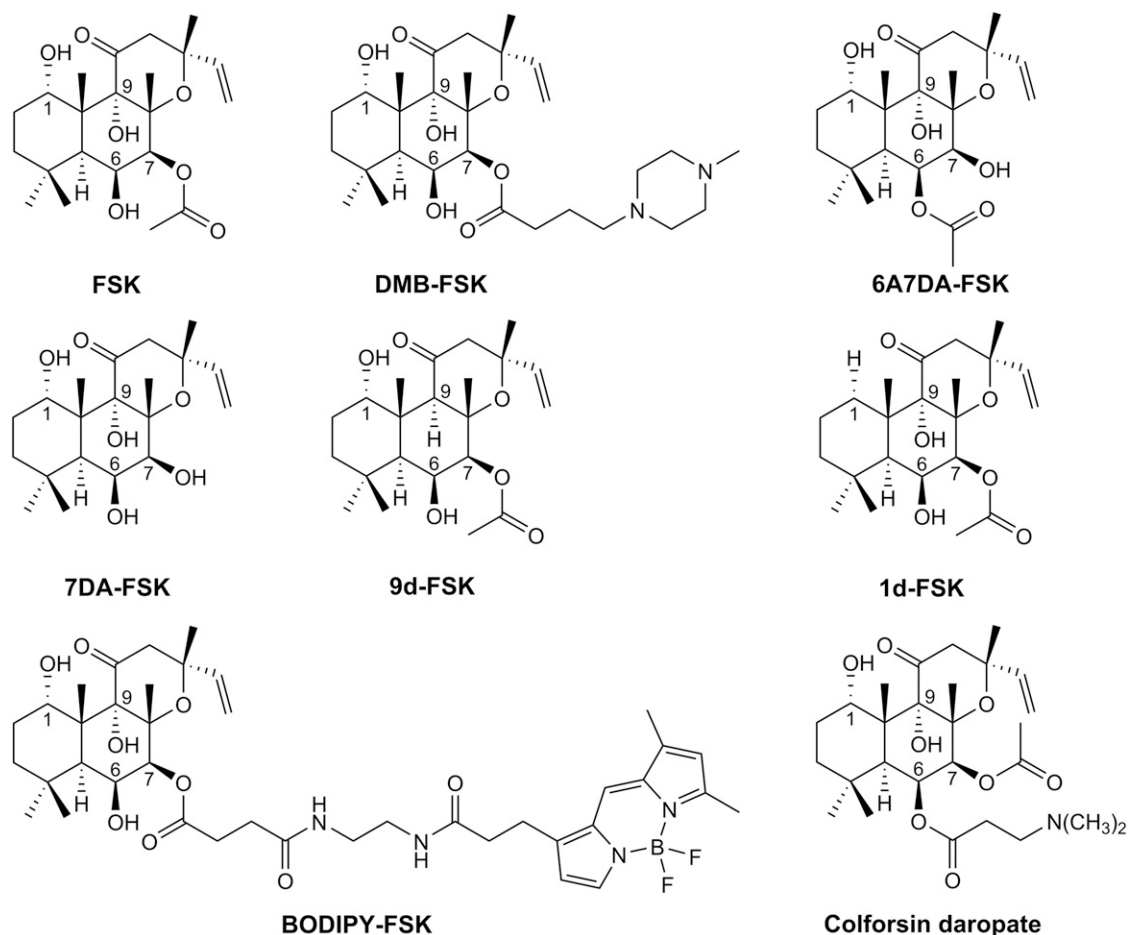


Fig. 12. Structures of diterpenes. Pharmacological data for selected diterpenes are listed in Table 5. FSK, forskolin; DMB, 7-deacetyl-7-(*N*-methylpiperazino-butiryloxy); 6A7DA, 6-acetyl-7-deacetyl; 7DA, 7-deacetyl; 9d, 9-deoxy; 1d, 1-deoxy; BODIPY, 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene. Diterpenes are highly lipophilic. To increase water solubility, hydrophilic substituents were introduced at the 6'-position (colforsin daropate) and the 7'-position (DMB-FSK) of the diterpene ring. In Japan, colforsin daropate is used clinically for treatment of heart failure. BODIPY-FSK is a fluorescent FSK derivative that shows inverse agonist activity at AC2 and partial agonist activity at ACs 1 and 5.

the actual activation step of the enzyme. 1-Deoxy-FSK prevents activation of 5C1:2C2 by FSK, supporting the view that the former compound still binds to the FSK binding site.

The most notable selectivity among FSK derivatives for AC isoforms was observed for BODIPY-FSK, characterized by a large bulky fluorescent substituent at the 7'-position of the diterpene ring. Although BODIPY-FSK is a partial activator of AC1 and AC5, BODIPY-FSK decreases the basal activity of AC2 (Pinto et al., 2008). AC2 is characterized by a high degree of ligand-independent constitutive activity (Pieroni et al., 1995; Pinto et al., 2008). By analogy to the situation described for GPCRs (Seifert and Wenzel-Seifert, 2002), BODIPY-FSK could be considered as inverse agonist at AC2. When Mn^{2+} is substituted by Mg^{2+} , potencies and efficacies change, the most remarkable difference being a profound increase in inverse agonistic efficacy of BODIPY-FSK at AC2 (Erdorf et al., 2011). These data also raise the question about the possible physiologic relevance of the high constitutive activity of AC2. The selectivity of BODIPY-FSK for AC2 inhibition, in conjunction with RT-PCR studies and analysis of MANT-nucleotides, was used to define AC2 as the functionally predominant AC isoform in rat B103 neuroblastoma cells (Kinast et al., 2012). Intriguingly, upon loss of hypoxanthine phosphoribosyl transferase, the causative enzyme defect of Lesch-Nyhan disease (Fu et al., 2014), AC2 function is substantially impaired (Kinast et al., 2012).

3. Off-target Effects of Diterpenes. Thus, although the selectivity of currently available FSK derivatives for defined AC isoforms is far from satisfying, proof of principle has been provided that ligand selectivity could be obtained, particularly by the introduction of large substituents. However, it should be kept in mind that FSK does not only bind to mACs but also to other important pharmacological targets such as glucose transporters, P-glycoprotein multidrug resistance transporters, ion channels, and nuclear receptors such as pregnane X receptor (Joost et al., 1988; Morris et al., 1991; Ding and Staudinger, 2005; Pavlikova et al., 2010; Kinast et al., 2012; Angel-Chavez et al., 2015). Such interactions of FSK are of concern for clinical application, specifically for long-term systemic treatment in conjunction with other drugs, increasing the risk of drug/drug interactions. Large dissociations between K_d values for [3H]FSK binding and EC_{50} values of FSK for AC activation support the notion that multiple FSK targets exist (Seamon et al., 1984). Limited structure-activity relationship studies have been undertaken to dissociate interaction of FSK analogs with ACs and off-target sites (Joost et al., 1988; Pfeuffer and Pfeuffer, 1989; Appel et al., 1992). When using BODIPY-FSK as a tool, nonspecific effects of the compound should be kept in mind as well (Kinast et al., 2012).

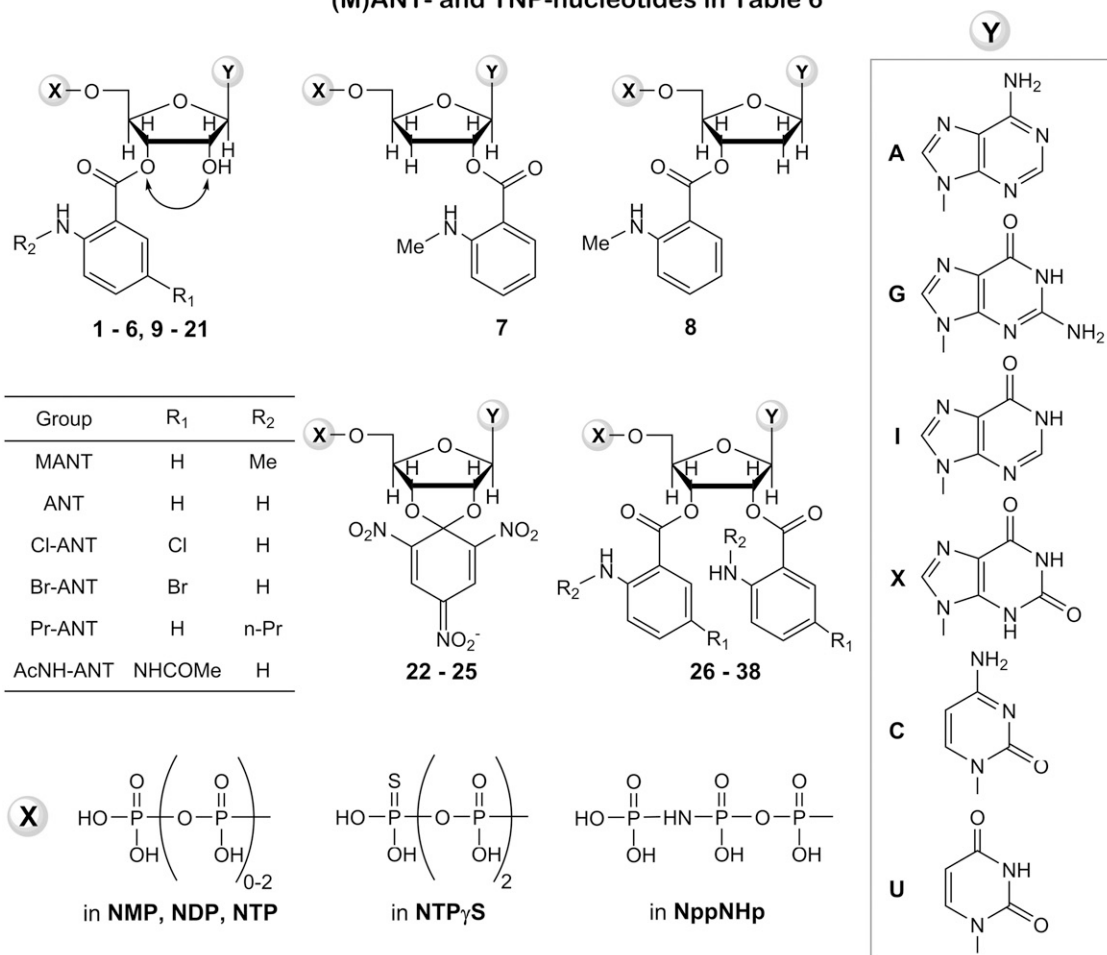
4. Clinical Applications of Diterpenes. A water-soluble FSK derivative, colforsin daropate hydrochloride, also referred to as NKH477 (Onda et al., 2001), is used for treatment of acute heart failure in Japan (Hosono, 1999) (Fig. 12). Colforsin exhibits moderate selectivity for AC5 relative to ACs 2 and 3, but its complete pharmacological profile at all mAC isoforms is not known (Toya et al., 1998; Onda et al., 2001). However, the primary goal of colforsin development was not as an AC5-selective inhibitor but rather to obtain a compound with high water solubility to prevent unwanted effects in the CNS. Colforsin daropate hydrochloride may also be useful for the treatment of septic syndrome and subarachnoid hemorrhage (Oishi et al., 2012; Suzuki et al., 2012). The local use of FSK for treatment of glaucoma is being explored, too (Majeed et al., 2015). Moreover, the dermatological application of FSK to protect the skin against ultraviolet light and induce persistent tanning is under investigation (Spry et al., 2009; Scott et al., 2012; Sikora et al., 2015). Furthermore, FSK derivatives are explored as anticancer compounds (Ponnam et al., 2014). Lastly, *Coleus forskohlii* extracts with varying and undefined FSK content are widely and uncritically used as dietary supplement for weight reduction (Rios-Hoyo and Gutierrez-Salmean, 2016). The available clinical evidence (Godard et al., 2005) is insufficient to recommend the use of FSK for treatment of obesity.

C. Adenylyl Cyclase Inhibitors

1. General Aspects. Most inhibitory compounds bind to the catalytic site. Such compounds may act either as noncompetitive, activity-dependent inhibitors, so-called P-site inhibitors, or as competitive inhibitors that are actually also activity-dependent (Seifert et al., 2012). Figure 13 shows representative competitive mAC inhibitors [(M)ANT- and TNP-nucleotides] and noncompetitive P-site inhibitors. For more structures and detailed descriptions of individual inhibitor profiles, the reader is referred to Seifert et al. (2012). So far, there is no known selective inhibitor for a given mAC isoform with clinical relevance. In contrast to the development of AC activators (see section B.1), the development of AC inhibitors requires a reasonably high catalytic activity to begin with, because otherwise inhibition cannot be detected analytically. Therefore, all typical AC inhibitor studies are conducted under optimal stimulatory AC assay conditions. Should an AC isoform-selective inhibitor be identified, it would have to be verified that the inhibitor and the corresponding *ADCY* gene knockout show very similar effects. Conceptually, this is an important control to exclude off-target effects of small molecule inhibitors.

The catalytic site possesses three pharmacophoric sites, i.e., the polyphosphate-binding site, the base-binding domain, and a hydrophobic pocket between the latter two sites (Mou et al., 2005, 2006; Hübner et al., 2011). In terms of inhibitor affinity, the hydrophobic

(M)ANT- and TNP-nucleotides in Table 6



Membrane-permeable P-site inhibitors in Table 7

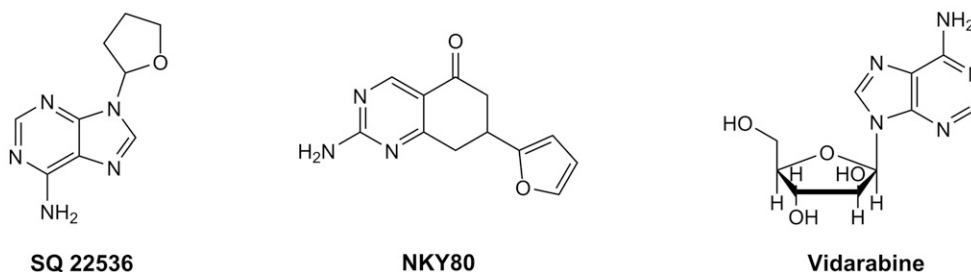


Fig. 13. Structures of representative inhibitors targeting the catalytic site of mACs. Pharmacological data for competitive inhibitors are listed in Table 6, and pharmacological data for noncompetitive P-site inhibitors are listed in Table 7. The (M)ANT group can spontaneously isomerize between the 2'- and 3'-ribosyl position, provided that there is only substituent and a free hydroxyl group. The different bases (A, adenine; G, guanine; I, hypoxanthine; X, xanthine; C, cytosine; U, uracil) constitute substituent Y at the ribosyl moiety. The different phosphate chains (mono-, di-, and triphosphate and hydrolysis-resistant phosphorothioates or β,γ -imidophosphates) constitute substituent X at the ribosyl moiety. (M)ANT- and TNP-nucleotides do not cross the plasma membrane and can only be used in cell-free systems or in electrophysiological studies in which compounds are injected into the cell via the patch pipette. Experimental problems with the membrane-permeable P-site inhibitors are the relatively low potency, insufficient selectivity and high lipophilicity, requiring the use of high concentrations of organic solvents.

pocket exhibits the largest impact on inhibitor affinity, followed by the phosphate-binding domain and the base-binding domain (Seifert et al., 2012). Accordingly, the catalytic site of ACs exhibits a broad base specificity and accepts numerous purine and pyrimidine bases, xanthine being an exception for extremely poor binding (Mou et al., 2006).

2. P-site Inhibitors. The first identified mAC inhibitors were P-site inhibitors; adenosine analogs that are typically noncompetitive or uncompetitive with respect to substrate ATP (Londos and Wolff, 1977). These compounds were characterized in the classic cell-free AC assay with [α -³²]ATP as substrate. Representative P-site inhibitors, ordered by potency, include:

2',5'-dideoxyadenosine-3'-tetrphosphate > 2',5'-dideoxy-3'-ATP > 2',5'-dideoxy-3'-ADP > 2',5'-dideoxy-3'-AMP > 2'-deoxy-3'-AMP > 3'-AMP > 2'-deoxyadenosine > adenosine (Johnson et al., 1989; Desaubry et al., 1996; Dessauer et al., 1999; Seifert et al., 2012). P-site inhibitors containing a phosphate residue are too hydrophilic to cross the plasma membrane and cannot be used in intact-cell studies. The requirement for an intact adenine ring reflects the fact that P-site inhibitors bind within the same enzyme pocket as substrate ATP (Tesmer et al., 1997; Tesmer et al., 2000). These inhibitors have revealed important insights into the mechanism of AC catalytic activity. Kinetic analysis of the forward and reverse (i.e., formation of ATP from cAMP and PP_i) reactions of mAC show that the cAMP product is preferentially released from the enzyme before PP_i (Dessauer and Gilman, 1997). In the basal state or under low levels of activity, the slow step of the reaction is dictated primarily by the cyclization of ATP to cAMP. But upon activation of the enzyme, the catalytic rate speeds up and the release of PP_i becomes the slow step in the reaction. P-site inhibitors bind to the catalytic pocket of AC and mimic a product-bound transition state of the enzyme that often includes bound PP_i. In the basal state, the catalytic site is generally open. Activators of the enzyme (e.g., G_{α_s} and FSK) increase the affinity between the C1 and C2 domains and result in a more closed conformation (Whisnant et al., 1996; Yan et al., 1996; Tesmer and Sprang, 1998). Structures of AC with bound P-site inhibitors exhibit an even further closed conformation. This explains the uncompetitive nature of their inhibition; they require substrate binding and catalysis to form an enzyme-PP_i-inhibitor dead-end complex. Moreover, P-site inhibitors are far more potent on stimulated forms of mAC, where release of PP_i is the slow step and the concentration of steady-state enzyme-PP_i complex increases (Dessauer and Gilman, 1997).

P-site inhibitors that have 3'-polyphosphate moieties, such as 2',5'-dideoxy-3'-ATP, bind in the absence of PP_i but still display noncompetitive kinetics (Desaubry et al., 1996; Desaubry and Johnson, 1998; Tesmer et al., 2000). The 3'-polyphosphates bind in the pyrophosphate pocket of the catalytic site and serve to increase the potency of these molecules by 100–1,000-fold. Because these inhibitors do not require bound PP_i, one might expect a competitive pattern of inhibition, as is observed for ATP analogs (Gille and Seifert, 2003). However, P-site inhibitors still require for binding a conformation that resembles a product-like transition state and thus are more potent on the active forms of the enzyme. The affinity of mAC for all P-site inhibitors increases in the presence of Mn²⁺ versus Mg²⁺, similar to the reduction of the K_m for ATP with Mn²⁺. The tight binding of these inhibitors at the active site renders these important tools for understanding the function of

these enzymes and the development of future cell-permeable inhibitors (Seifert et al., 2012).

3. *Structure-activity Relationships and Binding Modes of 2',(3')-O-(N-Methylantraniloyl)- and 2',3'-O-(2,4,6-Trinitrophenyl)-nucleotides.* MANT- and TNP-nucleotides explore all three pharmacophoric domains in ACs and are competitive inhibitors (Gille and Seifert, 2003). Some of the compounds studied exhibit nanomolar affinities that enabled their use for crystallographic studies (Mou et al., 2005, 2006; Hübner et al., 2011) and fluorescence spectroscopy studies monitoring ligand-specific conformational changes (Pinto et al., 2009, 2011; Suryanarayana et al., 2009a) with purified 5C1:2C2. Unfortunately, because of the fact that MANT-nucleotides bind to many proteins with a nucleotide binding site (see, e.g., Remmers et al., 1994; Franks-Skiba and Cooke, 1995; Scheidig et al., 1995; Dayan et al., 1997; Hiratsuka, 2003; Zielke et al., 2015), their potential as starting point for the development of AC isoform-selective drugs is very limited.

The binding modes of 2'- and 3'-MANT as well as TNP-substituted nucleotides at mACs are roughly similar to the binding mode of ATP (Fig. 11). In the complex of 5C1:2C2 with 3'-MANT-GTP (Mou et al., 2005), the ribosyl moiety adopts a 2'-endo conformation with an axial orientation of the MANT group (Fig. 14A). Its wedge-like fit between $\alpha 1$, $\alpha 2$, and the $\beta 5'$ - $\alpha 4'$ loop prevents the formation of the fully closed conformation. The phenyl ring is involved in hydrophobic interactions with L412 ($\alpha 2$) and van der Waals contacts with the backbone of W1020, G1021, and N1022 ($\beta 5'$ - $\alpha 4'$ loop). With a 3'-endo conformation of the ribosyl moiety corresponding to an equatorial 3'- and an axial 2'-orientation, 2'-MANT, and even 2',3'-bis-MANT nucleotides may bind in a similar mode.

The triphosphate conformation in 3'-MANT-GTP differs from that in ATP. In particular, α - and β -phosphates interact only with metal ions. The γ -phosphate forms three hydrogen bonds with the backbone NH functions of F400 and T401 ($\alpha 1$) and with K1065 ($\beta 7'$ - $\beta 8'$ loop), but does not contact R484 in contrast to ATP. The ribosyl moieties of 3'-MANT-GTP and ATP also interact differently with 5C1:2C2. In case of 3'-MANT-GTP, a weak hydrogen bond of the 2'-OH group with N1025 ($\alpha 4'$) and distant van der Waals contacts with the side chains of F400 ($\alpha 1$) and V1024 ($\alpha 4'$) are formed. The hydrogen bonds of the adenylyl moiety in the 5C1:2C2-ATP complex with K938 ($\beta 2'$) and D1018 ($\beta 5'$) are reproduced by the oxygen, the N1 atom and the 2-NH₂ group, respectively, of the guanine base. In both cases, the base adopts an *anti* conformation. In contrast, a *syn* position of the adenylyl moiety and no specific enzyme-base interactions are characteristic for the binding mode of 3'-MANT-ATP in complex with 5C1:2C2 (Mou et al., 2006).

TNP-ATP binds roughly similar to 3'-MANT-GTP (Mou et al., 2006), i.e., the pockets occupied by the three

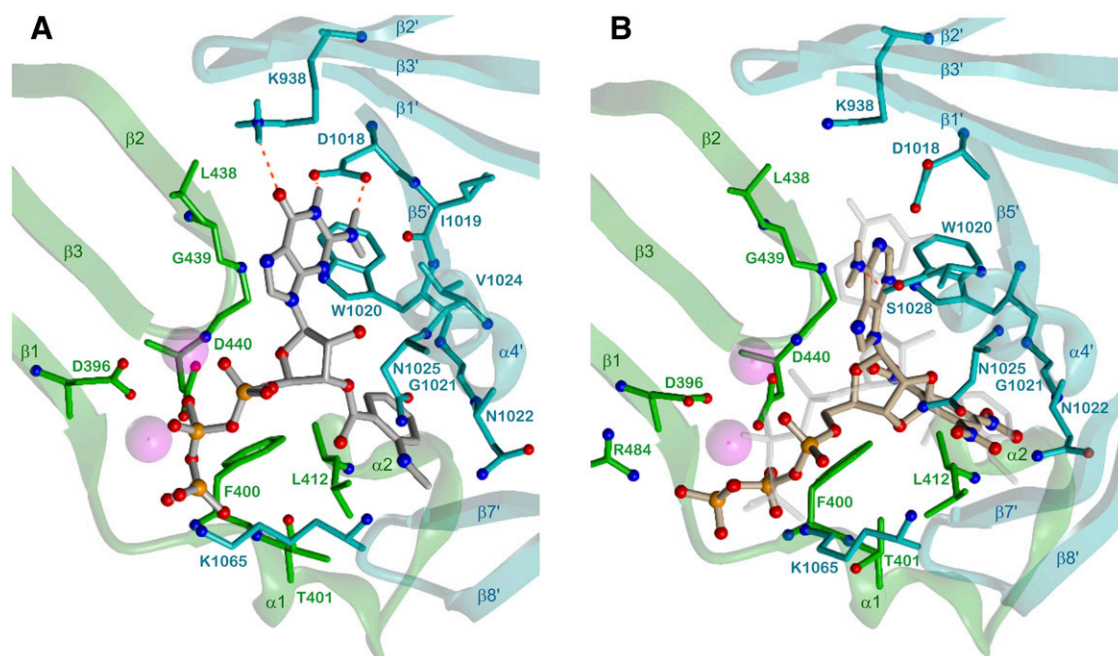


Fig. 14. Interactions of 5C1:2C2 with selected MANT- and TNP-nucleotides. 5C1, green; 2C2, cyan; heteroatom colors: N, blue; O, red; P, orange; Mn^{2+} , violet. Nucleobase-2C2 hydrogen bonds are drawn as red dashed lines, contacting amino acids (distance $< 3.5 \text{ \AA}$) and secondary structure elements are labeled. (A) Binding mode of 3'-MANT-GTP, model based on the crystal structure PDB 1TL7 (Mou et al., 2005) C and selected H atoms of 3'-MANT-GTP, gray. (B) Binding mode of TNP-ATP, model based on the crystal structure PDB 2GVD (Mou et al., 2006), C and selected H atoms of TNP-ATP, mauve. The gray shadow image shows 3'-MANT-GTP from a superposition of both binding sites (root-mean-square deviation of $C\alpha$ atoms, 0.44 \AA).

components nucleobase, triphosphate, and substituent are common (Fig. 14B). The fit of the trinitrophenyl ring between L412 ($\alpha 2$) and the backbone of W1020, G1021, and N1022 ($\beta 5'$ - $\alpha 4'$ loop) corresponds to that of the 3'-MANT substituent. The 2-nitro group is involved in a hydrogen bond with the side chain amide of N1022. N1025 ($\alpha 4'$) also interacts with the 2-nitro group and not with the ribosyl fragment, which is almost planar with two axial oxygens in 2'- and 3'-position. Interactions and conformation of the triphosphate moiety of TNP-ATP differ from those of 3'-MANT-GTP with respect to the β - and γ -phosphate. In case of TNP-ATP, the β -phosphate forms two hydrogen bonds with the backbone NH of F400 ($\alpha 1$), and the γ -phosphate interacts with R484 ($\beta 4$) and K1065 ($\beta 7'$ - $\beta 8'$ loop). In contrast to 3'-MANT-ATP, the adenine base in TNP-ATP adopts an *anti* position. A single hydrogen bond is formed between the 6-NH₂ group and the side chain of S1028 ($\alpha 4'$). Taken together, Fig. 14B is suggestive of flexible fits of MANT- and TNP-nucleotides to partially different components of the binding pocket as major cause of mAC promiscuity for competitive inhibitors.

Table 6 lists the structure-activity relationships for selected (M)ANT- and TNP-nucleotides at ACs 1, 2, and 5 as representatives of the three major mAC families, purified catalytic subunits 5C1:2C2, sAC and purified sGC $\alpha 1\beta 1$, also referred to as GC1. In general, AC2 is the least sensitive mAC isoform toward TNP- and (M)ANT-nucleotides, but selectivity for ACs 1 and 5 is at best 10-fold (Gille et al., 2004; Suryanarayana et al., 2009a;

Pinto et al., 2011). In addition, TNP- and (M)ANT-nucleotides are very potent sGC inhibitors (Gille et al., 2004; Suryanarayana et al., 2009a; Dove et al., 2014). The potent sGC inhibition by (M)ANT-nucleotides is not surprising in light of the high conservation of the catalytic domains of these enzymes (Sunahara et al., 1998; Dove et al., 2014). Given the beneficial clinical effects of allosteric sGC activators and sGC stimulators (Stasch et al., 2011, 2015), sGC inhibition would be clearly an off-target effect of mAC inhibitors (Dove et al., 2014). However, there is evidence for pharmacological distinction between mACs and sGC with (M)ANT- and TNP-nucleotides (Dove et al., 2014) (Table 6).

In addition to nucleotidyl cyclases, (M)ANT- and TNP-nucleotides bind to many other nucleotide-binding enzymes and signal-transducing guanine nucleotide-binding proteins (see e.g., Remmers et al., 1994; Franks-Skiba and Cooke, 1995; Scheidig et al., 1995; Dayan et al., 1997; Hiratsuka, 2003; Zielke et al., 2015), rendering AC specificity difficult. Application of (M)ANT- and TNP-nucleotides in intact cells is also impossible because the compounds are too hydrophilic to penetrate the plasma membrane. Electrophysiological experiments with intracellularly injected MANT-GTP pointed to a role of AC5 in the regulation of voltage-dependent calcium channels in cardiomyocytes (Rottlaender et al., 2007), but studies with the structurally closely related nucleotide MANT-ITP revealed unexpected off-target effects (Hübner et al., 2011b), although MANT-ITP is the most

TABLE 6
Inhibition of mACs, sAC, and sGC by (M)ANT- and TNP-nucleotides

Nucleotidyl cyclase activity was determined in the presence of Mn^{2+} . Reaction mixtures contained nucleotides analogs at concentrations from 10 nM to 100 μ M. Inhibition curves were analyzed by nonlinear regression and were best-fitted to monophasic sigmoidal curves. pK_i values are listed in the table. The structures of the inhibitors examined are shown in Fig. 13. Compounds 1, 3, 7, 10, 11, and 20 were studied at partially purified sAC from rat testis; compounds 22–25 were studied at the purified catalytic domain of human sAC. The Table has been modified from Table 1 in Dove et al. (2014). Data for mACs 1, 2, and 5 and 5C1:2C2 were taken from the literature as noted by the footnotes.

Cpd.	Inhibitor	AC1	AC2	AC5	5C1:2C2	sAC	sGC $\alpha_1\beta_1$ (GC1 ^a)
1	MANT-ATP	6.82 ^b	6.48 ^b	7.00 ^b	7.80 ^b	5.25 ^c	6.77
2	MANT-ITP	8.55 ^b	7.85 ^b	8.92 ^b	9.15 ^b	N.D.	6.31
3	MANT-GTP	7.05 ^b	6.21 ^b	7.28 ^b	7.74 ^b	>4.00 ^c	6.06
4	MANT-XTP	5.96 ^b	5.52 ^b	5.89 ^b	5.92 ^b	N.D.	6.95
5	MANT-CTP	6.82 ^b	6.16 ^b	6.82 ^b	8.04 ^b	N.D.	6.28
6	MANT-UTP	7.34 ^b	6.34 ^b	7.49 ^b	8.21 ^b	N.D.	6.30
7	2'-MANT-3'-dATP	6.33 ^b	6.27 ^b	7.19 ^b	7.05 ^b	4.60 ^c	7.78
8	3'-MANT-2'-dATP	6.49 ^b	5.32 ^b	6.44 ^b	6.72 ^b	N.D.	6.13
9	MANT-ATP γ S	6.80 ^d	6.42 ^d	7.11 ^d	N.D.	N.D.	6.88
10	MANT-ITP γ S	7.39 ^c	6.92 ^c	7.51 ^c	7.72 ^b	>4.00 ^c	6.03
11	MANT-GTP γ S	7.21 ^c	6.44 ^c	7.46 ^c	7.62 ^b	>4.00 ^c	6.33
12	ANT-ATP	6.89 ^b	6.19 ^b	6.92 ^b	7.77 ^b	N.D.	6.63
13	Cl-ANT-ATP	7.10 ^e	6.31 ^e	7.35 ^e	N.D.	N.D.	7.33
14	Cl-ANT-ITP	8.48 ^e	8.10 ^e	8.70 ^e	N.D.	N.D.	5.92
15	Br-ANT-ITP	8.15 ^e	7.66 ^e	8.34 ^e	N.D.	N.D.	6.22
16	Pr-ANT-ATP	6.36 ^e	5.96 ^e	6.44 ^e	N.D.	N.D.	6.80
17	Pr-ANT-ITP	7.66 ^e	7.17 ^e	8.00 ^e	N.D.	N.D.	6.41
18	AcNH-ANT-ATP	5.17 ^e	4.96 ^e	5.47 ^e	N.D.	N.D.	6.11
19	AcNH-ANT-ITP	6.85 ^e	6.41 ^e	7.43 ^e	N.D.	N.D.	6.22
20	MANT-AppNHp	6.80 ^c	6.31 ^c	6.92 ^c	7.70 ^c	5.72 ^c	6.77
21	MANT-GppNHp	6.96 ^c	6.18 ^c	7.01 ^c	7.47 ^c	N.D.	5.56
22	TNP-ATP	8.05 ^f	7.00 ^f	8.43 ^f	7.09 ^g	6.19 ^f	7.67
23	TNP-GTP	7.64 ^f	6.66 ^f	7.57 ^f	7.08 ^g	5.14 ^f	7.97
24	TNP-CTP	7.62 ^f	6.96 ^f	7.51 ^f	6.51 ^g	5.29 ^f	7.01
25	TNP-UTP	8.15 ^f	7.62 ^f	7.82 ^f	7.04 ^g	5.51 ^f	7.29
26	Bis-MANT-ATP	6.15 ^e	5.68 ^e	6.37 ^e	N.D.	N.D.	6.23
27	Bis-MANT-ITP	6.51 ^e	5.96 ^e	6.85 ^e	N.D.	N.D.	6.46
28	Bis-MANT-CTP	6.21 ^e	5.11 ^e	6.12 ^e	N.D.	N.D.	6.10
29	Bis-MANT-IDP	6.00 ^e	5.85 ^e	6.15 ^e	N.D.	N.D.	5.72
30	Bis-MANT-IMP	<3 ^e	<3 ^e	<3 ^e	N.D.	N.D.	<3
31	Bis-Cl-ANT-ATP	5.77 ^e	5.62 ^e	5.80 ^e	N.D.	N.D.	6.28
32	Bis-Cl-ANT-ITP	7.18 ^e	6.70 ^e	7.19 ^e	N.D.	N.D.	6.08
33	Bis-Br-ANT-ATP	N.D.	N.D.	N.D.	N.D.	N.D.	6.37
34	Bis-Br-ANT-ITP	7.68 ^e	7.15 ^e	7.82 ^e	N.D.	N.D.	6.20
35	Bis-Pr-ANT-ATP	4.74 ^e	4.44 ^e	4.74 ^e	N.D.	N.D.	5.44
36	Bis-Pr-ANT-ITP	6.36 ^e	5.85 ^e	6.60 ^e	N.D.	N.D.	5.91
37	Bis-AcNH-ANT-ATP	4.66 ^e	5.15 ^e	5.21 ^e	N.D.	N.D.	5.41
38	Bis-AcNH-ANT-ITP	5.77 ^e	5.25 ^e	6.32 ^e	N.D.	N.D.	5.97

N.D., not determined.

^aDove et al., 2014.

^bPinto et al., 2011.

^cGille et al., 2004.

^dGöttle et al., 2009.

^eGeduhn et al., 2011.

^fSuryanarayana et al., 2009a.

^gMou et al., 2006.

potent mAC inhibitor known so far (Hübner et al., 2011a). Intriguingly, bulky bis-MANT-nucleotides exhibit strong preference for the *Bordetella pertussis* AC toxin CyaA relative to mACs, constituting the basis for the development of bacterial toxin inhibitors (Geduhn et al., 2011) (Table 6).

4. Membrane-permeable Membrane Adenylyl Cyclase Inhibitors. The issues with impermeability of the plasma membrane for nucleotides can be avoided by applying membrane-permeable nucleotide prodrugs (Laux et al., 2004), but this strategy has not yet found broad application in the field, leaving many of the most potent AC inhibitors with little cellular utility. As an alternative strategy, membrane-permeable and hence lipophilic nucleoside analogs can be used in intact cell studies. In general, these compounds are much less

potent than MANT/TNP-nucleotides because they only explore the base-binding domain of ACs (Seifert et al., 2012; Brand et al., 2013). These compounds act as noncompetitive P-site inhibitors. The lipophilicity of compounds may require high concentrations of organic solvents in cell experiments so that nonspecific solvent effects become a problem (Brunskole Hummel et al., 2013), but this aspect has not been generally considered in cell studies.

Three compounds, 2-amino-7-(2-furanyl)-7,8-dihydro-5(6H)-quinazolinone (NKY80), 9-(tetrahydro-2-furanyl)-9H-purin-6-amine (SQ22,536), and vidarabine (also referred to as adenine 9- β -D-arabinofuranoside or 9- β -D-arabinofuranosyl-adenine), have been systematically studied at all nine mAC isoforms, several native membrane systems, and sAC (Brand et al., 2013;

Bitterman et al., 2013; Braeunig et al., 2013; Braeunig et al., 2014). Table 7 lists the pIC_{50} values of these compounds for inhibition of ACs and pK_i values for interaction with adenosine receptors. Some compounds have been studied with defined ACs by several independent research groups. Overall, there is reasonably good agreement in the obtained pIC_{50} values (Table 7). SQ22,536, NKY80, and vidarabine exhibit some degree of selectivity among AC isoforms, AC2 being relatively insensitive compared with ACs 5 and 6 (Bitterman et al., 2013). ACs 8 and 9 and sAC are very resistant to inhibition by SQ22,536, NKY80, and vidarabine. pIC_{50} values for sGC of the compounds listed in Table 7 are not available. A recent study reported on the identification of noncompetitive inhibitors with some selectivity for AC2 relative to ACs 1 and 5, compound 8-bromo-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1H-3-benzazepin-7-ol hydrobromide (SKF-83566) being a representative (Conley et al., 2013). However, SKF-83566 is also a potent dopamine D1 receptor antagonist (O'Boyle and Waddington, 1984).

cis-N-(2-phenylcyclopentyl)-azacyclotridec-1-en-2-amine hydrochloride (MDL 12,330A) was one of the first AC inhibitors to be introduced into experimental pharmacology (Hunt and Evans, 1980) and is still widely used. The compound is membrane-permeable and has been reviewed critically (Seifert et al., 2012). MDL 12,330A cannot be recommended for intact-cell studies because of its low potency and pleiotropic effects. The

compound has only been incompletely characterized so far at defined AC isoforms. MDL 12,330A inhibits a lysate of mAC with a pIC_{50} of 3.4 and purified sAC with a pIC_{50} of 3.6 (Bitterman et al., 2013). Thus, MDL 12,330A does not discriminate between mACs and sAC, and the potency of the compound is lower than the potency of many membrane-permeable P-site inhibitors (Table 7).

5. Adenylyl Cyclase 1 Inhibitors and Modulation of Calmodulin. A nucleotidyl cyclase that has attracted considerable attention as drug target is AC1. It was proposed that AC1 inhibition could be used to treat various neuropsychiatric disorders such as neuropathic pain (Wang et al., 2011a). 5-[2-(6-Amino-9H-purin-9-yl)ethyl]amino]-1-pentanol (NB001) was suggested to inhibit AC1 in intact cells selectively (Wang et al., 2011a), but this proposition was not confirmed with recombinant AC1 in membrane preparations (Brand et al., 2013). Thus, the precise mechanism of action of NB001 remains elusive.

AC1 forms a unique interface with its activator CaM compared with other mammalian CaM targets (Vorherr et al., 1993; Diel et al., 2008; Masada et al., 2012; Lübker and Seifert, 2015; Lübker et al., 2015a,b). Principally, such a unique protein-protein interaction interface could be exploited pharmacologically. In this context, it is interesting to note that calmidazolium chloride, which is very potent at inhibiting numerous CaM-regulated effectors, only very ineffectively inhibits AC1 (Lübker and Seifert, 2015). However, potent and

TABLE 7
Interaction of prototypical membrane-permeable P-site inhibitors with mACs, sAC, sGC, and adenosine receptors

AC activity was determined in the presence of Mg^{2+} and in the presence of stimulators ($G_{\alpha_{\text{S}}}$, $\text{GTP}_{\gamma}\text{S}$ and/or FSK) to ensure maximum catalytic activity and, hence, highest inhibitor potency possible. For ACs, pIC_{50} values are listed. For adenosine receptors, pK_i values are listed. Structures of P-site inhibitors are shown in Fig. 13. 2',5'-Dideoxy-3'-ATP (Table 8 and Fig. 15) is mechanistically related to the P-site inhibitors shown here.

AC	SQ22,536	NKY80	Vidarabine
AC1	4.27 ^a , 3.95 ^b , 3.92 ^c	4.04 ^a , 3.48 ^b	4.31 ^a , 3.86 ^b
AC2	3.71 ^a , 3.41 ^b , 3.17 ^c , 3.54 ^d	3.11 ^a , >3.00 ^b , >3.00 ^c , >3.00 ^d	3.61 ^a , 3.26 ^b , 3.15 ^c
AC3	3.96 ^a , 4.00 ^d	4.03 ^a , 3.64 ^e , 3.89 ^d	4.07 ^a
AC4	3.55 ^a	3.95 ^a	4.03 ^a
AC5	5.46 ^a , 5.02 ^b , 5.66 ^d	5.15 ^a , 4.09 ^b , 4.82 ^e , 5.08 ^d	5.67 ^a , 4.70 ^b , 5.00 ^e
AC6	5.25 ^a	4.80 ^a	5.34 ^a
AC7	3.38 ^a	3.09 ^a	3.64 ^a
AC8	>3.00 ^a , 3.92 ^c	>3.00 ^a	>3.00 ^a
AC9	>3.00 ^a	>3.00 ^a	>3.00 ^a
5C1:2C2	N.D.	N.D.	N.D.
sAC	>>3.00 ^f	>>3.00 ^f	>>3.00 ^f
sGC	N.D.	N.D.	N.D.
Mouse heart AC, wild type	5.20 ^b	4.37 ^b	4.98 ^b
Mouse heart AC, AC5 knockout	5.30 ^b	4.41 ^b	5.00 ^b
Rat heart AC, left ventricle	N.D.	4.48 ^g	4.73 ^g
Rat heart, right ventricle	N.D.	4.62 ^g	5.12 ^g
Adenosine A ₁ receptor	4.86 ⁱ	4.62 ⁱ	>4.00 ^h
Adenosine A _{2A} receptor	4.76 ^h	5.52 ^h	>4.00 ^h
Adenosine A ₃ receptor	>4.22 ^h	>4.00 ^h	>4.00 ^h

N.D., not determined.

^aBrand et al., 2013.

^bBraeunig et al., 2013.

^cJohnson et al., 1997.

^dOnda et al., 2001.

^eIwatsubo et al., 2004.

^fBitterman et al., 2013.

^gBraeunig et al., 2014.

^hKlotz and Kachler, 2016.

selective inhibitors of the AC1-CaM interaction have not yet been identified in a small-scale screening study (Lübker and Seifert, 2015). Calmidazolium also inhibits AC9, probably by binding to the catalytic site (Haunso et al., 2003). Currently, there is no clinical use of AC1 inhibitors.

Ageing processes are related to the oxidation of methionine residues in CaM (Bigelow and Squier, 2005), and oxidized CaM is less efficient than native CaM at activating AC1 (Lübker et al., 2015a,b). Thus, one could envisage that reduction of oxidized methionine residues in CaM by increased expression of methionine sulfoxide reductase is therapeutically useful in CNS diseases associated with impaired learning and memory (Moskovitz and Oien, 2010; Lübker et al., 2015a,b).

6. Adenylyl Cyclase 5 Inhibitors. Currently, there is great interest in AC5-selective inhibitors. Based on experiments with AC5 knockout mice (see section II. E.3.a), it has been proposed that selective AC5 inhibition may be beneficial in the treatment of heart failure and other age-related diseases (Vatner et al., 2013; Bravo et al., 2016). However, AC5 is broadly expressed in many organs, including kidney (Erdorf and Seifert, 2011) and brain (Kim and Han, 2009), so that substantial organ toxicity could emerge as a result of global AC5 inhibition (Table 3). Most notably, AC5 inhibition in the brain could result in Parkinson-like symptoms (Iwamoto et al., 2003; Seifert, 2014). In addition, the available competitive and noncompetitive AC inhibitors do not discriminate between AC5 and the closely related AC6 (Gille et al., 2004; Brand et al., 2013) (Table 7). This is supported by the observation that vidarabine shows very similar potency in heart membranes from wild-type mice and AC5 knockout mice (Table 7). The lack of discrimination between ACs 5 and 6 is problematic, because AC6 also plays an important functional role in the heart (Göttle et al., 2009; Tang et al., 2013).

Another complication constitutes the fact that several P-site inhibitors show significant affinity to adenosine receptors (Klotz and Kachler, 2016) (Table 7) and inhibit cellular phosphorylation cascades via an as yet unknown mechanism (Emery et al., 2013), so that interpretation of pharmacological data as AC5 inhibition is very problematic. In some instances, because of solubility issues and low compound potency, it has been impossible to obtain sufficiently high inhibitor concentrations in intact cells to observe AC inhibition (Brunskole Hummel et al., 2013). Similarly, it is questionable whether the required micromolar concentrations of P-site inhibitors can be obtained in the intact organism without off-target effects (Seifert, 2016). Overall, NKY80, SQ22,536, and vidarabine, which are very widely used as “selective mAC inhibitors” (or even more problematic as “selective AC5 inhibitors”) (e.g., Rangel-Barajas et al., 2011; Bravo et al., 2016; Liu and

Steketee, 2016; Wada et al., 2016; Wei et al., 2016), should be used with great caution as pharmacological tools, and any observed effects should be carefully checked for off-target actions (Seifert, 2014, 2016). It is particularly important to confirm that the AC inhibitor used actually prevents a GPCR- or FSK-mediated cAMP increase (Brunskole Hummel et al., 2013). Because vidarabine constitutes an FDA-approved antiviral drug, the clinical use of this compound for AC5 inhibition has been proposed (Vatner et al., 2013; Bravo et al., 2016). Unfortunately, vidarabine possesses a high potential for toxic effects so that systemic clinical use would potentially be dangerous (Seifert, 2014, 2016).

A newly emerging potential clinical application of AC5 inhibitors is the treatment of extrapyramidal movement disorders such as FDFM, caused by gain-of-function mutations in the *ADCY5* gene (Chen et al., 2012c, 2014b). Thus, reduction of the pathologically increased catalytic activity of the R418W AC5 mutant and A726T AC5 mutant may be beneficial. However, like with wild-type ACs, selectivity of inhibitors is a concern. As a first step toward the development of mutant-selective AC5 inhibitors, a comprehensive pharmacological characterization of AC5 mutants with the available tools (Figs. 12 and 13; Tables 6 and 7) is required.

7. Soluble Adenylyl Cyclase Inhibitors. In general, MANT- and TNP-nucleotides inhibit purified sAC much less potently than ACs 1, 2, and 5 and purified 5C1:2C2 and sGC (Table 6), but data sets are incomplete. Along the same line, membrane-permeable P-site inhibitors, often used in intact cell studies, inhibit sAC considerably less potently than most mAC isoforms (Table 7). The membrane-impermeable P-site inhibitor 2',5'-dideoxy-3'-ATP (Fig. 15) is less potent at inhibiting sAC than purified 5C1:2C2 and various mAC isoforms (Table 8), fitting to the data on membrane-permeable P-site inhibitors.

Considerable effort has been directed toward the development of sAC inhibitors to probe the role of sAC in cell physiology. Catechol estrogens are noncompetitive sAC inhibitors, binding to a hydrophobic region in the vicinity of the catalytic site (Steebhorn et al., 2005). 2-Hydroxyestradiol (2CE) is a prototypical compound of this class (Fig. 15). 2CE exhibits selectivity toward AC1, 2, and 5, but not AC7 (Table 8). The potency of 2CE in intact cells and cell-free systems differs for as yet unknown reasons (Steebhorn et al., 2005; Bitterman et al., 2013).

(±)-2-(1*H*-Benzimidazol-2-ylthio)propanoic acid 2-[(5-bromo-2-hydroxyphenyl)methylene]hydrazide (KH7) is the most widely used sAC inhibitor in intact-cell studies (Kumar et al., 2009; Schmid et al., 2010; Kolodecik et al., 2012; Bitterman et al., 2013; Dey et al., 2014; Hasan et al., 2014; Nickols et al., 2015; De Rasmio et al., 2015; Sosa et al., 2016). Caution must be exerted when

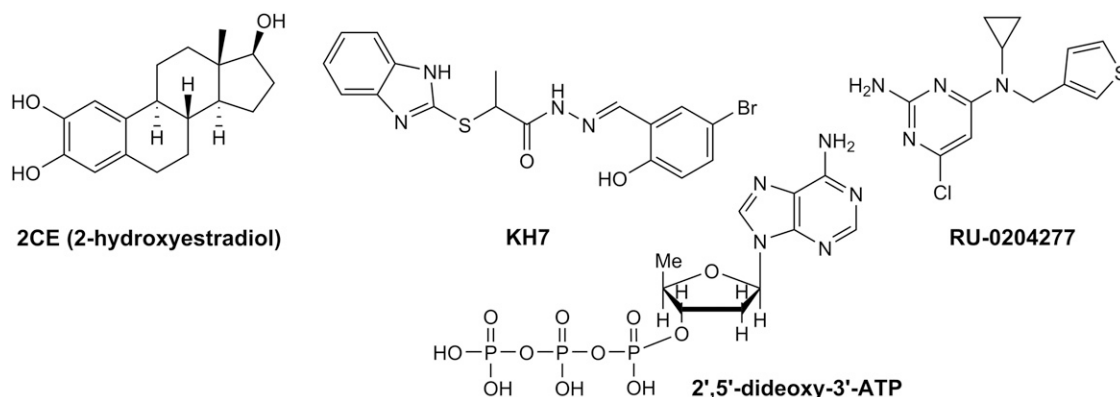


Fig. 15. Structures of representative inhibitors targeting sAC. Pharmacological data for inhibitors are listed in Table 8. All compounds are noncompetitive sAC inhibitors. 2CE, KH7, and RU-0204277 are membrane-permeable. KH7 has been broadly used in intact cell studies, but cell toxicity is of concern. RU-0204277 is a recently developed compound with less toxic liability than KH7 and binds to the bicarbonate site of sAC. 2CE exhibits different potencies at purified sAC and in intact cells. 2',5'-dideoxy-3'-ATP is a classic P-site inhibitor related to SQ 22536, NKY80, and vidarabine (for structures, see Fig. 13 and for pharmacological data see Table 7) that also potently inhibits mACs and cannot penetrate the plasma membrane.

interpreting intact-cell studies with KH7 because of toxic effects of this compound in mitochondria (Di Benedetto et al., 2013) and β cells (Tian et al., 2011). KH7 inhibits purified sAC with similar potency as 2CE and shows selectivity when examined against a lysate containing mACs (Table 8). However, data for individual mAC isoforms are not available. The exact binding site of KH7 on sAC is not known.

6-Chloro- N^4 -cyclopropyl- N^4 -[(thiophen-3-yl)methyl]pyrimidine-2,4-diamine (RU-0204277) has recently emerged as a novel sAC inhibitor from a high-throughput screening strategy (Ramos-Espiritu et al., 2016). RU-0204277 binds to the bicarbonate-binding site of sAC and exhibits potency comparable to the potencies of 2CE and KH7 (Ramos-Espiritu et al., 2016) (Table 8). At a concentration of 50 μ M, the substance did not affect ACs 1, 2, 5, 8, and 9. Given the IC_{50} for sAC of ~ 10 μ M, it will be necessary to examine inhibitor concentrations of 100–500 μ M to assess specificity more comprehensively. Initial studies indicate that

RU-0204277 is also useful for intact-cell studies and devoid of toxicity (Ramos-Espiritu et al., 2016).

Inhibition of sGC is a problematic property when aiming at the clinical application of mAC and sAC inhibitors (Stasch et al., 2011, 2015; Dove et al., 2014). Unfortunately, pIC_{50} values for sGC of the compounds listed in Table 8 are not yet available.

V. Conclusions and Future Directions

Since the initial identification of cAMP in biologic systems almost 60 years ago by Rall and Sutherland (1958), substantial progress has been made with respect to structure, expression, regulation, and compartmentation of both mACs and sAC. This knowledge constitutes the basis for the development and clinical application of mAC activators and inhibitors as well as sAC inhibitors. Despite our large increase in knowledge, there are still important gaps. Most importantly, a systematic pharmacological characterization of ACs is,

TABLE 8
Interaction of prototypical membrane-permeable sAC inhibitors and the membrane-impermeable P-site inhibitor 2',5'-dd-3'-ATP with sAC and mACs

pIC_{50} values are listed. Structures of prototypical sAC inhibitors and 2',5'-dideoxy-3'-ATP are shown in Figure 15. pIC_{50} values for ACs 3, 4 and 6 and sGC are not available. 2',5'-dideoxy-3'-ATP is mechanistically related to the P-site inhibitors documented in Fig. 13 and Table 7.

AC	2CE	KH7	RU-0204277	2',5'-dideoxy-3'-ATP
Purified sAC	$\sim 5.7^a$	5.52^b	$\sim 5.0^c$	6.16^d
5C1:2C2	N.D.	N.D.	N.D.	7.42^d
mAC lysate	$\sim 4^a$	no inhibition at 500 μ M ^b	N.D.	7.40^d
AC1	$\sim 4^a$	N.D.	no inhibition at 50 μ M ^c	7.43^d
AC2	$\sim 4^a$	N.D.	no inhibition at 50 μ M ^c	6.66^d
AC5	$\sim 4^a$	N.D.	no inhibition at 50 μ M ^c	7.43^d
AC7	$\sim 5.7^a$	N.D.	N.D.	N.D.
AC8	N.D.	N.D.	no inhibition at 50 μ M ^c	N.D.
AC9	N.D.	N.D.	no inhibition at 50 μ M ^c	N.D.

N.D., not determined. Specific experimental conditions for analysis of inhibitors varied in the different studies and are given in:

^aSteegborn et al. (2005);

^bBitterman et al. (2013);

^cRamos-Espiritu et al. (2016);

^dGille et al. (2004).

with one exception for a small group of membrane-permeable inhibitors (Brand et al., 2013), not available. The incomplete pharmacological mAC characterization has resulted in misconceptions regarding the supposed selectivity of certain compounds (Seifert, 2014, 2016; Bravo et al., 2016).

Most crystal structures were obtained with a 5C1:2C2 hybrid from different species, with parts of the cytosolic domains and the entire transmembrane domains missing. From the tremendous progress in GPCR crystallography (Venkatakrishnan et al., 2013; Piscitelli et al., 2015), it can be extrapolated that resolution of holo-mAC crystal structures with homologous cytosolic domains and transmembrane domains would substantially increase our knowledge on these enzymes, specifically with regard to hitherto unexplored ligand binding sites. This would require, as a first step, systematic testing and optimization of detergents that may stabilize holo-mACs (Cho et al., 2015). In case of GPCRs, this effort was ultimately successful but took almost 20 years (Rasmussen et al., 2007).

Although numerous experimental tools are available to the AC research community, lack of selectivity and potency of such compounds has often turned out to be a problem. Because of the structural differences between mACs (class IIIa) and sAC (class IIIb) (Linder and Schultz, 2003), the development of global mAC inhibitors and sAC inhibitors with selectivity against the other AC class may be feasible, but cross-inhibition of sGC, definitively an off-target effect (Stasch et al., 2011, 2015; Dove et al., 2014), has to be considered as well. From a medicinal chemistry point-of-view, the development of isoform-selective mAC inhibitors is particularly challenging because of the high degree of conservation of the catalytic core. One approach toward achieving this ambitious goal would be to pharmacologically address the poorly conserved transmembrane domains of mACs (Beltz et al., 2016), but this is not trivial for two reasons: First, it is difficult to conduct site-directed mutagenesis and chimeric studies with the transmembrane domains of mACs. But these studies constitute a first step toward delineation of potential ligand binding sites within transmembrane spans. Second, it is expected that ligands binding to the transmembrane domains would be highly hydrophobic, but this would also increase the risk of off-target effects considerably (Daniel, 2003).

Very few, if any, studies from the pharmaceutical industry on the development on mAC and sAC inhibitors have been published, in marked contrast to the situation regarding allosterically acting NO-dependent sGC stimulators and NO-independent sGC activators (Stasch et al., 2011, 2015; Friebe et al., 2015). One reason for these differences may be the intrinsic difficulties from the medicinal chemistry perspective. However, another reason for this situation is certainly

the fact that the pathophysiological relevance of a given mAC isoform for specific human diseases is much less clear than for sGC. The complex and overlapping expression pattern of mACs makes it difficult to obtain clinically useful pharmacological modulation without toxicity. One solution to the problem could be approaches to apply mAC inhibitors and activators locally. Such a strategy would, however, limit possible clinical applications to locally accessible organs such as the eye, skin, and airways. sAC inhibition could be a strategy to achieve the elusive goal of a male contraceptive (Roth and Amory, 2016), but the proposed broad expression and function of sAC beyond the male reproductive system (Steebhorn, 2014; Levin and Buck, 2015) would probably result in many side effects. It remains to be determined whether the recently introduced microRNA approach (Zhuang et al., 2014) constitutes a clinically useful approach to target mACs.

There are some similarities between ACs and PDEs as drug targets, covered in *Pharmacological Reviews* a number of years ago (Bender and Beavo, 2006). ACs and PDEs exist in multiple isoforms that have different biochemical properties, functions, and tissue distribution (Conti and Beavo, 2007). For PDE4 and PDE5 inhibitors, important clinical applications have already been established (Francis and Corbin, 2005; Garnock-Jones, 2015), and a number of novel therapeutic applications of selective PDE inhibitors are emerging (Heckman et al., 2015; Lee et al., 2015). Given the fact that any given AC and PDE possess a unique tissue distribution, one could envisage unique therapeutic applications of isoform-selective AC activators and inhibitors.

The role of defined AC isoforms in human diseases is just beginning to emerge. Proof-of-principle for a pathophysiological role of an AC isoform was recently provided by linking gain-of-function mutations in *ADCY5* to a extrapyramidal movement disorders (Chen et al., 2012c, 2014b, 2015b). These data also raise the question whether development of mutant-specific AC isoform inhibitors is useful. The development of mutant-specific protein kinase inhibitors has been highly successful for targeted cancer therapy (Dhillon, 2016).

The recent advances in our knowledge on the pathophysiological roles of AC isoforms call for more studies on links between *ADCY* gene polymorphisms and human diseases. In addition, upregulation or downregulation in the expression and function of wild-type AC isoforms in human diseases must be studied in greater detail. At the mRNA/RT-PCR level, such studies are possible, but at the protein level, the paucity of specific antibodies constitutes a bottleneck. Once a role of AC isoforms in human diseases is better defined, we hope that the pharmaceutical industry is willing to take the risk for such AC inhibitor and activator development programs that, in principle, are feasible.

Acknowledgments

The authors thank Drs. Erich Schneider (Hannover Medical School) and Jochen Schultz (University of Tübingen) for critical reading of the manuscript and Annette Stanke for careful compilation of the reference list and formal edition of the manuscript. A special thanks to the external reviewers for thoughtful comments and suggestions.

Authorship Contributions

Wrote or contributed to the writing of the manuscript: Dessauer, Watts, Ostrom, Conti, Dove, and Seifert.

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