CHAPTER SEVENTEEN

Molecular, Enzymatic, and Cellular Characterization of Soluble Adenylyl Cyclase From Aquatic Animals

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Contents

Abstract

The enzyme soluble adenylyl cyclase (sAC) is the most recently identified source of the messenger molecule cyclic adenosine monophosphate. sAC is evolutionarily conserved from cyanobacteria to human, is directly stimulated by HCO_3^- ions, and can act as a sensor of environmental and metabolic CO_2, pH, and HCO_3^- levels. sAC genes tend to have multiple alternative promoters, undergo extensive alternative splicing, be translated into low mRNA levels, and the numerous sAC protein isoforms may be present

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in various subcellular localizations. In aquatic organisms, sAC has been shown to mediate various functions including intracellular pH regulation in coral, blood acid/base regulation in shark, heart beat rate in hagfish, and NaCl absorption in fish intestine. Furthermore, sAC is present in multiple other species and tissues, and sAC protein and enzymatic activity have been reported in the cytoplasm, the nucleus, and other subcellular compartments, suggesting even more diverse physiological roles. Although the methods and experimental tools used to study sAC are conventional, the complexity of sAC genes and proteins requires special considerations that are discussed in this chapter.

ABBREVIATIONS

A/B	acid/base
BSA	bovine serum albumin
C1	catalytic domain 1
C2	catalytic domain 2
cAMP	3′,5′-cyclic adenosine monophosphate
CAs	carbonic anhydrases
cDNA	complementary deoxyribonucleic acid
dCEs	derivatives of catechol estrogens
DDA	2′, 5′-dideoxyadenosine
DTT	dithiothreitol
EC_{50}	half maximal effective concentration
ELISA	enzyme-linked immunosorbent assay
EST	expressed sequence tag
Fsk	forskolin
FW	forward primer
GM-130	Golgi matrix protein 130
GPCR	G protein-coupled receptor
GSPs	gene-specific primers
GTPγS	guanosine 5′-*O*-[gamma-thio]triphosphate
IBMX	3-isobutyl-1-methylxanthine
IC_{50}	half maximal inhibitory concentration
IPTG	Isopropyl beta-D-thiogalactopyranoside
KH7	(*E*)-2-(1*H*-benzo[*d*]imidazol-2-ylthio)-*N*′-(5-bromo-2-hydroxybenzylidene)propanehydrazide
LRE1	6-chloro-*N*4-cyclopropyl-*N*4-(2-thienylmethyl)-2,4-pyrimidinediamine, RU-0204277
PBS	phosphate-buffered saline
PDEs	phosphodiesterases
PMSF	phenylmethylsulfonyl fluoride
PVDF	polyvinylidene difluoride
qPCR	quantitative/real-time PCR
RACE	rapid amplification of cDNA ends
RASL-Seq	RNA-mediated oligonucleotide annealing, selection, and ligation with next-generation sequencing

RNA-seq	RNA sequencing
RT	reverse transcriptase
RV	reverse primer
sAC	soluble adenylyl cyclase
sAC_{FL}	full-length soluble adenylyl cyclase
sAC_t	truncated soluble adenylyl cyclase
SDS/PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBS-T	tris-buffered saline and polyethylene glycol sorbitan monolaurate
tmACs	transmembrane adenylyl cyclases
TPR	tetratricopeptide
Tris	tris(hydroxymethyl)aminomethane
UTR	untranslated region

1. INTRODUCTION

The soluble adenylyl cyclase (sAC, *adcy10*) is a Class III adenylyl cyclase that is evolutionarily conserved from cyanobacteria to human (Buck, Sinclair, Schapal, Cann, & Levin, 1999; Chen et al., 2000). It catalyzes the cyclization of adenosine triphosphate (ATP) into 3′,5′-cyclic adenosine monophosphate (cAMP), the ubiquitous messenger molecule that regulates virtually every aspect of physiology by inducing posttranslational modifications on target proteins. A unique characteristic of sAC over other adenylyl cyclases is that its activity is stimulated by HCO_3^- (Buck et al., 1999; Chen et al., 2000; Litvin, Kamenetsky, Zarifyan, Buck, & Levin, 2003; Tresguerres, Parks, et al., 2010). Furthermore, because HCO_3^- is typically in equilibrium with CO_2 and H^+, sAC can functionally associate with carbonic anhydrases (CAs) to also sense CO_2 and H^+ (reviewed in Tresguerres, Levin, & Buck, 2011).

Another fascinating aspect of sAC is its complexity both at the gene and protein levels. Mammalian sAC genes have multiple alternative promoters and undergo extensive alternative splicing (Chen et al., 2014; Farrell et al., 2008; Geng et al., 2005); both characteristics are also seen in coral (Barott, Barron, & Tresguerres, 2017), suggesting they are common to sACs from all animals. The two better characterized mammalian sAC variants are truncated sAC (sAC_t), an ~50 kDa protein containing the two catalytic domains essential for cAMP producing activity, and full-length sAC (sAC_{FL}), which additionally contains an ~140 kDa C-terminus region with yet unidentified functions. Putative regulatory domains in sAC's C-terminus include P-loop, leucine zipper, and tetratricopeptide (TPR) domains (Buck et al., 1999; Steegborn, 2014).

sAC_t and sAC_{FL} have the same half maximal effective concentration (EC_{50}) for HCO_3^- and half maximal inhibitory concentration (IC_{50}) for inhibitors; however, V_{max} is ~20-fold lower for sAC_{FL} as a result of an auto-inhibitory region that is absent in sAC_t (Chaloupka, Bullock, Iourgenko, Levin, & Buck, 2006). Other reported mammalian sAC variants include ~30, ~45, ~70, ~80, and ~130 kDa proteins (Chen et al., 2014; Geng et al., 2005; Stessin et al., 2006). In addition to potential different regulatory properties, the sAC variants may have distinct subcellular localizations with specific physiological roles. Indeed, sAC has been reported in the cytoplasm, in the nucleus, inside mitochondria, and associated with various other intracellular structures (Acin-Perez et al., 2009; Zippin et al., 2004; Zippin, Levin, & Buck, 2001). Those multiple localizations are consistent with the concept of intracellular cAMP signaling microdomains (Cooper, 2003; Schwencke et al., 1999; Zaccolo & Pozzan, 2002).

sAC from aquatic organisms also has alternative promoters and multiple splice variants (Barott et al., 2017; Tresguerres, Barott, Barron, & Roa, 2014), and has been reported in the cytoplasm and nuclei of fish cells (Roa & Tresguerres, 2017). However, sACs from aquatic organisms have a few differences from mammals. In shark and ray, the most abundant sAC protein is ~110 kDa (Roa & Tresguerres, 2016, 2017; Tresguerres, Parks, et al., 2010), and in coral it is ~94 kDa (Barott, Venn, Perez, Tambutté, & Tresguerres, 2015). Both sACs contain the two catalytic and the P-loop domains; the additional 16 kDa in shark sAC does not have any identifiable functional domains. The EC_{50} for HCO_3^- also differs among animals: it is ~10 m*M* for coral sAC (Barott et al., 2013), ~5 m*M* for shark sAC (Tresguerres, Parks, et al., 2010), and ~20 m*M* for mammals (Buck et al., 1999; Chen et al., 2000; Litvin et al., 2003) and hagfish (Wilson, Roa, Cox, Tresguerres, & Farrell, 2016). The species-specific EC_{50} matches the normal [HCO_3^-] in fluids of the respective animal, which makes sAC a suitable physiological acid/base (A/B) sensor (reviewed in Tresguerres, 2014).

The interest on sAC from aquatic organisms is severalfold: (1) to study the evolution of A/B sensing by identifying amino acid motifs that confer the species-specific EC_{50} for HCO_3^-; (2) to study the evolution of cAMP signaling microdomains; (3) to understand and predict physiological responses to A/B disturbances related to aquaculture and environmental stress such as acidification, warming, and feeding; and (4) as models for biomedicine, taking advantage of the more pronounced A/B disturbances, higher sAC

mRNA, and other technical advantages found in aquatic animals compared to mammals (reviewed in Tresguerres, 2014; Tresguerres et al., 2014).

This chapter outlines some strategies to study sAC at the gene, enzyme, protein, and cellular levels (summarized in Table 1). Specifically, it highlights important considerations regarding gene cloning, production of recombinant protein, measuring cAMP production in tissue homogenates and purified protein, identifying sAC protein variants, and determining their intracellular localizations. Although the main focus is on aquatic organisms, the techniques and advice presented here likely apply to other organisms.

2. GENE

sAC genes are complex and unusual (for example, some introns can be >3500 bp), have multiple alternative promoters, undergo multiple alternative splicing, and are typically transcribed at low levels. As a result, characterizing sAC genes and mRNAs is challenging. Although large-scale -omics techniques are becoming increasingly popular and cheaper, the complexity of sAC does not mesh well with bioinformatics analyses based on genomes and large-scale transcriptomic studies. This is especially true for many aquatic organisms that do not have sequenced and annotated genomes, or have transcriptomes with moderate coverage and annotation quality. In our experience, predicted sAC nucleotides sequences rarely match the actual sequences elucidated by cloning. Our success characterizing sAC at the nucleotide level has been directly proportional to the quality of genomic information available in the species of question: we have cloned one mRNA encoding for shark (*Squalus acanthias*) sAC from an expressed sequence tag (EST) database (Tresguerres, Parks, et al., 2010), five mRNAs coding for coral (*Pocillopora damicornis*) sACs using transcriptomic databases as reference for primer design (Barott et al., 2017), and >20 mRNAs encoding rainbow trout (*Oncorhynchus mykiss*) sACs (C. Salmerón and M. Tresguerres, unpublished), for which excellent quality genome and transcriptomes are available (Berthelot et al., 2014; Salem, Rexroad, Wang, Thorgaard, & Yao, 2010). However, even for human, trout, and mice (Chen et al., 2014; Farrell et al., 2008; Geng et al., 2005) it is necessary to empirically confirm putative transcripts using targeted reverse transcriptase (RT) and rapid amplification of cDNA ends (RACE) PCRs. Some helpful considerations are listed below.

Table 1 Summary of Goals, Challenges, and Strategies Associated With Studying sAC at the Gene, Enzyme, Protein, and Cellular Levels

1. Introduction			
Soluble adenylyl cyclase (sAC) (*adcy10*) is stimulated by HCO_3^- to produce cAMP and is an evolutionarily conserved acid/base sensor. Because sAC is complex at the gene, mRNA, and protein level, common laboratory techniques usually require extensive optimization			
Level	Goals	Challenges	Tools and Approaches
2. Gene	Characterize sAC genes and mRNA splice variants. Identify motifs responsible for differential sensitivity to HCO_3^- and pharmacological inhibitors. Design stimulators based on structural studies. Identify potential regulatory domains	Extensive alternative splicing and multiple promoters. Low mRNA abundance. Unusually long introns	Well-annotated genome and high coverage transcriptomes. Cloning from tissues with high sAC mRNA abundance. Primers specific for splice variants. Multiple rounds of PCR, nested PCR, RACE-PCR
3. Enzymatic activity	Characterize sAC kinetics and detect sAC in specific tissues/cells. Species-specific and evolutionary studies on acid/base sensing	Protein production and purification. Finding appropriate cofactors and assay conditions. tmACs and PDEs as confounding factors. Measuring cAMP production	sAC sources: purified recombinant protein, immunoprecipitation, tissue homogenates, cells. Extensive enzyme assay optimization. Use of sAC, tmAC, and PDE-specific pharmacological inhibitors. cAMP detection: two-column assay, ELISA, mass spectrometry
4. Protein	Identify sAC and sAC variants in specific tissues, cells and subcellular compartments	Multiple isoforms. Limited availability of markers of subcellular compartments in nonmodel species	Generic and isoform-specific antibodies for Western blot and immunolocalization studies. Expression of fluorescently tagged sAC in cell systems
5. Cellular studies	Characterize sAC's roles in cells and cAMP signaling microdomains	Distinguish between sAC and tmACs, and interactions with PDEs. Lack of robust gene downregulation techniques in nonmodel species. Compensation of function under sAC inhibition	Gather information about sAC at the nucleotide, enzymatic, and protein levels as described in the previous rows. Design-specific cell, tissue, and whole animal experiments to test sAC roles under acid/base relevant conditions; look for responses sensitive to sAC genetic and/or pharmacological knockdown. Using two pharmacological inhibitors is recommended. Use care interpreting results using PDE and tmAC agonists and antagonists as well as cAMP analogs

2.1 RNA Isolation and cDNA Synthesis

a. For cloning sAC mRNAs from animals we recommend isolating RNA from mature testis because it typically contains the highest mRNA abundance among all tissues. As a trade-off, testis contains multiple somatic, germ, and sex cells in which alternative splicing is especially prevalent (Elliott & Grellscheid, 2006; Yeo, Holste, Kreiman, & Burge, 2004), which can complicate sequencing and analyses.

b. We have obtained best results with fresh samples and stored in RNAlater or equivalent. Samples snap-frozen in liquid N_2 immediately after dissection also are acceptable (but RNAlater is preferred).

c. Due to the low expression of sAC transcripts, mRNA purification is essential and therefore it is important to isolate as much total RNA as possible. A ratio of 100 mg of tissue for 1 mL of TRIzol Reagent or equivalent could be used as a reference during isolation optimization.

d. We recommend precipitating RNA with isopropyl alcohol at −80°C overnight, and doing two washes with 75% ethanol before drying the pellet.

e. After total RNA isolation, it is essential to quantify each sample using an absorbance- or fluorescence-based nucleic acid quantification method. Also assess the integrity of total RNA by running a total RNA sample (e.g., 200 ng to 1 μg) in an agarose gel or equivalent method. If the 28S and 18S ribosomal RNA bands are not prominent and sharp, do not proceed.

f. We successfully cloned sAC from a variety of organisms ranging from coral to trout using Poly(A) RNA purification MAG kit (Ambion™) (Barott et al., 2017; Tresguerres, Parks, et al., 2010; Salmerón and Tresguerres, unpublished). As reference, we recommend using at least 90 ng of purified mRNA from trout mature testes for making cDNA.

g. We tested different polymerases (e.g., Platinum Taq DNA Polymerase (Invitrogen™), Phusion High-Fidelity PCR Master Mix (Thermo Scientific™)), and observed higher amplification rate and success with high-fidelity polymerases.

h. Many invertebrate animals do not possess prominent testes as in vertebrates; however, in our experience they tend to have generally higher sAC mRNA levels so whole animal (e.g., coral) or various nongonadal tissues (e.g., mollusks) are usually acceptable sources. For cloning sAC from coral, an ~2 cm fragment yields enough RNA. The coral skeleton must be crushed using a chilled mortar and pestle into a fine powder, which can then be homogenized in TRIzol Reagent.

2.2 Cloning

sAC multiple promoters, extensive alternative splicing, and low mRNA abundance are problematic for cloning experiments. In some cases, confirming the presence of mRNA coding for the catalytic domains of sAC might suffice. However, cloning full-length cDNAs encoding sAC splice variants and identifying and quantifying expression of cDNAs coding for specific sAC splice variants requires extensive optimization and almost every trick in the book.

To clone full-length sAC cDNAs, we recommend to first search for sAC cDNA sequences for the target species in genomic, transcriptomic, and protein databases using already cloned sAC sequences from the same or a related species as the query. For fish, we recommend using sAC from dogfish shark (*S. acanthias*) (ACA52542) (Tresguerres, Parks, et al., 2010) and sACs from rainbow trout (*O. mykiss*) (MF034907–MF03490727, MF670431, and MF511189); sAC from *P. damicornis* (KX910691, KY853034, KY853037, KY853039, KY853041) (Barott et al., 2017) and *Acropora yongei* (MG269969–MG269972) might be used for coral. However, in our experience predicted complete cDNA sequences based on bioinformatics analyses are not trustworthy, most likely due to "glitches" resulting from the presence of multiple splice variants. Thus, if the aim is to identify full-length sAC cDNAs, it is essential to clone and sequence them using traditional approaches. Results from the searches will follow into one of the three following categories:

a. *sAC cDNA sequences containing 5′ and 3′ gene untranslated regions (UTRs):* These are particularly helpful for cloning sACs because they often are part of intronic regions and therefore helpful for designing mRNA splice variant-specific primers (Fig. 1). We have had better success by synthetizing the cDNA with SuperScript III First-Strand Synthesis System (Thermo Scientific™) with reverse gene-specific primers (GSPs) in the 3′ UTR region of sAC cDNA, instead of Oligo(dT) primers. Another strategy to improve sAC mRNA detection is by doing a second PCR using the product of the first PCR as template (at different dilutions) and nested primers. PCR products using sAC primers on UTRs typically produce multiple bands in agarose gel electrophoresis (e.g., Fig. 1B, lane 2). Rather than nonspecific products, those potentially are different sAC cDNAs so we recommend cutting, purifying, and sequencing each band.

b. *sAC cDNA sequences not containing UTRs:* In this case 5′ and 3′ RACE-PCR is necessary (e.g., using 5′ and 3′ RACE System for Rapid

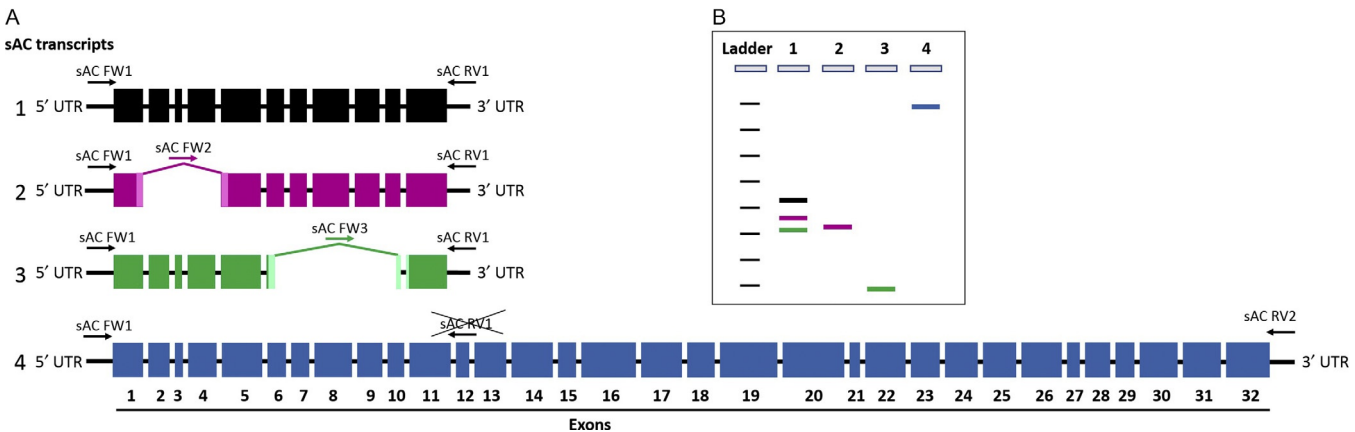

Fig. 1 Identification of four sAC splice variants by PCR. (A) Exons and introns are represented by *boxes and lines*, respectively. Primers used for PCR are indicated above each sAC transcript. sAC forward primers (sAC FW): sAC FW1 is common to all sAC transcripts, and sAC FW2 and sAC FW3 bind within the exon–exon junction of the splicing event of sAC transcripts 2 and 3, respectively. sAC reverse primers (sAC RV): sAC RV1 binds to sAC variants 1–3 (but not 4), and sAC RV2 is exclusive for sAC variant 4. (B) Electrophoresis simulation of PCRs using different primer sets for sAC (DNA bands and sAC variants are color coded). *Lane 1*: sAC FW1 and sAC RV1 yield bands of different sizes corresponding to sAC variants 1–3 (*black*, *magenta*, and *green*, respectively). *Lane 2*: sAC FW2 and sAC RV1 only amplify sAC variant 2 (*magenta*). *Lane 3*: sAC FW3 and sAC RV1 only amplify sAC variant 3 (*green*). *Lane 4*: sAC FW1 and sAC RV2 exclusively amplify sAC variant 4 (*blue*).

Amplification of cDNA Ends (Thermo Scientific™)). The required GSPs should be designed against regions close to the putative UTRs.

c. *No results:* PCRs using degenerated primers for well-conserved nucleotide regions based in alignments of cloned sAC sequences from different species are an option. However, we never had success using this approach, again possibly due to the combination of low sAC mRNA abundance and multiple splice variants.

2.3 mRNA Quantification

If full-length cDNAs for various sAC splice variants are known, the transcriptional expression of each of them can be quantified by regular or quantitative/real-time PCR (qPCR) using the following different strategies (more details in Camacho Londoño & Philipp, 2016; Leparc & Mitra, 2007):

a. Primers spanning exon–exon junctions (Fig. 1).

b. Flanking PCR (primers for constitutive exons flanking a spliced region).

c. Seminested PCR. Using three primers: (1) an "external" forward primer #1, localized 5′ upstream of a canonical exon, (2) a reverse primer #2, localized within an exon–exon junction of the splicing event, and (3) a second "internal" forward primer in an exonic region between primers #1 and #2. An initial PCR uses primers #1 and #2, and a second PCR uses a 1:100 dilution of the first PCR as template, and primers #2 and #3.

d. The abundance of splice variants can be quantified based on the relative abundance of qPCR products obtained with variant-specific and common primers.

In this "-omics" era, it is tempting to use RNA sequencing (RNA-seq) both for identifying sAC splice variants and for quantifying their expression. However, this method is not recommended for genes with low mRNA abundance such as sAC; quantification using variations such as targeted RNA-Seq and RASL-Seq requires prior knowledge of the sequences of interests (reviewed in Hrdlickova, Toloue, & Tian, 2017).

3. ENZYMATIC ACTIVITY

Biochemical characterization of purified sAC has so far been done for sAC from human (Geng et al., 2005; Jaiswal & Conti, 2003; Litvin et al., 2003), rat (Buck et al., 1999; Chaloupka et al., 2006; Chen et al., 2000; Jaiswal & Conti, 2003), shark (Tresguerres, Parks, et al., 2010), cyanobacteria (Chen et al., 2000), and *Chloroflexus* bacteria (Kobayashi, Buck, & Levin, 2004). sAC activity has also been characterized in semipurified sAC from

mouse (Farrell et al., 2008) and sea urchin sAC (Nomura, Beltrán, Darszon, & Vacquier, 2005), as well as in tissue homogenates from shark (Roa & Tresguerres, 2017; Tresguerres, Parks, et al., 2010), ray (Roa & Tresguerres, 2016), hagfish (Wilson et al., 2016), coral (Barott et al., 2013), and diatom (Tresguerres et al., 2014). Common biochemical characteristics include the requirement of ATP, Mg^{2+}, and another cation (Ca^{2+} or Mn^{2+}) to sustain HCO_3^- stimulation. Furthermore, all sACs are strongly stimulated by millimolar Mn^{2+} concentrations, the property that originally suggested the existence of distinct cAMP producing enzymes "soluble" in the cell cytoplasm (Braun, 1991; Braun & Dods, 1975; Braun, Frank, Dods, & Sepsenwol, 1977; Gordeladze & Hansson, 1981; Neer, 1978; Neer & Murad, 1979). Although it is not clear whether Mn^{2+} is physiologically relevant, robust Mn^{2+}-stimulated cAMP production remains useful as initial biochemical evidence about the presence of sAC in a sample. Other important differences between sAC and the classic hormone and G protein-coupled receptor (GPCR)-regulated transmembrane adenylyl cyclases (tmACs) from animals include sAC's lower affinity for ATP (which may be related to a role in sensing ATP levels; Zippin et al., 2013), and its insensitivity to tmAC pharmacological agonists such as forskolin (Fsk) and GTPγS (Buck et al., 1999; Chen et al., 2000).

There are three well-characterized cell permeable sAC inhibitors with different degrees of specificity and associated side effects in different types of assays. Derivatives of catechol estrogens (dCEs) such as 2- and 4-hydroxyestradiol inhibit purified sAC with $IC_{50} \sim 2–50\,\mu M$ (Bitterman, Ramos-Espiritu, Diaz, Levin, & Buck, 2013; Steegborn et al., 2005; Tresguerres, Parks, et al., 2010), but research on purified protein raised concerns they could also inhibit tmACs at similar concentrations (Steegborn et al., 2005). However, subsequent research on cells determined the IC_{50} of dCEs for sAC is $\sim 100\,\mu M$, and that it does not affect cAMP production by tmACs (Bitterman et al., 2013) so dCEs are a valid option for in vivo research. The small molecule KH7 inhibits sAC with higher affinity than dCEs both in purified protein ($IC_{50} \sim 3–10\,\mu M$) (Bitterman et al., 2013; Ramos-Espiritu et al., 2016; Tresguerres, Parks, et al., 2010) and cell assays ($IC_{50} \sim 25\,\mu M$) (Bitterman et al., 2013). Furthermore, KH7 does not affect tmAC activity in vitro or in vivo (Bitterman et al., 2013). However, under certain experimental conditions KH7 may affect mammalian mitochondrial function in unspecific manner (De Rasmo et al., 2016; Di Benedetto, Scalzotto, Mongillo, & Pozzan, 2013) (although not in coral; Barott et al., 2017). The most novel sAC-specific small molecule inhibitor is LRE1, which has similar low IC_{50} of $\sim 10\,\mu M$ both in vitro

and in vivo and does not seem to have unspecific effects on mitochondria (Ramos-Espiritu et al., 2016). KH7 and dCEs have been shown to inhibit sAC from mammals (Hess et al., 2005), fish (Roa & Tresguerres, 2016; Tresguerres, Parks, et al., 2010), sea urchin (Beltrán et al., 2007), and coral (Barott et al., 2013) with similar IC_{50}; to our knowledge LRE1 has only been tested on mammals so far.

3.1 Recombinant Protein

Enzymatic assays on recombinant sAC protein are used to characterize enzyme kinetics, responsiveness to HCO_3^-, the efficacy of known inhibitors, and to screen for novel stimulators and inhibitors. Production and purification of recombinant sAC proteins can be done using a number of standard methods (Structural Genomics Consortium et al., 2008). Some considerations to produce, purify, and determine sAC enzymatic activity include:

a. Despite its name, only the shorter sAC variants that only include one or both catalytic domains are "soluble" proteins. The larger sAC variants are typically found in the "particulate" fraction, likely due to their association with multiple other proteins. For example, in sea urchin sperm sAC coimmunoprecipitates with >10 proteins of the plasma membrane and axoneme (Nomura & Vacquier, 2006).

b. sAC responses to HCO_3^-, metals, and pharmacological inhibitors are largely determined by the two catalytic domains (Chaloupka et al., 2006; Litvin et al., 2003). Thus, kinetic parameters such as EC_{50} and IC_{50} can be studied using sAC variants that lack the long C-terminus region, which are easier to produce in bacteria and have more robust activity. However, regulatory aspects of the P-loop, leucine zipper, TPR, and other domains must be studied on the longer sAC proteins, which should be produced in eukaryotic expression systems such as yeast, insect, or mammalian.

c. Because cAMP is a universal signaling molecule, sAC recombinant proteins can have toxic effects on the expression system, impair growth, or result in production of inclusion bodies. Some factors that help mitigate those harmful effects on bacteria include culturing at low temperature (10–20°C) and carefully regulating gene expression, for example, by optimizing the amount of arabinose or IPTG.

3.2 Tissue Homogenates and Cellular Fractions

sAC enzyme activity assays on homogenates and cell fractions can be used to confirm if sAC is present in a given tissue or subcellular compartment; they

are particularly useful when the sAC cDNA sequence of the organism is not known or mRNA levels are too low for RT-PCR, and when no antibodies are available to survey for sAC protein. In some cases, tissue homogenates can additionally be used as surrogates for recombinant protein to provide an initial characterization of sAC enzyme kinetics (Barott et al., 2013; Tresguerres, Parks, et al., 2010; Wilson et al., 2016).

After dissection from the animal, samples must be immediately homogenized and assayed, or flash frozen in liquid N_2 and stored at −80°C. Tissue homogenization may be done using a variety of methods (Goldberg, 2008). We prefer pulverizing the tissue in liquid N_2 using pestle and mortar, followed by suspension in homogenization buffer (250 m*M* sucrose, 100 m*M* Tris pH 7.5, and 100 μg/mL PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin). Ratio of sample to buffer should be between 1:5 and 1:10 weight (mg) to volume (μL). The mix is then further homogenized by sonication (2 ×, 15 s each, on ice) (Tresguerres, Parks, et al., 2010) or in a Dounce homogenizer (Wilson et al., 2016). After pelleting down large debris (500 × *g*, 10 min, 4°C), the supernatant is saved (crude homogenate) and can be used in sAC activity assays or processed further for cell fractionation using standard methods (e.g., see Roa & Tresguerres, 2017 for isolation of nuclei). For coral, we found it sufficient to remove and homogenize the tissue from the skeleton using an artist's air airbrush or by scraping with a toothbrush into homogenization buffer (Barott et al., 2013).

3.3 cAMP Activity Assay

The activity assay is based on the production of cAMP from ATP in the presence of appropriate cofactors. The basic assay buffer contains 150 m*M* NaCl, 100 m*M* Tris pH 7.5, 1 m*M* dithiothreitol (DTT), 5 m*M* ATP, and 5 m*M* Mg^{2+} or Mn^{2+}. This amount of Mn^{2+} induces maximum sAC stimulation, which is typically >10-fold higher compared to Mg^{2+}-sustained activity. However, those high Mn^{2+} levels are not physiological, and do not sustain HCO_3^- stimulation (probably because sAC is already maximally stimulated).

The physiological conditions that sustain HCO_3^- stimulation in vivo vary from species to species. For example, mammalian sACs require millimolar concentrations of Mg^{2+} and Ca^{2+} and similar ATP levels (Litvin et al., 2003). However, shark sAC requires about 10-fold higher Mg^{2+} concentration than ATP (20 and 2.5 m*M*, respectively) and must be supplemented with 0.5 m*M* Mn^{2+} (Tresguerres, Parks, et al., 2010). For sAC from new species, we recommend trying different concentrations and combinations of Mg^{2+}, Ca^{2+}, and Mn^{2+}.

The kinetics of HCO_3^- stimulation must be done over a range that is physiologically relevant to the species in question. Most water-breathing animals experience lower HCO_3^- levels compared to air breathers; we advise testing the following HCO_3^- concentrations: 0, 1, 2.5, 5, 7.5, 10, 15, 20, 40 m*M*. This may be followed by more detailed studies around the EC_{50}. The use of 100 m*M* Tris ensures those HCO_3^- concentrations do not have a major effect on pH. However, a pH dose–response curve over the range 7–9 is advisable (this could be done by combining appropriate amounts of 100 m*M* Tris–HCl and Tris–base).

Inhibitors such as dCE, KH7, LRE1, and 3-isobutyl-1-methylxanthine (IBMX) are usually dissolved in DMSO. The concentration of DMSO must be identical in every reaction, and in no case it should exceed 2%.

Tissue homogenates contain sAC and its native cofactors, but also phosphodiesterases (PDEs) and ATPases, which degrade cAMP and hydrolyze ATP, respectively. Addition of 500 μ*M* IBMX into the assay buffer inhibits PDEs, while 20 m*M* creatine phosphate and 100 U/mL creatine phosphokinase regenerate ATP thus maintaining constant levels throughout the assay (the effect of cAMP production on ATP levels is negligible). Tissue homogenates also contain tmACs, which produce cAMP and can introduce noise and significantly contribute to background cAMP levels. sAC activity can be differentiated from tmAC's using specific inhibitors for sAC (dCE, KH7, LRE1) and tmACs (e.g., 2′,5′-dideoxyadenosine (DDA)) (Roa & Tresguerres, 2016, 2017).

3.4 cAMP Quantification

Production of cAMP can be quantified using several methods, which vary in time involvement, cost, accuracy, precision, sensitivity, and specificity. The most specific detection method is the two-column adenylyl cyclase assay which requires radiolabeled [α-^{32}P]ATP and [^{3}H]cAMP (Salomon, 1979), or [^{3}H]adenine if used to measure cAMP accumulation in cells (Levin & Reed, 1995). The two-column assay is preferred for characterizing adenylyl cyclase kinetics because of its specificity, accuracy, and precision; this method was used for mammalian sAC (Chen et al., 2000; Litvin et al., 2003) and also for confirming the exceptionally high sAC activity in coral tissues (Barott et al., 2013). Some disadvantages are the need for radiolabeled reagents (with their associated hazards and detection equipment), its low sensitivity that requires samples with high cAMP producing activity, and being labor intensive and time consuming.

The most versatile cAMP detection method is enzyme-linked immunosorbent assay (ELISA) based. It can be used for characterizing activity of purified protein, homogenates, and cells, as well as for measuring cAMP concentration in tissues. In addition to versatility, its advantages include relative short time and simplicity, and high sensitivity. An "acetylated" format increases sensitivity and specificity; however, not even that format is as accurate, precise, or specific as the two-column assay. These issues can be reduced by increasing the number of replicates, ensuring the readouts are in the linear portion of the standard curve, and diluting samples to avoid interference of divalent metals and ATP with binding. Additionally, cAMP standards can be dissolved in the presence of equivalent concentrations of metals and ATP resulting in multiple standard curves each specific for each condition. When used correctly, ELISA detection of cAMP is a very powerful method that has been used to characterize and confirm sAC activity in recombinant protein (Tresguerres, Parks, et al., 2010), immunoprecipitated protein (Nomura et al., 2005), and tissue homogenates and cell fractions (Barott et al., 2013; Roa & Tresguerres, 2016, 2017; Tresguerres, Parks, et al., 2010), as well as to measure cAMP levels in coral throughout the day/night cycle (Barott et al., 2013).

The newest cAMP detection method combines high-capacity sample analyses with mass spectrometry (Ramos-Espiritu et al., 2016). In addition to high-throughput screening, it has high specificity for cAMP, it can be used to simultaneously measure cAMP and ATP levels, and has a large dynamic range. However, its disadvantages include inferior sensitivity compared to the two-column assay, a requirement for large amounts of purified sAC protein, and not being useful for cell accumulation assays. In addition, this method requires expensive and complex equipment, which effectively restricts its use to highly specialized and well-funded medical and biotechnology research.

4. PROTEIN

The extensive alternative splicing described in Section 2 results in multiple protein isoforms. While fascinating from evolutionary and physiological perspectives, this adds an additional layer of complexity for studying sAC at the protein level. One of those issues is generating and validating specific antibodies, because most antigenic regions will be shared by multiple protein variants (Fig. 2); this situation is analogous to primer design for PCR (Fig. 1). Furthermore, anti-sAC antibodies are likely to produce multiple

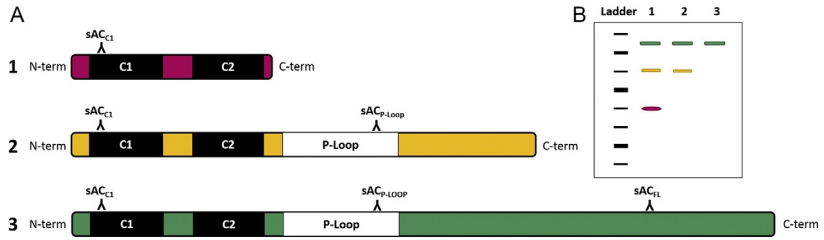

Fig. 2 Antibody design and detection of three sAC protein isoforms by Western blot. (A) C1 and C2, catalytic domains 1 and 2; P-Loop, P-loop domain. Antibodies against rtsAC's are indicated by an inverted Y. Anti-sAC_{C1} antibody detects all sAC isoforms. Anti-$sAC_{P\text{-}Loop}$ antibody detects sAC isoforms containing the P-loop domain. Anti-sAC_{FL} antibody only detects sAC full-length. (B) PAGE–Western blot simulation using the three anti-sAC antibodies (protein bands and sAC isoforms are color coded). *Lane 1*: anti-sAC_{C1} antibody yields bands of three different sizes corresponding to sAC isoforms 1–3 (*purple*, *yellow*, and *turquoise*, respectively). *Lane 2*: anti-$sAC_{P\text{-}Loop}$ antibody yields bands of two different sizes corresponding to sAC isoforms 2 and 3 (*yellow* and *turquoise*, respectively). *Lane 3*: anti-sAC_{FL} exclusively detects sAC variant 3 (*turquoise*).

bands in Western blots that, even if demonstrated to disappear by peptide preabsorption, often cast doubts about their specificity. Similarly, immunostaining will label multiple variants throughout the cell (potentially in various subcellular compartments) or it may differentially label sAC variants that provide better antigen access to the antibodies (due to folding and microenvironment conditions such as number and type of proteins associated to sAC).

Pan-specific anti-sAC antibodies against peptides in the catalytic domains are suitable tools for many research goals; however, the potential detection of multiple sAC variants must be considered (Fig. 2). To tease apart different variants, our approach is to generate different antibodies against different parts of the protein. To increase the chances of antibodies to work both in Western blots and immunohistochemistry, we recommend choosing antigenic peptides that are exposed at the surface of the protein, hydrophobic, with high disorder value (a measure of how similar the linear peptide is compared to its natural conformation in the protein), and positioned near the C- or N-terminus. In addition, the peptide needs to induce a string immune response (which is in part determined by its dissimilarity to proteins from the host animal where the antibodies are produced). Considering those restrictions, it clearly is not possible to generate antibodies against every part of the protein, and thus designing antibodies against every sAC isoform is unfeasible. Our strategy has been to generate antibodies against three distinctive sAC regions: catalytic domain one or two, the P-loop, and near the N-terminus of the full-length sAC protein (Fig. 2). Combining results from

the three antibodies should allow deducing which sAC variants are present in a certain sample, and where within a cell. For example, antibodies against the catalytic domains will detect all bands in Western blot and label all intracellular localizations where sAC is present; antibodies against the N-terminus will only detect the larger molecular weight bands in Western blots and label those intracellular locations where sAC_{FL} is present (but not sAC_t or equivalents), and so on with other antibodies.

Once antibodies are generated and properly validated (Bordeaux et al., 2010), they are among the most powerful tools for studying sAC presence in specific tissues (Roa & Tresguerres, 2016, 2017; Tresguerres, Parks, et al., 2010), cell types (Barott et al., 2017; Roa & Tresguerres, 2016, 2017), and subcellular compartments (Roa & Tresguerres, 2017). In biomedicine, specific anti-sAC antibodies are even used as diagnostic tools in dermatopathology (Magro, Crowson, Desman, & Zippin, 2012; Zippin, Chadwick, Levin, Buck, & Magro, 2010). Our standard protocols for immunodetection of sAC from coral, shark, and bony fish are listed below. These conditions can be used in initial studies for other species, but keep in mind each new species may require additional optimization.

4.1 Western Blotting (Optimized for Various Coral and Fish Tissues)

a. Obtain a crude homogenate as described in Section 3.2.
b. Measure protein concentration using the Bradford assay or similar.
c. Combine the sample with an equal volume of 2× Laemmli buffer (with 5% β-mercaptoethanol, freshly added). Heating at 70°C for 15 min tends to give better results for larger sAC variants, while 95°C for 5 min is usually better for shorter sAC variants.
d. Separate 20 μg of total protein by SDS/PAGE (7%–10% polyacrylamide gel) and transfer onto a polyvinylidene difluoride (PVDF) membrane. To ensure transfer of large molecular weight sAC variants, we recommend overnight transfer at 4°C.
e. Block nonspecific binding sites with blocking buffer (Tris-buffered saline with 0.1% Tween 20 (TBS-T) with 5% fat-free milk (weight:volume), 1 h at room temperature.
f. Incubate with primary antibody diluted in blocking buffer, overnight at 4°C. We use our anti-sAC custom-made antibodies made in rabbit at the following concentrations: 0.006 μg/mL (coral), 3 μg/mL (shark), 0.6 μg/mL (trout). Wash with TBS-T 3×, 20 min.

g. Incubate with secondary antibodies diluted in blocking buffer, 1 h at room temperature. We use horseradish peroxidase-linked goat anti-rabbit antibodies (BioRad™) (1:10,000 dilution). Wash with TBS-T 3 ×, 20 min.

h. Visualize using method of choice. Unlike most proteins that typically yield a single band in Western blots, sAC is likely to produce multiple bands. Those bands should not be ruled out as "background noise," as they may be sAC splice variants.

i. Band specificity must be confirmed by peptide preabsorption control, which requires incubating the primary antibodies with excess antigen peptide (300 × on a molar basis) in blocking buffer, overnight at 4°C before proceeding to step f. Primary antibodies without peptide should be handled and applied to the same sample in parallel. We recommend loading two sets of increasing concentrations of total protein side by side in the same gel, cutting the PVDF membrane in half, and incubate one half (containing one set of lanes) with preabsorbed antibodies, and the other half (containing the other set) with antibodies without antigen peptide (see figure 1A in Roa, Munévar, & Tresguerres, 2014). Another control should omit the primary antibodies.

4.2 Immunocytochemistry (Optimized for Rainbow Trout Cell Line RT-W1 (ATCC CRL-2523))

a. Grow cells on glass bottom culture dishes coated with collagen until semiconfluence (14–18°C). Wash with sterile growth media without serum for 5 min.

b. (Optional for mitochondria labeling) Incubate in 200 n*M* MitoTracker (Invitrogen™) in sterile growth media without serum, 25 min at 18°C (color must be compatible with fluorescent secondary antibodies). Wash the cells with sterile phosphate buffer solution (PBS) at room temperature.

c. Fix in 3.7% paraformaldehyde in PBS, 10 min at room temperature. Wash with PBS.

d. Permeablize cells with 0.5% Triton X-100 in PBS, 5 min at room temperature. Wash with PBS, 2 ×, 30 s.

e. Block nonspecific-binding sites with blocking buffer (10 mg/mL bovine serum albumin in PBS), 1 h at room temperature.

f. Incubate with primary antibody diluted in blocking buffer, overnight at 4°C. We use our custom-made antibodies made in rabbit against rainbow trout sAC at concentrations between 1 and 3 μg/mL. Wash with PBS 3 ×, 5 min.

g. Incubate with secondary antibodies (e.g., Alexa fluorophore-conjugated goat antirabbit), diluted 1:500 in blocking buffer for 2 h at room temperature in the dark. For visualization of nuclei, Hoechst 33342 dye (Invitrogen™) can be added (1 μg/mL) in the mix. Wash with PBS 3 ×, 5 min. Visualize in fluorescence microscope.

h. Controls should include omission of primary antibody and peptide preabsorption control. The latter requires incubating the primary antibodies with excess antigen peptide (300 × on a molar basis) in blocking buffer, overnight at 4°C before proceeding to step f. Another dish with cells must be treated with primary antibodies handled in identical manner (but without antigen peptide) and imaged under the same conditions and exposure times.

i. (Optional) we have simultaneously labeled other proteins by incubating cells with anti-sAC antibodies together with variety of mouse monoclonal antibodies such as anti-α-tubulin 12G10 antibody from the Iowa Hybridoma Bank (0.1 μg/mL) and anti-Golgi matrix protein 130 (GM-130) from BD Biosciences™ (2.5 μg/mL).

4.3 Immunohistochemistry (Coral, Various Shark, and Fish Tissues)

We have successfully immunolocalized sAC in tissue paraffin sections (Roa & Tresguerres, 2016, 2017; Tresguerres, Parks, et al., 2010; Wilson et al., 2016) as well as in cryosections (Tresguerres, Levin, et al., 2010); protocol details can be found in those publication. Some things to consider include:

a. Tissue fragments must be immersed in ice-cold fixative immediately after dissection and incubated on a circular shaker or rotator mixer at 4°C. Overnight incubation is a good starting point, but the time might have to be optimized for each tissue to ensure fixation while avoiding over-fixation. Thinner and smaller samples require less fixation time.

b. For fish samples, we routinely fix samples in 0.2 mol/L cacodylate buffer, 3.2% paraformaldehyde, 0.3% glutaraldehyde, pH 7.4 (Electron Microscopy Sciences™). However, we have also had success fixing fish intestine in 4% paraformaldehyde in PBS (Tresguerres, Levin, et al., 2010), and coral tissue in 3% paraformaldehyde in S22 buffer (Barott et al., 2017).

c. After deparaffinization and initial tissue hydration, incubation in 1% SDS in PBS (10 min, room temperature) may help retrieve antigen sites (Roa & Tresguerres, 2016, 2017; Tresguerres, Parks, et al., 2010).

d. Perform the same controls described earlier for immunocytochemistry.

5. CELLULAR STUDIES

The information gathered using the techniques described earlier is essential for designing experiments to establish the role(s) of sAC in specific cell types and organs: enzymatic assays inform the A/B conditions likely to stimulate sAC in vivo, the efficacy of sAC inhibitors, and the presence of tmACs, and mRNA, and Western blotting and immunocytochemistry experiments establish the sAC isoforms that are expressed and where they are located within a cell. Unfortunately, sAC gene knockout and knockdown are still not feasible in the majority of aquatic animals. Pharmacological inhibition of sAC is currently the best tool to infer sAC's physiological roles in nonmodel aquatic animals. Pharmacological inhibitors have been used in experiments related to a large variety of physiological processes including intracellular pH measurements (Barott et al., 2017), sperm motility (Hess et al., 2005) and acrosome reaction (Beltrán et al., 2007), transepithelial NaCl absorption (Tresguerres, Levin, et al., 2010), blood pH regulation (Tresguerres, Parks, et al., 2010), translocation of proteins from the cell cytoplasm to the membrane (Roa & Tresguerres, 2016), and heart beat rate (Wilson et al., 2016), to name a few examples. Whenever possible, we recommend first confirming each inhibitor is specific for the sAC from the species in question. We also recommend using more than one sAC inhibitor, because their different structures and mechanisms of action minimize the chances of obtaining the same unspecific effect.

Traditional approaches to study cAMP-related processes in cells have included inhibition of PDEs to maximize responses, addition of cell permeable cAMP analogs, and stimulation of tmACs with Fsk. Our advice is to reinterpret (and in some cases repeat) those types of experiments taking into account the current cAMP microdomain model. Specifically, PDE inhibition and cAMP analogs (which tend to be nonhydrolyzable) likely result in cAMP diffusion into microdomains that are not relevant under normal conditions, and Fsk stimulates cAMP production by tmAC to nonphysiologically levels that also have the potential to act on nonphysiological microdomains (Fig. 3).

In many cases, pharmacological sAC inhibition does not cause any noticeable effect under control conditions. We believe this is due to sAC having a role in sensing deviations from an A/B set point, and in eliciting responses to correct them. Accordingly, sAC inhibitors tend to induce larger effects under conditions in which sAC is stimulated, typically resulting in blocking a certain response to A/B stress.

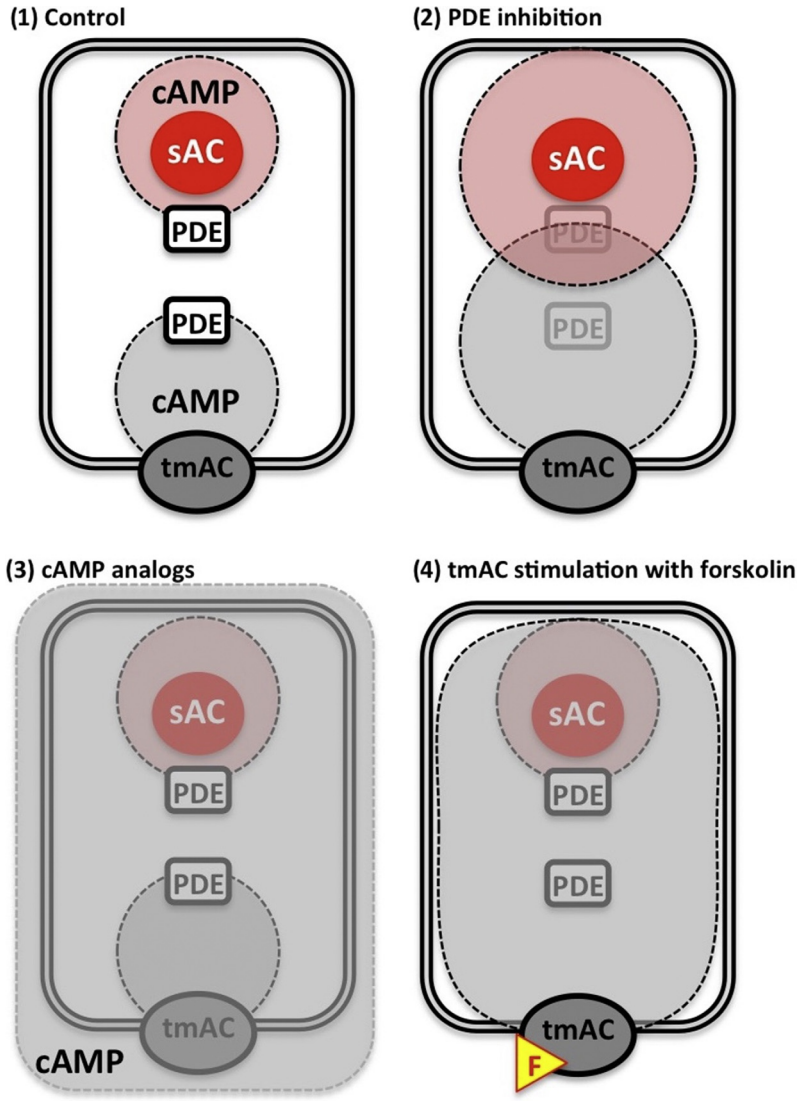

Fig. 3 Pharmacological manipulation of cAMP levels in cells. (1) cAMP signaling microdomains under control conditions. Soluble adenylyl cyclase (sAC) and transmembrane adenylyl cyclases (tmACs) generate cAMP at focal points. Phosphodiesterases (PDEs) hydrolyze cAMP therefore restricting its diffusion. Protein kinase A, exchange protein activated by cAMP, and cyclic nucleotide-gated channels are regulated by cAMP in each microdomain and modulate the activity of specific downstream proteins (none of which are depicted in these cartoons). For simplicity only one sAC- and one tmAC-mediated microdomain are shown, but cells might have several of each in different cell regions. (2) sAC activity can be pharmacologically inhibited using derivatives of catechol estrogens (dCEs),

(Continued)

6. SUMMARY AND CONCLUSIONS

The complexity of sAC at the gene and protein levels requires extensive optimization of molecular biology and biochemical techniques. A detailed characterization of sAC gene structure and regulation, protein isoforms, and subcellular localization is essential to be able to design and interpret experiments to study its physiological roles in various parts of the cell. The existing knowledge about sAC can and should be used as frame of reference for studies on sAC from new species; however, we recommend cloning sAC gene(s), characterizing dose response curves for HCO_3^- and inhibitors, and performing immunolocalization studies in the organisms of choice before proceeding to functional studies.

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Fig. 3—Cont'd KH7 and LRE1 (see text for details). (3) tmACs activity can be pharmacologically inhibited using DDA, among other drugs. (4) The broad PDE inhibitor 3-isobutyl-1-methylxanthine (IBMX) prevents cAMP degradation and thus can magnify the cAMP signaling cascade; however, it may result in nonphysiological responses due to abolition of cAMP microdomains. (5) Cell permeable cAMP analogs also increase cAMP levels inside cells; however, they may simultaneously act on multiple microdomains. (6) Stimulation of tmAC activity with forskolin (Fsk) specifically increases cAMP in those microdomains; however, it might reach nonphysiologically high levels that might overwhelm PDE activity, again acting on other microdomains that are not physiologically relevant.

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