Intracellular cAMP signaling by soluble adenylyl cyclase

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Soluble adenylyl cyclase (sAC) is a recently identified source of the ubiquitous second messenger cyclic adenosine 3′,5′ monophosphate (cAMP). sAC is distinct from the more widely studied source of cAMP, the transmembrane adenylyl cyclases (tmACs); its activity is uniquely regulated by bicarbonate anions, and it is distributed throughout the cytoplasm and in cellular organelles. Due to its unique localization and regulation, sAC has various functions in a variety of physiological systems that are distinct from tmACs. In this review, we detail the known functions of sAC, and we reassess commonly held views of cAMP signaling inside cells.

Kidney International (2011) **79,** 1277–1288; doi:10.1038/ki.2011.95; published online 13 April 2011

KEYWORDS: cyclic AMP; intracellular pH; intracellular signal

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Received 23 November 2010; revised 7 January 2011; accepted 1 February 2011; published online 13 April 2011

Adenylyl cyclase (AC) is the effector molecule of one of the most widely utilized signal-transduction pathways. Its product, cyclic adenosine 3′,5′ monophosphate (cAMP), modulates cell growth and differentiation in organisms from bacteria to higher eukaryotes. In animals, a seemingly ubiquitous membrane-associated AC activity is encoded by a family of transmembrane adenylyl cyclases (tmACs), which mediate cellular responses to extracellular stimuli. In mammals, nine distinct tmAC genes differing in their patterns of expression and regulatory properties have thus far been identified. These tmACs are widely studied in a variety of laboratories.

A second type of AC activity in mammals was described in 1975.[1] A soluble source of AC activity was detected in the testis and predicted to be molecularly distinct from tmACs.[2,3] The activity was thought to be dependent on manganese[1] and found to be insensitive to G-protein[4] and forskolin[5] regulation. A biochemically related activity was detected in spermatozoa; however, it was loosely associated with membranes.[1] This membrane associated, soluble adenylyl cyclase (sAC)-like activity was thought to be stimulated by sodium bicarbonate.[6–9] However, the molecular nature, biochemical regulation, and physiological function of sAC remained unclear until the sAC protein was purified and cloned in 1999.[10] The catalytic domains of sAC are related to bicarbonate-sensing ACs from cyanobacteria,[10,11] suggesting conservation of function of these cyclases as bicarbonate sensors throughout evolution.

GENOMIC ORGANIZATION, STRUCTURE, AND BIOCHEMISTRY

There is a single functional sAC gene in the human genome (ADCY10), comprising 33 exons that encompasses greater than 100 kb; however, it seems to utilize multiple promoters,[12,13] and sAC mRNA undergoes extensive alternative splicing.[12–15] Full-length mammalian sAC (sAC_{fl}) is comprised by two heterologous catalytic domains (C1 and C2), which constitute the 50 kDa amino terminus of the protein. The additional ~140 kDa C terminus of sAC_{fl} includes several putative regulatory domains, such as an autoinhibitory region[16] and canonical P-loop and leucine zipper sequences.[10] The minimal functional sAC variant, termed sAC_t, is a truncated form almost exclusively comprised of C1 and C2.[10,15] While sAC_t cAMP-forming activity is several

times stronger than it is for sAC_{fl}, both C1- and C2-containing sAC proteins are stimulated by HCO_3^- and are sensitive to all known selective sAC inhibitors (reviewed in ref. 17).

sAC activity requires two divalent metal cations in the catalytic active site of the enzyme to coordinate binding and cyclizing of adenosine 5′ triphosphate (ATP). sAC is most active in the presence of Mn^{2+}, a distinctive feature that led to its discovery in mammals,[1] and which also applies to sea urchin,[18] shark,[19] and cyanobacterial sAC-like cyclases.[11,20,21] However, it is not clear whether the physiological intracellular [Mn^{2+}] concentration would support sAC activity in mammals or in other species. *In vivo*, Mg^{2+} sAC activity can be modulated by Ca^{2+} (which increases the affinity for ATP of mammalian sAC) and by HCO_3^- (which releases ATP–Mg^{2+} inhibition and increases V_{max} of mammalian sAC)[22] (reviewed in ref. 17). The catalytic mechanism of cAMP production by sAC-like enzymes and its activation by HCO_3^- has been elucidated for CyaC, an AC from the cyanobacterium *Spirulina platensis*.[23] The key amino-acid residues implicated in catalysis are conserved in cyanobacterial and mammalian sAC. In the structure- and kinetics-based model, ATP, with Ca^{2+} bound to its γ-phosphate, coordinates with specific residues in the sAC catalytic center. This results in an 'open sAC state'. Then, the second divalent metal, a Mg^{2+} ion, binds to the α-phosphate of ATP, leading to a distinct set of catalytic residue interactions referred to as the 'closed state'. This change, from the 'open' to 'closed' states, induces esterification of the α-phosphate with the ribose in adenosine and the concomitant release of the β- and γ-phosphates ('cyclizing'). HCO_3^- stimulates the enzyme's V_{max} by fostering the allosteric change that leads to active site closure, recruitment of the catalytic Mg^{2+}, and rearrangement of the phosphates in the bound ATP.[23] A human sAC_t crystal structure reported in a patent application (WO 2007/010285) is consistent with the proposed catalytic mechanism and bicarbonate regulation.

The source of HCO_3^- regulating sAC could be external to the cell (body fluids or environment) or metabolically generated (Figure 1). Intra- and extracellular carbonic anhydrases (CAs) are in many cases essential for rapid hydration of CO_2 into HCO_3^- that activates sAC (reviewed in ref. 24). For example, in a number of epithelia and endothelia, elevated [HCO_3^-] stimulates ion and fluid transport, and in a subset of these examples, sAC has been shown to be involved (see below). But sAC was also shown to be modulated by metabolically generated CO_2/HCO_3^- inside the matrix of the mitochondria.[25–27] In diverse systems, such as the endocrine pancreas, neuronal cells, and neutrophils, sAC activity can be activated by elevations in free intracellular Ca^{2+}.

Molecular studies predict the existence of sAC isoforms, which contain only the C2 catalytic domain, generated by alternative splicing and/or alternate promoter utilization.[12–14] When heterologously expressed in insect Sf9 cells, some of these sAC variants localized to different regions of the cell, in a manner that suggested that the non-catalytic C-terminal domain favors association with the plasma membrane.[28] However, it is not clear how these isoforms could generate cAMP with just one catalytic domain, and recombinant C2-only sAC isoforms had no detectable activity under the conditions tested.[13,29]

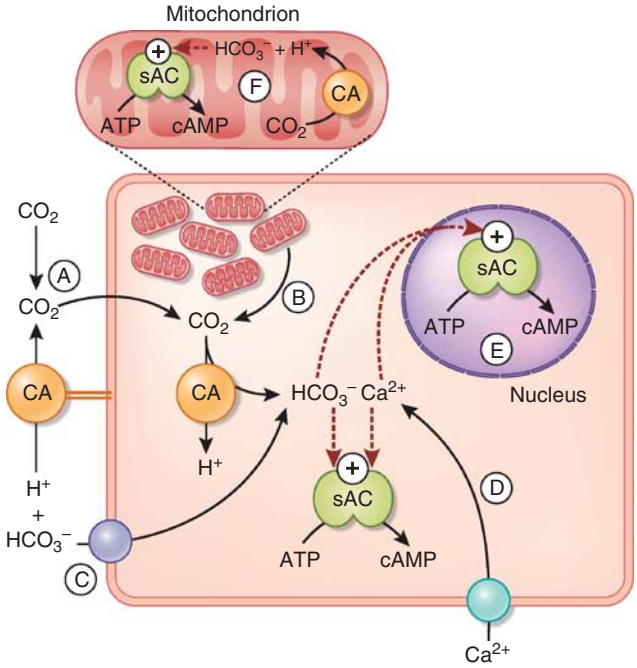


Figure 1 | Activation of soluble adenylyl cyclase (sAC) by HCO_3^- and Ca^{2+}. Cytosolic sAC can be activated by HCO_3^- derived from carbonic anhydrase (CA)-dependent hydration of (A) external and (B) metabolic CO_2 and/or (C) HCO_3^- that enters through membrane-transporting proteins (purple icon) such as anion exchangers, Na^+/HCO_3^- cotransporters (NBCs), or cystic fibrosis transmembrane conductance regulators. sAC can also be activated by (D) Ca^{2+} entering the cell through membrane transporters (turquoise icon) such as voltage-dependent Ca^{2+} channels or potentially by Ca^{2+} release from the endoplasmic reticulum or mitochondria (not depicted). (E) HCO_3^- and Ca^{2+} can potentially activate sAC in the nucleus. (F) sAC inside mitochondria has been shown to be activated by metabolically generated CO_2 through CA. See text for details. ATP, adenosine 5′ triphosphate; cAMP, cyclic adenosine 3′,5′ monophosphate.

Specific base substitutions in the human *sAC* gene have been linked to absorptive hypercalciuria (deficient renal and intestinal Ca^{2+} absorption) and to low spinal bone density.[30] To date, the only reported phenotype of the existing sAC-knockout (KO) mouse model is male infertility due to an inability to activate flagellar movement on ejaculation and failure to undergo a maturation process known as 'capacitation'.[31–33] However, results from other pH-sensing proteins suggest that additional phenotypes may be revealed under stressful conditions where sAC is required for sensing and compensation.[34] In addition, the existing sAC-KO mouse only removes the exons encoding the C1 domain; it retains the C2 domain and the non-catalytic C terminus, and it appears to include a putative alternative promoter.[12] Therefore,

it is possible that C2-containing proteins are responsible for essential functions or that they compensate for the lack of C1- and C2-containing sAC isoforms. Testing this hypothesis awaits generation of KO mice, which specifically disrupt the C2 domain.

sAC orthologs have been functionally characterized in gills of the dogfish shark,[19] and potential roles have been suggested for sAC in sea urchin sperm[18,35,36] and in the intestine of teleost (bony) fishes.[37] The recent surge in genomic and transcriptomic information confirmed sAC orthologs to be present in most metazoan phylogenetic groups.

MICRODOMAIN ORGANIZATION OF cAMP SIGNALING

Dr Earl Sutherland was awarded the Nobel Prize for identifying cAMP as the mediator of cellular control of metabolic activity.[38] In the 50 years since he discovered this prototypical second messenger, cAMP signaling has been described in organisms as diverse as bacteria and mammals. However, in a seeming conundrum, cAMP has also been implicated in a wide variety of often-contradictory physiological processes, including different aspects of metabolism, proliferation, apoptosis, differentiation, migration, development, ion transport, pH regulation, and gene expression. Only recently has it become clear how this single second messenger could simultaneously mediate so many processes. In current models of cyclic nucleotide signal transduction, cAMP is locally generated within independently regulated microdomains (most recently reviewed in ref. 39). This new microdomain model depends upon A-kinase-anchoring proteins, which tether protein kinase A (PKA) to specific locations inside cells,[40,41] and phosphodiesterases (PDEs), which degrade cAMP and act as barriers for cAMP diffusion[42–44] to avoid unregulated cross-communication between microdomains. The modern cAMP microdomain model also requires multiple sources of cAMP distributed at the cell membrane and throughout the cell (Figure 2).

A need for cAMP microdomains was first postulated in cardiomyocytes, when it was observed that distinct hormones elicited unique responses through cAMP in a single cell type.[45] Subsequently, fluorescence resonance energy transfer (FRET)-based and biophysical methods that enable measuring cAMP concentrations *in situ* revealed that cAMP levels are not uniform within cells (recently reviewed in refs 46,47). The existence of membrane-proximal cAMP microdomains was definitively demonstrated in neurons[48] and cardiomyocytes,[49] and artificial, localized production of second messenger[50–52] supports the model that cAMP acts in locally restricted microdomains.

In most membrane microdomains, cAMP is likely generated by the classic tmACs, which are regulated by heterotrimeric G-proteins downstream from hormonally regulated G-protein-coupled receptors (GPCRs). As their name implies, tmACs are obligatory transmembrane proteins, and their mode of activation demands that they be localized on the plasma membrane in close proximity to the GPCR. Recently, it was found that tmACs in thyroid follicles can still signal as they co-sort with ligand-bound GPCRs on endosomes during receptor internalization[53,54] (reviewed in refs 43, 44).

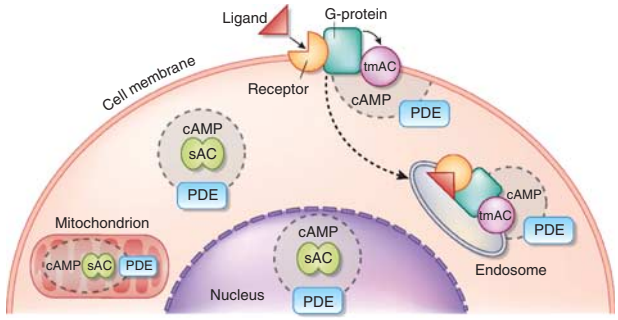


Figure 2 | Intracellular cyclic adenosine 3′,5′ monophosphate (cAMP) signaling microdomains. cAMP signaling occurs in discrete intracellular compartments such as the membrane vicinity, focal points throughout the cytoplasm, mitochondria, and the nucleus. Each microdomain contains (1) a source of cAMP (soluble adenylyl cyclase—sAC or transmembrane adenylyl cyclase—tmAC); (2) phosphodiesterases (PDE) that act as barriers for cAMP diffusion; and (3) cAMP targets such as protein kinase A (PKA) or exchange proteins activated by cAMP (EPAC) (not illustrated). tmAC cAMP signaling occurs in response to various extracellular ligands and it requires modulation by G-protein-coupled receptors and heterotrimeric G-protein. The most widely described tmAC-dependent microdomain occurs at the cell membrane, but additional intracellular tmAC-dependent microdomains occur in endosomes after internalization. sAC present throughout the cytoplasm and in organelles, such as mitochondria, nucleus, mid-bodies, and centrioles, define other microdomains. Additional regulation might involve the movement of sAC between compartments. See text for details.

Classically, research on cAMP signaling relied on the use of membrane-permeant cAMP analogs, the potent, pan-tmAC activator, forskolin, and/or the broad specificity PDE inhibitor, 3-isobutyl-1-methylxanthine (IBMX). In light of the cAMP microdomain model, results with each of these reagents needs re-evaluation because their effects do not reflect biologically meaningful second messenger responses. Cyclic nucleotide analogs have multiple targets and they competitively inhibit PDEs,[55] which will disrupt the integrity of signaling microdomains. Forskolin stimulation will produce unphysiologically potent and prolonged cAMP changes. For example, in rat insulinoma INS-1 cells, forskolin induces a continuous increase in cAMP accumulation, reaching maximum values of ~10-fold after 30 min.[56] This contrasts sharply with the effect of glucagon-like peptide-1, a physiological activator, that induces ~2.5-fold activation after 5–10 min, but which returns to basal levels after 30 min.[57] Similarly, the use of IBMX will eliminate the inter-microdomain PDE barriers permitting cAMP diffusion. Use of forskolin and IBMX is more akin to the toxic effects of cAMP-elevating toxins, such as the edema toxin of anthrax or cholera or pertussis toxins. Inhibition of PDEs with IBMX includes an additional complication because it prevents degradation of both cAMP and cyclic guanosine monophosphate (cGMP). Therefore, any effect obtained with IBMX

could be due to increased activity of cAMP or cGMP from any microdomain, or even to inhibition of cross-regulation between cAMP and cGMP pathways.[58]

Mammalian sAC is distributed to discrete locations throughout the cell. It is found diffusely distributed in the cytoplasm and at the centrioles and mid-body,[59] and inside the nucleus[59–62] and mitochondria.[26,27,59,63] Each of these locations contain known targets for cAMP; thus, sAC represents an additional source of cAMP inside cells that can produce the second messenger locally and activate nearby cAMP effectors.[64–66] The nuclear and mitochondrial sAC-defined cAMP microdomains have been functionally characterized. Nuclear sAC is capable of phosphorylating cAMP response element-binding protein (CREB),[61,67] and this microdomain might be related to the gene regulation in solid tumor cells observed with alkaline cytoplasmic pH (reviewed in ref. 68). Mitochondrial sAC modulates oxidative phosphorylation in response to CO_2/HCO_3^- generated by the tricarboxylic acid cycle,[26,27] and cytoplasmic sAC translocates to the mitochondria during acidosis/ischemia to promote the mitochondrial apoptotic pathway.[63]

sAC ROLES THROUGHOUT THE BODY

Testis and sperm

The most widely accepted role of sAC in mammals is in male fertility.[32,33] During spermatogenesis, sAC mRNA is first detectable in mid-pachytene spermatocytes and shows a strong upregulation in the later stages of spermiogenesis.[69] sAC_{fl} protein is only detectable by immunohistochemistry in late pachytene spermatocytes (end of meiotic prophase I).[70] However, because the antibodies utilized were against the carboxy terminus of sAC_{fl}, it is possible that other sAC variants are present and have a role at earlier stages. Sperm continue maturation during transit through the epididymis, and they are stored in the cauda epididymis, where $[HCO_3^-]$ is significantly lower than in plasma and seminal fluids.[71] The diminished luminal bicarbonate concentration in the epididymis is maintained by sAC regulation of V-type H^+-ATPases (VHAs) in a process described in greater detail below. Although morphologically mature, these stored epididymal sperm still do not have the 'capacity' to fertilize an egg.[72] Upon ejaculation, the stored sperm are mixed with seminal and prostatic fluids, where $[HCO_3^-]$ suddenly rises to $\sim$25 mmol/l.

In mature sperm, sAC is the sole producer of cAMP in response to elevations in $[HCO_3^-]$,[32,33] which varies dramatically in the environments sperm encounter during the reproductive process.[71] The ejaculated sperm acquire fertilization competence during transit through the female reproductive tract. This critical process involves many changes that are collectively grouped under a single term, 'capacitation'. Among the first definable events in capacitation is the entrance of Ca^{2+} and HCO_3^- into sperm, which activate cAMP production by sAC. In the short term, this activates asymmetrical flagellar beat frequency resulting in vigorous forward sperm motility. Extended activation of PKA in the presence of cholesterol acceptors leads to the prototypical pattern of tyrosine phosphorylation that represents a molecularly defined hallmark of capacitation.[73–78] Physiologically, the end results of capacitation are sperm hyperactivation of motility and the ability to perforate the egg's zona pellucida by the acrosome reaction.

The existing sAC-KO mouse model, which deletes the exons encoding the C1 domain (sAC-C1 KO), removes the two characterized C1- and C2-containing isoforms, sAC_t and sAC_{fl}.[33] The phenotype of sAC-C1 KO sperm includes defects in flagellum movement resulting in lack of motility,[31–33] an aberrant tyrosine phosphorylation pattern during capacitating conditions,[33] and the inability to fertilize an egg.[32,33] Loss of these C1 and C2 isoforms also resulted in a morphological aberration, termed flagellar angulation (tail bending),[32,33] which may be the result of diminished metabolic capacity.[31]

In addition to responding to HCO_3^-, sAC is essential for the acceleration of flagellar beat frequency in response to adenosine analogs and catecholamine agonists.[79] The catecholamine stimulatory mechanism is not clear, but it does not seem to be mediated by bicarbonate, as it occurs when HCO_3^- is omitted from the medium. Both agonists and antagonists of β-adrenergic receptors stimulated flagellar beat frequency and there was no discrimination between *l*-(−) and *d*-(+) catecholamine isomers, arguing against a conventional (that is, G-protein coupled) adrenergic receptor. Modulation by adenosine also seems independent from GPCRs and tmACs,[79,80] and instead may rely on the entrance of adenosine by specific transporters to promote sAC-generated cAMP accumulation by unknown direct or indirect mechanisms.[80]

Somatic functions of sAC

Initially, sAC's role in sperm biology was thought to be its only function. This was largely due to the relatively low expression of sAC mRNA and protein in other mammalian tissues,[12,69,81] to the absence of any other overt phenotype besides male infertility in the sAC-C1 KO mice[32] and to the absence of sAC in the sequenced genomes of fruit fly (*Drosophila melanogaster*) and roundworm (*Caenorhabditis elegans*).[82] However, more sensitive mRNA and protein analytical techniques and the explosion in genome sequencing during the last decade reveal that sAC is widely expressed in animals and is found in virtually every animal phyla. Thus, the putative existence of sAC-C2 isoforms coupled with the likely need to stress the system to unmask additional sAC functions presumably explains the lack of additional obvious sAC-C1 KO phenotypes.

KIDNEY

The presence of sAC in the kidney was hinted in an early study that described HCO_3^--stimulated cAMP-forming activity in rat kidney (medulla > cortex) homogenates.[83] Subsequent to its molecular isolation,[10] sAC mRNA was detected in the kidney by RNA Array[13] and reverse

transcriptase-polymerase chain reaction,[12,13,84] and sAC protein was identified in the kidneys by western blotting[11–13] and immunohistochemistry.[85,86] Taken together, these studies suggested the presence of several sAC splice variants in kidney. The immunohistochemistry revealed that sAC (or at least a subset of the sAC variants) are preferentially expressed in cells of the medullary and cortical thick ascending loop of Henle (TAL), in cells of the distal tubule (DT) and in cells of the collecting duct (CD).[85,86]

Renal corpuscle and proximal convoluted tubule

Immunohistochemical studies using the monoclonal antibody R21 (directed against an epitope in coding sequence exon 5) did not detect sAC either in the renal corpuscle or in the proximal convoluted tubule (PCT).[85,86] However, unpublished results from our laboratory using polyclonal antibodies against the C terminus of sAC revealed strong immunostaining in the PCT, and positive immunostaining in some glomeruli (L.R.L. and J.B., unpublished observations). Studies of sAC function in the PCT await confirmation of its presence in these regions of the nephron, for example, by laser-capture micro-dissection followed by transcriptomic or proteomic studies. Potential roles for sAC in the PCT include interaction with other pH/CO_2/HCO_3^- responsive enzymes proposed to regulate salt and fluid absorption in the PCT, such as Pyk2 or an as yet undefined tyrosine kinase.[87–89] pH/CO_2/HCO_3^- sensing throughout the body, including kidney, was recently reviewed elsewhere.[24]

Thick ascending loop of Henle

The TAL actively absorbs NaCl (and to a much lesser extent, Ca^{2+} and Mg^{2+}), and it is responsible for urine concentration during antidiuresis and for urine dilution during diuresis (reviewed in ref. 90). The bulk of NaCl absorption across cells of the TAL takes place by apical $Na^+/Cl^-/2Cl^-$ cotransporters (NKCC),[91–94] energized by basolateral Na^+/K^+-ATPases (recently reviewed in ref. 95). sAC is present in the TAL,[85,86] in both medullary and cortical segments,[85] and even though there are, as yet, no functional studies of sAC in native TAL, there is evidence supporting its ability to regulate both Na^+/K^+-ATPase (see below for description of sAC regulation in $mpkCCD_{c14}$ cells, immortalized cells derived from the mouse cortical CD) and NKCCs.

The intestine of marine teleost fish absorbs NaCl by cellular mechanisms similar to those in the TAL, and it has traditionally been used as a model for NaCl absorption.[96–100] In the toadfish intestine, it was recently shown that HCO_3^- stimulates NaCl absorption (estimated from short-circuit current measurements), seemingly by sAC-dependent regulation of NKCC2 and/or Na^+/K^+-ATPase.[37] Several lines of evidence suggest that sAC may have a similar role in the TAL: (1) mild metabolic alkalosis reduces the diuretic, natriuretic, and chloruretic effects of bumetanide by ~40, ~21, and ~25%, respectively,[101] suggesting a stimulatory effect of HCO_3^- on NaCl and water absorption; and (2) NKCC2 has been demonstrated to be phosphorylated and shuttled into the apical membrane of the TAL in response to vasopressin[102] and cAMP,[103,104] in a PKA-dependent manner.[104]

Distal tubule

In this review, we use 'DT' to collectively refer to the distal convoluted tubule, connecting tubule, and cortical CD.[105] Overall, the DT reabsorbs ~10% of the filtered NaCl, and it is also important for Mg^{2+} and Ca^{2+} reabsorption and for K^+ secretion and reabsorption.[105] In addition, A- and B-type intercalated cells (ICs) are responsible for metabolic compensation of systemic ('blood') acid/base (A/B) status.[106]

The pioneering research about sAC as a sensor and regulator of A/B-related ion transport was performed in the epididymis, which shares its embryonic origin with the renal nephron.[107] In addition, both epithelia have acid-secreting cells that are functionally similar (termed 'clear cells' in the epididymis and A-type ICs in the nephron DT). The epididymis is often used for functional studies on acid secretion as a surrogate model for the DT, because it is easier to isolate and perfuse compared to the nephron, and it is simpler due to the absence of any cell type corresponding to the countering B-type ICs.

In order to maintain sperm quiescence, the lumen of the epididymis has low pH and low [HCO_3^-].[71] This is achieved by H^+ pumping by apical VHA.[108] Elevations in luminal pH or [HCO_3^-] are transmitted to the inside of the clear cells either by Na^+/HCO_3^- cotransporters or by hydration/dehydration into CO_2, catalyzed by extracellular and intracellular CAs.[85,109] Intracellular HCO_3^- activates sAC, which promotes the insertion of VHAs into the apical membrane and the development of extensive apical microvilli, leading to increased apical H^+ secretion into the epididymis lumen.[85] Thus, sAC in clear cells senses elevations in luminal pH and [HCO_3^-] and restores, by VHA translocation and H^+ pumping, the original low pH and [HCO_3^-] luminal values. On the basis of the effects of pharmacological inhibitors, the immediate downstream target of sAC-generated cAMP appears to be PKA, while exchange protein directly activated by cAMP does not seem to have a role on the VHA apical translocation.[110] Downstream of PKA, the a-subunit of the VHA is a potential phosphorylation target.[111] The stimulatory effect of sAC/cAMP/PKA on VHA apical accumulation is counterbalanced by an inhibitory effect of AMP-activated kinase (AMPK)[111] (Figure 3). This is not the only time where the effects of PKA and AMPK are antagonistic,[111–114] and if the two kinases are localized within the same signaling microdomain, cAMP and its degradation product, AMP, could function as a timing mechanism. AMPK is regulated by an increase of AMP at the expense of an ATP; in a two-step reaction catalyzed by any ATPase and adenylate kinase, one ATP is converted into one AMP, and this change stimulates AMPK activity (reviewed in ref. 115). The same change in the AMP/ATP ratio is effected, also in two steps, in cAMP signaling cascades. In the first step, an ATP is converted into cAMP via an AC, and in the second step, cAMP is hydrolyzed into AMP by a PDE. Thus,

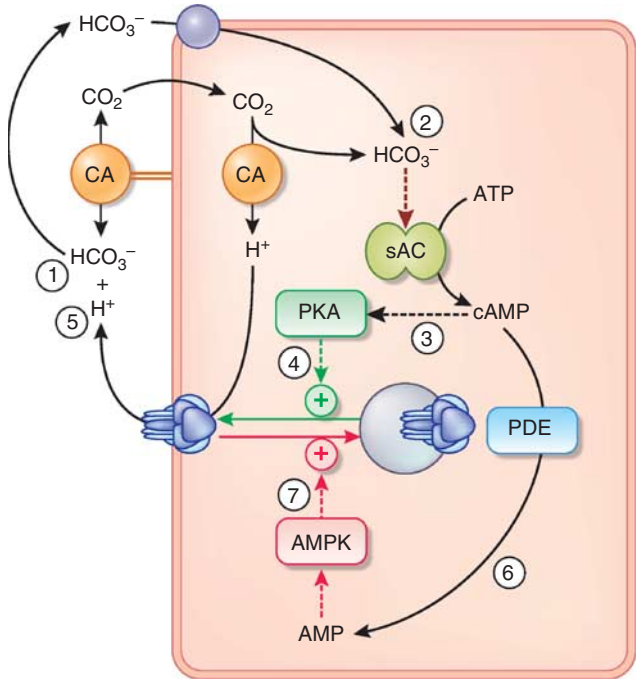


Figure 3 | Regulation of V-type H^+-ATPase (VHA) translocation by soluble adenylyl cyclase (sAC) and AMP-activated kinase (AMPK). (1) Extracellular HCO_3^- enters the cell through transporter proteins (purple icon) or is dehydrated into CO_2, a reaction catalyzed by an extracellular carbonic anhydrase (CA). CO_2 would then diffuse into the cell, where it is hydrated into H^+ and HCO_3^- by an intracellular CA. (2) The elevated intracellular [HCO_3^-] activates sAC (3) to produce cAMP, which promotes (through PKA) (4) the insertion of VHA-containing vesicles into the cell membrane. (5) Membrane inserted VHAs secrete H^+, which counteract the original alkalosis. (6) cAMP is hydrolyzed by phosphodiesterase (PDE) into AMP, which can (7) by stimulation of AMPK, inhibit the PKA-mediated effects. This hypothetical mechanism, involving sequential activation of PKA and AMPK, could serve as a self-regulating circuit.

PKA and AMPK regulation of VHA translocation may function as a timer. Activation by cAMP and PKA could be automatically terminated by AMPK subsequent to PDE hydrolysis of the cAMP into AMP. This 'clock' may function in other systems where PKA and AMPK are antagonistic. AMPK is a known modulator of metabolic pathways, and we have shown that sAC-generated cAMP modulates metabolic activity in pancreatic β-cells[57] and astrocytes,[116] and that it functions as a metabolic sensor inside mammalian mitochondria.[26,27]

The role of sAC was subsequently examined in the renal DT. sAC is present in A- and B-type ICs,[84] as well as in principal cells.[84,86] Immunofluorescent and immunogold staining shows that sAC is most abundantly present in the apical pole of A-type ICs, and in the basolateral and apical poles of B-type ICs.[84] sAC and VHA not only co-localize in both types of ICs, but they also co-immunoprecipitate from rat kidney homogenates.[84] Functional studies in renal A-type ICs basically mimicked those from the clear cells from the epididymis; that is, apical VHA accumulation and microvilli elongation dependent on cAMP and PKA,[113,117] possibly involving direct phosphorylation of the VHA a-subunit.[118] Importantly, equivalent results were obtained in kidney slices,[113] after intravenous cAMP infusion through the femoral vein,[117] and in isolated ICs.[117] Although direct effects of increased external pH and/or [HCO_3^-] on these processes have not yet been demonstrated, chronic CA inhibition with acetazolamide, which increases HCO_3^- delivery to the DT,[119,120] did stimulate the apical microvilli elongation (as well as the number of A-type ICs).[119] Interestingly, as in epididymal clear cells, AMPK activity opposes the role played by sAC-generated cAMP; it inhibits the VHA apical translocation and the development of microvilli in A-type ICs.[113]

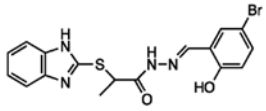
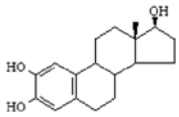
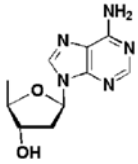
The physiological role of sAC in renal B-type ICs is less clear, especially as intravenous cAMP infusion had no clear effect on VHA intracellular localization.[117] However, sAC has been shown to have a regulatory role on VHA translocation to the basolateral membrane of cells involved in a base-secreting physiological process in a non-mammalian system.[19] In aquatic animals, the gills (and not the kidneys) are the principal A/B regulatory organs,[121] and the cellular and molecular mechanisms governing H^+ and HCO_3^- transport are remarkably similar. In the dogfish shark gill epithelium, base-secreting cells normally have VHA in cytoplasmic vesicles, where they are presumably inactive.[122] Upon blood alkalosis, VHA-containing vesicles exhibit sAC-,[19] CA-,[123] and microtubule-dependent[124] VHA translocation to the basolateral membrane, where VHA absorbs H^+ into the blood and energizes HCO_3^- secretion. Pharmacological inhibition of sAC prevents VHA translocation both *in vitro* and *in vivo*,[19] suggesting that dogfish sAC is both necessary and sufficient as a sensor and regulator of systemic blood A/B homeostasis. Elucidating this mechanism was possible because of the ultrastructure of the basolateral membrane of shark base-secreting cells (which is heavily infolded, but lacks an elaborated tubulovesicular system[122,125]) and because of the feeding physiology of the dogfish shark, which involves a pronounced post-feeding blood alkalosis.[123,126]

Another, yet unexplored, potential target of sAC activity in B-type ICs is the anion exchanger Pendrin, which co-localizes with sAC at the apical region.[84] Interestingly, dogfish pendrin also seems to be present in shark gill base-secreting cells.[121]

The potential role of sAC in principal cells of the DT has been studied in confluent polarized mouse cortical CD ($mpkCCD_{c14}$) cells. These cells, which express ENaC and Na^+/K^+-ATPase in their apical and basolateral membranes, respectively, and exhibit hormonal regulation of Na^+ transport similar to *in vivo* models, are considered most similar to DT principal cells.[127,128] Pharmacological and sAC small interfering RNA (siRNA) manipulations revealed a potential role of sAC in regulating transepithelial Na^+ transport in these cultured cells, both in basal and in forskolin- or aldosterone-stimulated conditions.[86]

Finally, specific base substitutions in the *sAC* gene correlate with familial absorptive hypercalciuria,[30] a genetically

Table 1 | Pharmacological inhibitors of adenylyl cyclases

Name	Structure	Specificity	Ref.
KH7		sAC-specific inhibitor	33
Catechol estrogens, such as 2-hydroxyestradiol		Selective for sAC relative to tmACs	85,161
P-site ligands, such as 2′5′dideoxyadenosine		Potently inhibits tmACs Weakly inhibits sAC	57,150,153,162

Abbreviations: sAC, soluble adenylyl cyclase; tmAC, transmembrane adenylyl cyclase.

inherited disease characterized by an excess of Ca^{2+} in urine due to inadequate reabsorption in the DT and/or in the intestine.[129] However, a defined role for sAC in Ca^{2+} absorption has not yet been investigated in cells or organs.

Conclusions: kidney

The A/B status of plasma and filtrated fluid affect several ion-transporting processes in the nephron, several of which are regulated by cAMP. Because sAC is present throughout the nephron, sAC is a good candidate to integrate external (tubular fluid) and internal (plasma, renal interstitium) cues with their appropriate responses through cAMP signaling. In addition, confirmed or proposed roles for sAC in other tissues raises the possibility that sAC regulation might be involved in additional processes including Ca^{2+} signaling and gene expression.

EYE

In both corneal endothelium[130] and cilliary body,[131] HCO_3^- stimulates fluid secretion. A role for sAC was first suggested by the observation that HCO_3^- stimulates cAMP production in homogenates from both tissues.[83] Subsequent to its molecular isolation, sAC was confirmed to be present in primary cultures of bovine corneal endothelial cells, and sAC activation increased cystic fibrosis transmembrane conductance regulator (CFTR)-dependent secretion of Cl^-, HCO_3^-, and/or ATP.[132] Although these studies were performed before the advent of sAC-selective inhibitors, all data suggest that cAMP produced by sAC stimulates PKA phosphorylation of apical CFTR, thus increasing apical Cl^- permeability.[132,133] It was also demonstrated in cultured corneal endothelial cells that higher [HCO_3^-] in the cultured medium increased sAC expression.[133]

A role for sAC has also been proposed in retinal ganglion cells. In a subset of retinal ganglion cells, periodic depolarizations, acting through a Ca^{2+}-dependent cAMP/PKA cascade, are critical for proper circuit development.[134] Mice lacking the Ca^{2+}-sensitive tmACs (AC1 and AC8) still displayed depolarization-induced Ca^{2+}-dependent PKA transients, which were only inhibited after pharmacological inhibition of all mammalian ACs, including sAC.[135]

AIRWAYS

Calu-3 cells, a cancer cell line derived from bronchial submucosal glands,[136] express sAC_{fl} and sAC_t mRNA, although only a ~50 KDa band (consistent with sAC_t) is detectable at the protein level.[137] cAMP production in Calu-3 cells is stimulated by HCO_3^-, an activity which is inhibited by a selective sAC inhibitor (2-CE) but not by a tmAC-selective, P-site inhibitor[137,138] (see Table 1 for a summary of AC inhibitors). In cell-attached patch-clamp experiments, HCO_3^- stimulated CFTR single-channel activity in a 2-CE-sensitive manner, suggesting that sAC regulates CFTR in Calu-3 cells.[137] In addition, switching from HCO_3^--free to HCO_3^--containing buffer significantly increases CFTR mRNA and protein levels in a sAC-dependent manner.[138] These conditions also increased phosphorylation of nuclear CREB. *In vivo*, regulation of CFTR by sAC in airway glands is probably related to the secretion of airway surface liquid and mucus.

Airway epithelial cells express multiple sAC mRNA and protein variants, including putative sAC-C2-only isoforms.[14] One particular sAC variant (of ~50 kDa) was demonstrated by a combination of western blotting and immunocytochemistry to be present in cilia. Apical application of HCO_3^- to culture-differentiated human airway epithelial cells increased cAMP production by sAC; this sAC-generated cAMP stimulated PKA, which increased ciliary beat frequency.[14] Thus, sAC seems poised to sense changes in CO_2/HCO_3^- concentration in airways during normal and disease conditions and coordinate the clearance of mucus. For example,

this form of sAC-dependent regulation appears to be adversely affected in airway epithelia from cystic fibrosis patients, possibly contributing to their mucociliary dysfunction.[139] Finally, stimulation of ciliary beat frequency by ethanol also depends on PKA and sAC,[140] although the link between ethanol and sAC activation is not yet clear.

Because HCO_3^- stimulation of ciliary beat frequency is diminished in cultured cells from cystic fibrosis patients,[139] HCO_3^- probably enters ciliated cells through CFTR. Therefore, the relationship between sAC and CFTR in airway epithelial cells is complex and it may involve sAC regulation of CFTR expression and activity (as shown in Calu-3 cells) as well as CFTR regulation of sAC (as shown in ciliated cells).

PANCREAS

There are proposed roles for sAC in both exocrine and endocrine pancreas. In intrahepatic trees, sAC is preferentially expressed in cholangiocytes of large bile ducts,[141] which are specialized bile-secreting, secretin-responsive, epithelial cells. Pharmacological inhibition (by KH7) or siRNA down-regulation of sAC significantly abolishes the HCO_3^--induced stimulation of fluid secretion.[141] Similar inhibitions were found upon application of acetazolamide and H89, suggesting that CA and PKA are upstream and downstream, respectively, of sAC. These authors proposed that sAC sustains basal levels of cAMP and fluid secretion during the interdigestive phase, while gastrointestinal hormones and cholinergic and β-adrenergic agonists acting through GPCRs-tmACs mediate regulated phases of cAMP and fluid secretion. Because Cl^- secretion by cholangiocytes of large ducts depends on apical CFTR,[142] this might represent another case of CFTR regulation by sAC.

β-Cells in the endocrine pancreas release insulin in response to various stimuli, including hormones, neurotransmitters, and blood glucose levels. It was known for decades that an increase in external glucose concentration stimulates cAMP production while modulating the release of insulin.[143] However, the source of this cAMP remained unknown[144] until recently. On the basis of the studies on INS-1E cells using selective inhibitors and siRNA, sAC is responsible for the glucose-induced cAMP production, while tmAC(s) mediates responses to incretins such as glucagon-like peptide-1 (ref. 57). Glucose-induced activation of sAC is dependent upon entry of Ca^{2+} into the cell,[57] which may be synergistic with glucose metabolism-dependent elevations in intracellular $[HCO_3^-]$ and/or ATP. sAC-generated cAMP is essential for the increased activation of extracellular signal-regulated protein kinases 1 and 2 observed during high-glucose conditions; the contribution of sAC-generated cAMP on insulin release has not yet been reported.

DIGESTIVE TRACT

Transient and sustained Cl^- and K^+ secretions in the distal colonic epithelium, which determine the rate of fluid secretion in relation to food digestion, water conservation, and intestinal flushing, are subject to sympathetic modulation. Addition of epinephrine to isolated colonic mucosa induces rapid and transient Cl^- secretion, which is followed by sustained K^+ secretion. On the basis of the differential responses with inhibitors selective for sAC (KH7) or for tmACs (ddAdo), the rapid response is dependent on β_2-adrenergic receptors and tmACs, while the sustained response likely relies on HCO_3^-, β_1-, and β_2-adrenergic receptors and sAC.[145]

In marine bony fish, intestinal HCO_3^- secretion and NaCl and water absorption are essential for hypo-osmoregulation.[146,147] sAC has been proposed to coordinate HCO_3^- secretion with NaCl absorption by monitoring intracellular levels of CA-generated HCO_3^- and activating membrane ion-transporting proteins.[37] As explained above, a similar mechanism might be occurring in the mammalian TAL.

BRAIN AND NERVOUS SYSTEM

sAC-dependent processes have been hinted at or established in choroid plexus, neurons, and astrocytes. In choroid plexus, CO_2 metabolism has long been linked to cerebrospinal fluid secretion.[148] sAC mRNA,[149] protein,[11] and activity[83] have been demonstrated in chorioid plexus, and it is straightforward to hypothesize that bicarbonate regulation of sAC has a role in cerebrospinal fluid homeostasis.

Astrocytes express several sAC splice variants[116] (some or all of), which are involved in a novel mechanism of metabolic coupling between neurons and astrocytes. Elevation of $[K^+]$ at the extracellular space caused by neuronal activity depolarizes the cell membrane of nearby astrocytes and induces HCO_3^- entry through electrogenic Na^+/HCO_3^- cotransporters. The elevation in $[HCO_3^-]_i$ activates sAC, which leads to glycogen breakdown, enhanced glycolysis and generation, and release of lactate for use by the neighboring 'active' neurons for energy.

sAC has been shown to be present in developing neurons, where, depending on the origin of the neuron, it was located in cell bodies, dendrites, axons, and/or growth cones.[150] One proposed role for sAC in developing neurons is to regulate growth cones and promote axonal growth. In cultured dorsal root ganglion and spinal commissural neurons, sAC inhibition, either by small-molecule inhibitors selective for sAC (KH7 or catechol estrogens) or sAC-specific RNA interferace, blocked netrin-1-induced growth cone elaboration and axonal growth.[150] Both responses were mimicked by sAC overexpression. In an apparent conundrum, the existing sAC-KO mouse model (sAC-C1 KO) does not display any of the phenotypic defects in the ventral spinal commissure, which are a hallmark of netrin-1 deficiency.[81,150]

sAC was also shown to be essential for responses to the prototypical neurotrophin, nerve growth factor (NGF). PC12 cells, which are derived from rat adrenal medulla, are used as a model for neuronal differentiation, because they develop neuron-like characteristics when treated with NGF or with pituitary adenylyl cyclase-activating peptide. Both NGF and pituitary adenylyl cyclase-activating peptide stimulate axon generation by stimulation of the small G-protein Rap1. It had

long been established that pituitary adenylyl cyclase-activating peptide stimulates Rap1 and axonegenesis through cAMP generated by the GPCR-G protein–tmAC pathway,[151] but the NGF-stimulating mechanism, and whether it involved cAMP, remained unclear.[152] The confirmation that PC12 cells express sAC and the observation that sAC inhibition by small molecules or siRNA blocked NGF-induced activation of Rap1 suggested that sAC is also involved in axon growth in response to NGF.[153] Similar to the studies demonstrating calcium involvement in glucose-induced sAC activation in pancreatic β-cells, NGF stimulation of sAC in PC12 cells is dependent upon calcium.

IMMUNE CELLS

Calcium regulation of sAC and signaling by the effector Rap1 were also found to have a role in the inflammatory response in neutrophils. sAC mRNA was found to be abundant in human leukocytes,[13] and neutrophils represent the most abundant type of white blood cell. Immunostaining and western blotting of highly purified neutrophils confirmed sAC protein presence.[154] sAC in neutrophils was essential for tumor necrosis factor-induced release of H_2O_2 (respiratory outburst). And, as seen in PC12 cells in response to NGF, sAC activation was shown to be dependent upon elevated intracellular Ca^{2+} and the proximal target of sAC-generated cAMP was Rap1.

BONE

Several sAC splice variants are present in osteoclasts and osteoblasts,[13] and mutations in the human *sAC* gene correlate with low spinal bone density.[30] Calcification by osteoblasts is intrinsically connected to HCO_3^- and Ca^{2+}, and the A/B status greatly influences mineralization.[155–157] Although these elements suggest a key role for sAC in bone biology, to date the only reported role of sAC in bone is in osteoclastogenesis.[28] Differentiation of RAW264.7 cells into osteoclasts (estimated from tartrate-resistant acid phosphatase staining and activity) is maximum in the absence of HCO_3^- in the medium, and it is sharply inhibited in the presence of 12 or 24 mmol/l HCO_3^-. Experiments using the sAC inhibitor 2CE or siRNA suggested that sAC is important for inhibiting osteoclast differentiation in high external [HCO_3^-], although interpretation of results is somewhat confused by similar inhibitory effects during (non-physiological) HCO_3^--free conditions. Bone density in cultured mouse calvaria was similarly promoted by high [HCO_3^-] in a 2CE-sensitive manner,[28] indicating that HCO_3^--sensing sAC is a physiologically relevant regulator of bone formation and/or reabsorption.

sAC EXPRESSION IN OTHER TISSUES, OF AS YET UNKNOWN FUNCTIONS

sAC mRNA and/or protein has been reported in almost every other tissue.[13,69] However, apart from the systems described above, in most cases the role of sAC has not been elucidated yet. Some interesting cases include placenta,[13,30,158] carotid body[159] (potential roles reviewed in ref. 24), and embryos.[69,160] Other organs and tissues where sAC mRNA has been detected include liver, muscle, thymus, spleen,[13,69] and ovary.[69]

CONCLUSIONS

sAC is the most recently identified source of cAMP inside animal cells, and it is directly modulated by HCO_3^- and Ca^{2+}. This allows sAC to function as a sensor of the external and intracellular A/B status, as well as a sensor of metabolically generated HCO_3^- from CO_2. In addition, sAC can be secondarily modulated by hormones that lead to increase intracellular [Ca^2] or [HCO_3^-]. Because cAMP is a ubiquitous intracellular signaling messenger, the potential physiological effects subjected to sAC modulation are multiple and they include protein directly sensitive to cAMP-like cyclic nucleotide-gated ion channels, exchange proteins activated by cAMP, as well as proteins sensitive to exchange proteins activated by cAMP signaling and PKA phosphorylation.

DISCLOSURE

All the authors declared no competing interests.

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