CO$_2$/HCO$_3^-$ and Calcium-regulated Soluble Adenylyl Cyclase as a Physiological ATP Sensor*

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**Background:** The affinity of soluble adenylyl cyclase (sAC) for its substrate ATP suggested that it might be sensitive to fluctuations in ATP.

**Results:** In sAC-overexpressing and glucose-responsive cells, sAC-generated cAMP reflects intracellular ATP levels.

**Conclusion:** sAC can be an ATP sensor inside cells.

**Significance:** sAC serves as a metabolic sensor via regulation by three cellular metabolites: ATP, bicarbonate, and calcium.

The second messenger molecule cAMP is integral for many physiological processes. In mammalian cells, cAMP can be generated from hormone- and G protein-regulated transmembrane adenylyl cyclases or via the widely expressed and structurally and biochemically distinct enzyme soluble adenylyl cyclase (sAC). sAC activity is uniquely stimulated by bicarbonate ions, and in cells, sAC functions as a physiological carbon dioxide, bicarbonate, and pH sensor. sAC activity is also stimulated by calcium, and its affinity for its substrate ATP suggests that it may be sensitive to physiologically relevant fluctuations in intracellular ATP. We demonstrate here that sAC can function as a cellular ATP sensor. In cells, sAC-generated cAMP reflects alterations in intracellular ATP that do not affect transmembrane AC-generated cAMP. In β cells of the pancreas, glucose metabolism generates ATP, which corresponds to an increase in cAMP, and we show here that sAC is responsible for an ATP-dependent cAMP increase. Glucose metabolism also elicits insulin secretion, and we further show that sAC is necessary for normal glucose-stimulated insulin secretion in vitro and in vivo.

In mammalian cells, the ubiquitous second messenger cAMP can be generated by two distinct forms of adenylyl cyclase: a family of transmembrane adenylyl cyclases (tmACs)$^2$ and soluble adenylyl cyclase (sAC). tmACs mediate cAMP-dependent responses downstream from hormones and neurotransmitters via G protein-coupled receptors and heterotrimeric G proteins. The nine members of the tmAC gene family share a common structural organization; they possess 12 transmembrane-spanning domains and localize to a variety of subcellular organelles and compartments (2). sAC activity is stimulated by Ca$^{2+}$ (3, 4) and directly regulated by bicarbonate (HCO$_3^-$) (4, 5). Due to the ubiquitous presence of carbonic anhydrases, which instantaneously equilibrate HCO$_3^-$ with carbon dioxide (CO$_2$) and pH, sAC functions as a physiological CO$_2$/HCO$_3^-$/pH$_e$ sensor (6).

As originally described, the in vitro activity of sAC isolated from mammalian testis was thought to require millimolar concentrations of ATP (7). After cloning and heterologously expressing sAC, we determined its $K_m$ for ATP, under physiological conditions, to be ~1 mM (4). Because intracellular ATP concentrations are in the millimolar range, we hypothesized that intracellular sAC activity and cAMP generation should be affected by even small physiologically relevant fluctuations of ATP. β cells secrete insulin in response to an elevation of blood glucose. This glucose-stimulated insulin secretion (GSIS) requires glycolytic and mitochondrial oxidative glucose metabolism (8). In β cells, glucose metabolism is coupled to an increase in the cytosolic ATP/ADP concentration ratio, which leads to the closure of ATP-sensitive K$^+$ ($K_{ATP}$) channels. $K_{ATP}$ channel closure depolarizes the cell membrane, leading to an influx of Ca$^{2+}$, which triggers insulin secretory granule exocytosis. Metabolism of glucose also produces CO$_2$ from the decarboxylation of pyruvate and the subsequent metabolism of

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2 The abbreviations used are: tmAC, transmembrane adenylyl cyclase; sAC, soluble adenylyl cyclase; sACt, truncated isoform of sAC; GSIS, glucose-stimulated insulin secretion; IBMX, 3-isobutyl-1-methylxanthine; FSK, forskolin; KO, knock-out.
acetyl-CoA in the Krebs cycle. Thus, glucose metabolism in β cells leads to the production of three intracellular signaling messengers (CO₂, ATP, and Ca²⁺) that are able to regulate sAC activity (9–11). We demonstrated previously that sAC is present in β cell-like insulinoma INS-1E cells and that it is responsible for generating cAMP in response to elevated glucose (9). We further showed that the glucose-dependent influx of Ca²⁺ is essential for sAC activation. Here, we explored whether intracellular sAC activity may also be sensitive to intracellular fluctuations in ATP.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—To create stably sAC-transfected HEK293 cells, HEK293 cells were transfected with plasmid containing the sAC, cDNA (12) and placed under selection pressure with gentamycin. After selection, single clones were established via dilution. Once single clones were grown for multiple generations, gentamycin was removed from the medium. Overexpression of sAC was periodically confirmed by Western blot or activity assay. INS-1E cells were cultured as described previously (13).

**In Vitro Cyclase Assays**—Adenylyl cyclase activity was measured in whole cell extracts (50 μg of protein) from HEK293 or sAC-stable cell lines in buffer containing 100 mM Tris-HCl (pH 7.5) in the presence of 1 mM ATP, 5 mM MgCl₂, and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) with or without 25 mM NaHCO₃ or 50 μM forskolin (FSK) for 15 min at 30 °C. cAMP generated was quantitated by enzyme immunoassay-correlate ELISA (Enzo Life Sciences).

**Intracellular ATP and cAMP Determinations**—For inhibition of ATP production, HEK293 or sAC-stable cell lines were treated for 10 min with the indicated drugs in the presence of 0.5 mM IBMX. For glucose stimulation of HEK293 and sAC-stable cell lines, cells were starved of glucose by incubation in glucose-free DMEM for 1 h prior to the experiment, and then at time 0, cells were changed into DMEM containing the indicated amount of glucose in the presence of 0.5 mM IBMX. Intracellular ATP was measured using exogenously supplied luciferase and luciferin (Promega). The intracellular cAMP concentration was measured in the same lysate using enzyme immunoassay-correlate ELISA. To measure cAMP accumulation due to the addition of HCO₃⁻ (see Fig. 1B), cells are grown in HEPES-buffered DMEM under ambient CO₂ for 1 h prior to HCO₃⁻ addition (and growth in 5% CO₂).

**Insulin Release from INS-1E Cells**—Insulin release from INS-1E cells was performed as described previously (14). INS-1E cells were incubated for 1 h in Krebs-Ringer buffer/HEPES in the presence of 2.5 mM glucose, followed by incubation in Krebs-Ringer buffer/HEPES with 2.5 or 16 mM glucose in the presence of either the vehicle control (Me₂SO or methanol) or the indicated addition. Secreted insulin was measured by insulin ELISA (LINCO). If cAMP was to be concomitantly measured, 0.5 mM IBMX was included in all incubations.

**RNAi**—RNAi oligonucleotides were purchased from Qiagen (high performance 2-for-1 silencing) and reconstituted at 20 μM following the manufacturer’s instructions. The sAC2 RNAi oligonucleotide (TCGGAGCATGATTGAAATCGA) was transfected into INS-1E cells using Opti-MEM I (Gene Therapy Systems). Western analysis was performed using anti-sAC monoclonal antibody R21. The same membrane was stripped and reprobed with anti-actin antiserum for normalization. Band intensities were quantitated using the Alpha Innotech FluorChem imaging system and normalized to actin. Control (non-specific oligonucleotide) lane intensity was set to 100%; duplicate transfections were averaged.

**Mouse Islet Isolation**—Because metabolic phenotypes are often affected by multiple genetic loci and are sensitive to strain differences, we backcrossed the Sacytm1Lex allele into the C57BL/6 genetic background and analyzed mutant mice following the 10th generation of backcrossing; we refer to these mice (i.e. Sacytm1Lex/Sacytm1Lex in the C57BL/6 genetic background) as sAC-C1 KO mice. WT and sAC KO C57BL/6 mice were used and allowed food and water ad libitum. After CO₂ asphyxiation, the pancreases were surgically removed, and the islets were isolated by collagenase digestion. The islets were cultured in RPMI 1640 medium with 11.1 mM glucose for 24 h prior to use.

**Insulin Secretion by Mouse Pancreatic Islets under Perifusion Conditions**—Krebs–Ringer buffer/HEPES containing 129 mmol/liter NaCl, 5 mmol/liter NaHCO₃, 4.8 mmol/liter KCl, 1.2 mmol/liter KH₂PO₄, 2.5 mmol/liter CaCl₂, 1.2 mmol/liter MgSO₄, and 10 mmol/liter HEPES (pH 7.4) supplemented with 0.1% bovine serum albumin was used for the studies. 20 islets were placed into each of the 70-μl perifusion chambers. An equilibration period of 30 min of perifusion with Krebs–Ringer buffer and 5 mM/liter glucose at 37 °C was followed by the test period. Samples for insulin measurement were collected at 1-min intervals at a flow rate of 1 ml/min. Insulin in the perfusate samples was measured by radioimmunoassay using a charcoal separation method (15).

**Intraperitoneal Glucose Tolerance Test**—After a 14–16-h overnight fast with free access to water, adult (3–7 months old) male sAC-C1 KO mice (Sacytm1Lex/Sacytm1Lex) and heterozygous littermates (Sacytm1Lex/Sacy⁺) were injected intraperitoneally with 1 g/kg glucose (20% solution), and blood was collected from the tail vein to measure glucose and insulin.

**Body Fat**—Body fat, lean tissue, and body water were determined via quantitative magnetic resonance (16).

**Immunohistochemistry of Pancreatic Sections**—Gross necropsy was performed on six WT, six heterozygous, and six sAC-C1 KO mice (three males and three females of each genotype). Pancreases from WT and sAC-C1 KO mice were fixed in 10% formalin and embedded in paraffin. Sections were immunostained using anti-insulin antibody (Cell Signaling) at 1:400 in heat induced epitope retrieval solution 1 (HIER1, Leica Microsystems) and visualized using alkaline phosphatase with mixed red substrate.

**RESULTS**

**Generation and Characterization of sAC-overexpressing Cell Lines**—Thus far, characterization of sAC-overexpressing cell lines has examined its *in vitro* activity using either native sAC protein purified or immunoprecipitated from testis (2, 7, 12, 17) or recombinant sAC enzyme (3–5, 12). These studies demonstrated that the affinity of sAC for its substrate ATP is ~1 mM, which is close to the levels found inside cells (18). To examine
whether cellular sAC activity might be sensitive to fluctuations of ATP inside cells, we required a cellular system in which we could measure cAMP generation due to sAC. In most cells and tissues, sAC is expressed alongside tmACs, making it difficult to discern the specific contribution of sAC to total cAMP generation. The only exceptions are male germ cells and testis cytosol, where sAC is expressed alongside tmACs, making it difficult to discern the specific contribution of sAC to total cAMP generation. In most cells and tissues, sAC is expressed alongside tmACs, making it difficult to discern the specific contribution of sAC to total cAMP generation. In most cells and tissues, sAC is expressed alongside tmACs, making it difficult to discern the specific contribution of sAC to total cAMP generation. In most cells and tissues, sAC is expressed alongside tmACs, making it difficult to discern the specific contribution of sAC to total cAMP generation.

To study the effect of intracellular ATP concentration fluctuations on the activity of the different forms of adenyl cyclase, we measured cAMP accumulation when cellular ATP levels were diminished. The pharmacological ATP synthase inhibitor oligomycin lowered intracellular ATP levels in both parental and sAC-overexpressing cells, with a similar dose dependence (Fig. 2A). Although FSK-stimulated cAMP accumulation in parental cells (which reflects tmAC activity) was not appreciably affected (Fig. 2B, black line), in concert with the decrease in intracellular ATP (Fig. 2A).

We confirmed that the decreased sAC-dependent accumulation was due to alterations in ATP by using other means of inhibiting ATP production. 2-Deoxyglucose inhibits glycolysis,
FIGURE 2. Cellular sAC activity is dependent on intracellular ATP levels. Intracellular ATP (A, C, E, and G) and cAMP (B, D, F, and H) accumulated in parental HEK293 cells and the sAC-stable clones 1-1, 3-2, and 4-4 were measured 10 min after incubation in 0.5 mM IBMX and the indicated concentrations of oligomycin (A and B), 2-deoxyglucose (2DG; C and D), rotenone (E and F), and azide (G and H). For HEK293 cells, in all intracellular measurements, 50 μM FSK was included to stimulate cellular tmACs. Values were normalized to each cell line in the absence of ATP inhibitors. Over all experiments, HEK293 cells in the presence of 50 μM FSK averaged 14,016 ± 2498 relative absorbance units for ATP and 5.75 ± 1.5 pmol of cAMP/2.5 × 10^5 cells/10 min; sAC-stable clone 1-1 averaged 12,748 ± 1899 relative absorbance units for ATP and 2.10 ± 0.33 pmol of cAMP/2.5 × 10^5 cells/10 min; sAC-stable clone 3-2 averaged 12,851 ± 1688 relative absorbance units for ATP and 3.49 ± 0.37 pmol of cAMP/2.5 × 10^5 cells/10 min; and sAC-stable clone 4-4 averaged 12,076 ± 1829 relative absorbance units for ATP and 2.54 ± 0.39 pmol of cAMP/2.5 × 10^5 cells/10 min.
ATP Sensing by CO$_2$/HCO$_3^-$ and Ca$^{2+}$-regulated sAC

**FIGURE 4.** CAMP levels in INS-1E cells are dependent on intracellular ATP levels. A–C, intracellular ATP, accumulation of intracellular CAMP, and insulin release, respectively, in INS-1E cells in the presence of 16 mM glucose, 0.5 mM IBMX, and the indicated concentrations of azide in the presence (◇) or absence (□) of 28 μM KCl. D, intracellular CAMP in INS-1E cells accumulated in the presence of 0.5 mM IBMX and the indicated concentrations of azide in the presence or absence of a partially inhibitory concentration of KH7 (30 μM; IC$_{50}$ = 27 μM (55)). Data presented are averages from duplicate determinations of a representative experiment performed at least three times.

whereas rotenone and azide inhibit Complexes I and IV of the respiratory chain, respectively. In all cases, down-regulation of intracellular ATP diminished the accumulation of cAMP in sAC-overexpressing cell lines (i.e. sAC-dependent CAMP accumulation) while leaving the levels of FSK-stimulated cAMP accumulation in HEK293 cells (i.e. tmA-C-dependent CAMP) virtually unchanged (Fig. 2, C–H). Thus, consistent with its in vitro affinity for its substrate ATP, cellular sAC activity is sensitive to changes in intracellular ATP levels, whereas tmACs, which exhibit much higher affinities for substrate ATP, appear to be insensitive to these fluctuations in intracellular ATP.

**Overexpression of sAC Confers Glucose Responsiveness to HEK293 Cells**—sAC activity in the sAC-overexpressing cells also responded to glucose-induced increases in intracellular ATP. Feeding glucose to starved parental or sAC-overexpressing cells raised the intracellular ATP concentration (Fig. 3A).

![Image](image-url)

**FIGURE 3.** Overexpression of sAC confers glucose responsiveness to HEK293 cells. Intracellular ATP (A) and cAMP (B) accumulated in parental HEK293 cells (□) and sAC-stable clones 1-1 (◇), 3-2 (○), and 4-4 (○) were measured after a 20-min incubation in the presence of 0.5 mM IBMX and the indicated concentrations of glucose. Values were normalized to each cell line in the absence of glucose. For ATP, HEK293 cells in the presence of 50 μM FSK averaged 8473 relative absorbance units; sAC-stable clone 1-1 averaged 3923 relative absorbance units; and sAC-stable clone 3-2 averaged 3870 relative absorbance units; and sAC-stable clone 4-4 averaged 3923 relative absorbance units. Values for cAMP are as given in Fig. 2.

Although this increase had no effect on FSK-stimulated cAMP levels in parental HEK293 cells, feeding glucose to starved sAC-overexpressing cells resulted in a 2-fold elevation of cAMP accumulation (Fig. 3B). Thus, overexpressing CO$_2$/HCO$_3^-$, ATP$^-$, and Ca$^{2+}$-$^-$-responsive sAC converted cells that do not modulate CAMP in response to glucose (i.e. HEK293 cells) into glucose-responsive cells. Interestingly, the ATP levels in the sAC-overexpressing cell lines were elevated relative to those in the parental HEK293 cells (Fig. 3A); this could reflect a contribution from increased expression of intramitochondrial sAC, which has been shown to stimulate ATP production (23–26).

**cAMP Levels in INS-1E Cells Reflect Intracellular ATP Levels**—We next explored whether sAC might sense physiologically relevant changes in ATP in pancreatic β cells, which are naturally glucose-responsive. In high glucose, β cells have elevated ATP and cAMP levels (10, 27). We demonstrated previously that sAC is expressed in insulinoma INS-1E cells and that the glucose-induced rise in cAMP in INS-1E cells (9, 28, 29) is due to sAC in a Ca$^{2+}$-$^-$-dependent manner (9). As expected, in high glucose, the ATP levels were sensitive to the respiratory chain inhibitor azide (Fig. 4A), and similar to the CAMP generated in sAC-overexpressing HEK293 cells, diminishing ATP levels with azide also blunted the CAMP accumulation (Fig. 4B) and insulin release (Fig. 4C) in INS-1E cells. Although the sAC-specific inhibitor KH7 inhibits glucose-dependent CAMP (9), it did not alter the azide-insensitive CAMP pool (Fig. 4D), suggesting that sAC is responsible for ATP-dependent CAMP generation.

**Glucose-induced CAMP Generated by sAC Is Dependent on Both ATP and Ca$^{2+}$**—We showed previously that the glucose-induced CAMP generation in INS-1E cells due to sAC is dependent on the Ca$^{2+}$ influx subsequent to K$_{ATP}$ channel closure (9). The addition of high concentrations of extracellular KCl depolarizes the β cell membrane independent of ATP (10), and we showed previously that KCl-induced Ca$^{2+}$ influx is sufficient to activate sAC (9). In high glucose, KCl addition did not further stimulate CAMP accumulation (Fig. 4B) or insulin release (Fig. 4C) presumably because INS-1E cells are maximally depolarized. Reducing ATP levels with azide, which will diminish glucose-dependent depolarization, allowed KCl addition to enhance CAMP accumulation and insulin release. However, the sAC-dependent CAMP accumulation was still dependent on cellular ATP levels. The addition of ≥2 mM azide completely blocked the KCl-dependent depolarization-induced CAMP accumulation (Fig. 4B). Thus, in β cell-like INS-1E cells, glucose-stimulated sAC activity is dependent on both Ca$^{2+}$ (9) and ATP (Fig. 4).

**sAC Is Necessary for Physiological Glucose-induced Insulin Release in Vitro and in Vivo**—In INS-1E cells, as in β cells, glucose metabolism leads to insulin release (13) in an ATP-, Ca$^{2+}$-, and CAMP-dependent manner (Fig. 4) (10). We tested whether sAC might contribute to GSIS as an ATP- and Ca$^{2+}$-dependent source of CAMP. INS-1E cells incubated in high glu-
cose (16 mM) secreted more insulin than when they were grown in low glucose (2.5 mM) (Fig. 5). This GSIS was blocked by the sAC-selective inhibitor KH7 in a concentration-dependent manner (Fig. 5A) but not by KH7.15 (Fig. 5B), which is a compound structurally related to KH7 but inert against sAC (30).

Because KH7 has been ascribed cAMP-independent metabolic effects (25, 31), we confirmed its effects in INS-1E cells using a structurally unrelated sAC-selective small molecule inhibitor, catechol estrogen (32), and sAC-specific RNAi. Like KH7, catechol estrogen inhibited GSIS (Fig. 5B) in a concentration-de-
pendent manner (Fig. 5C), whereas sAC-specific RNAi, which resulted in ~50% knockdown of sAC protein (Fig. 5D), diminished glucose-induced cAMP generation (Fig. 5E) and insulin secretion (Fig. 5F) by ~50%.

We next examined the role of sAC in insulin release using sACKO mice (Sacytm1Lex/Sacytm1Lex), sAC null mice were originally described to exhibit male-specific sterility (19, 20). More recently, after backcrossing the Sacytm1Lex allele into the C57BL/6 genetic background, these sAC-C1 KO mice were shown to also exhibit increased intraocular pressure (33) and altered metabolic communication between astrocytes and neurons (34). Gross necropsy revealed no differences in pancreatic morphology between WT, heterozygous, and sAC-C1 KO animals, and immunohistochemical analyses confirmed that the islets in sAC-C1 KO pancreas had a normal structure and no overt morphological change compared with pancreatic islets from WT or heterozygous littermates (Fig. 5G). In isolated islets, elevated glucose leads to two phases of insulin release (35): a first phase, characterized by a peak reached after 3 min, is followed by a sustained second phase, which is apparent after 10 min (Fig. 5H). In islets isolated from sAC-C1 KO mice (20, 36), elevated glucose elicited both the first and second phases of insulin secretion; however, the rate of insulin secretion from the sAC-C1 KO islets was diminished, but not abolished, in both phases. Diminished GSIS was confirmed in vivo in sAC-C1 KO mice compared with their heterozygous littermates. In an intraperitoneal glucose tolerance test performed on sAC-C1 KO mice and their heterozygous littermates (i.e. Sacytm1Lex/Sacy−/−), sAC-C1 KO mice (n = 8) displayed elevated glucose and delayed glucose clearance (Fig. 5I) and diminished insulin release (Fig. 5J) compared with heterozygous littermates (n = 8). Thus, CO2/HCO3−/pH−, Ca2++, and ATP-regulated sAC is needed for normal glucose-stimulated insulin release in mice. Along with their altered glucose kinetics, 4.5-month-old male sAC-C1 KO mice (27.93 ± 1.85 g, n = 10) weighed more than their WT male littermates (24.92 ± 0.49 g, n = 5; p < 0.001) and exhibited an elevated percentage of body fat (5.0 ± 0.64% for sAC-C1 KO mice compared with 2.6 ± 0.53% for WT littermates; p < 0.001). It is unclear whether their elevated weight is related to the altered GSIS or whether there are additional metabolic defects in mice missing CO2/HCO3−/pH−, Ca2++, and ATP-regulated sAC that contribute to their increased body fat and weight.

**DISCUSSION**

We demonstrated previously that the in vitro activity of sAC is sensitive to physiological levels of ATP (4), and we confirmed here that, inside cells, sAC activity reflects fluctuations in ATP levels. As the primary source of energy in cells, ATP is the currency of life, and nature has evolved numerous mechanisms for sensing ATP to ensure that cells have sufficient energy stores. Many known ATP sensors are active when ATP levels are low. For example, when ATP is low, the ratio of ADP or AMP to ATP increases, and the AMP kinase is activated to stimulate cellular metabolism (37). Similarly, the nitric oxide-responsive soluble guanylyl cyclase is inhibited by high ATP levels (38), as is the KATP channel in pancreatic β cells. Closure of KATP channels by high ATP depolarizes the β cell, eliciting a Ca2+ influx that leads to insulin secretion (27). Our data reveal that sAC is a different kind of ATP sensor; sAC is less active when ATP levels are diminished. Thus, ATP sensing by sAC seems to be designed to ensure that certain physiological processes proceed only when ATP is sufficient. In addition to its millimolar Km for substrate ATP, in vitro sAC activity is inhibited when ATP levels are very high (4); thus, sAC might serve as an ATP sensor, allowing certain pathways to proceed only when ATP levels are at an optimal level. Also, because intramitochondrial sAC regulates the electron transport chain (23–26), sAC represents an ATP sensor that can regulate ATP generation.

With this study, sAC has now been shown to sense metabolism inside cells via three physiological signals: CO2/HCO3− (24, 34), Ca2+ (9, 25), and ATP. In the brain, sAC is most abundant in astrocytes (34, 36). Neuronal activity stimulates HCO3− transport in a nearby astrocyte, and the consequent CO2/HCO3− elevation stimulates sAC, which promotes the astrocyte to “feed” the active neuron (34). sAC is also found in the mitochondrial matrix (2, 24–26), where it is regulated by tricarboxylic acid cycle-derived CO2/HCO3− (24) and by cytoplasmic Ca2++ (25). Intramitochondrial sAC-generated cAMP increases the rate of flux through the electron transport chain, linking nutrient utilization with ATP production (24–26). Finally, sAC senses glucose metabolism in pancreatic β cells (9).

In β cells, glucose metabolism leads to the production of all three intracellular messengers (CO2, ATP, and Ca2++) that regulate sAC (3–5). We demonstrated previously in INS-1E cells that glucose-dependent activation of sAC requires elevation of intracellular Ca2++ via voltage-dependent Ca2++ channels (9). We have shown here that sAC also senses the glucose-elicted rise in intracellular ATP; thus, INS-1E cell sAC senses at least two of the intracellular messengers generated by glucose metabolism. The third glucose-derived signal, metabolically generated CO2, has been suggested to be important for insulin release (39), and we have shown that, inside mitochondria, metabolically generated CO2 modulates sAC activity (24). However, it is difficult to discern whether CO2 and HCO3− contribute to the regulation of sAC in INS-1E cells because CO2 and Ca2++ are linked to ATP levels via sAC. Tricarboxylic acid cycle-derived CO2 (24) and cytoplasmically derived Ca2++ (25) stimulate intramitochondrial sAC to increase ATP production. Other specialized tissues, such as the liver and adipose tissue, are also sensitive to alterations in nutritional availability. These tissues sense changes in their metabolism and respond by inducing gene expression or release of hormones. cAMP levels are associated with these metabolic changes, and the second messenger is implicated in the regulation of both hormone release and gene expression within these tissues. In the liver, sAC is found in both mitochondria (24) and the nucleus (40). We already know that intramitochondrial sAC links nutritional availability with activity of the electron transport chain (23–25). Nuclear sAC regulates the activity of CREB (cAMP-responsive element-binding protein), and it is intriguing to speculate that metabolic regulation of nuclear sAC might provide a link between nutritional availability and changes in gene expression.

It remains unclear how sAC-generated cAMP affects insulin release. Agents that elevate cAMP via stimulation of tmACs, such as the incretin hormones GIP and GLP-1, potentiate GSIS
ATP Sensing by CO\(_2\)/HCO\(_3\)- and Ca\(^{2+}\)-regulated sAC

(41), but this appears to be distinct from glucose-stimulated cAMP (10, 41). Early studies proposed that cAMP derived from glucose metabolism directly mediated the action of glucose to elicit insulin secretion (42, 43). These studies were largely forgotten until recent in vivo imaging using cAMP biosensors revealed that glucose stimulates compartmentalized cAMP production in β cells (28, 29, 44). What had yet to be determined is exactly how this cAMP production is stimulated by glucose metabolism (10). As mentioned above, sAC-defined microdomains regulate activation of transcription factors in the nucleus (40) and mitochondrial metabolism (24–26). sAC also plays an evolutionarily conserved role mobilizing vesicles containing the proton-pumping vacuolar ATPase, allowing for the movement of protons out the appropriate side of polarized epithelia (6, 45–47). In β cells, elevated cAMP is linked to the mobilization and fusion of insulin-containing secretory granules (29, 48–54), and sAC, which is localized throughout the cytoplasm of INS-1E cells (9), is well situated to provide the cAMP that mobilizes insulin secretory vesicles. Organization into discrete microdomains means that these possible roles are not mutually exclusive; nuclear, mitochondrial, or cytoplasmic sAC, regulated by Ca\(^{2+}\), HCO\(_3\)-, and/or ATP, may make unique or complementary contributions to insulin synthesis, ATP levels, or insulin secretion.

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REFERENCES

1. Kamenetsky, M., Middelhaute, S., Bank, E. M., Levin, L. R., Buck, J., and Steegborn, C. (2006) Molecular details of cAMP generation in mammalian cells: a tale of two systems. J. Mol. Biol. 362, 623–639
2. Zippin, J. H., Chen, Y., Nahirney, P., Kamenetsky, M., Wuttke, M. S., Fischman, D. A., Levin, L. R., and Buck, J. (2003) Compartmentalization of bicarbonate-sensitive adenylyl cyclase in distinct signaling microdomains. PFAEB J. 17, 82–84
3. Jaiswal, B. S., and Conti, M. (2003) Calcium regulation of the soluble adenylyl cyclase expressed in mammalian spermatozoa. Proc. Natl. Acad. Sci. U.S.A. 100, 10676–10681
4. Litvin, T. N., Kamenetsky, M., Zarifyan, A., Buck, J., and Levin, L. R. (2003) Kinetic properties of “soluble” adenylyl cyclase. Synergism between calcium and bicarbonate. J. Biol. Chem. 278, 15922–15926
5. Chen, Y., Kann, M. J., Litvin, T. N., Iourgenko, V., Sinclair, M. L., Levin, L. R., and Buck, J. (2000) Soluble adenylyl cyclase as an evolutionarily conserved bicarbonate sensor. Science 289, 625–628
6. Bresguerres, M., Buck, J., and Levin, L. R. (2010) Physiological carbon dioxide, bicarbonate, and pH sensing. Pflugers Arch. Eur. J. Physiol. 460, 953–964
7. Braun, T. (1991) Purification of soluble form of adenylyl cyclase from mammalian spermatozoa. Proc. Natl. Acad. Sci. U.S.A. 88, 1329–1333
8. Jensen, M. V., Joseph, J. W., Ronnebaum, S. M., Burgess, S. C., Sherry, A. D., and Newgard, C. B. (2008) Metabolic cycling in control of glucose-stimulated insulin secretion. Am. J. Physiol. Endocrinol. Metab. 295, E1287–E1297
9. Ramos, L. S., Zippin, J. H., Kamenetsky, M., Buck, J., and Levin, L. R. (2008) Glucose and GIP-1 stimulate cAMP production via distinct adenylyl cyclases in INS-1E insulinoma cells. J. Gen. Physiol. 132, 329–338
10. Tengholm, A. (2012) Cyclic AMP dynamics in the pancreatic β-cell. LIPS J. Med. Sci. 117, 355–369
11. Zippin, J. H., Levin, L. R., and Buck, J. (2001) CO\(_2\)/HCO\(_3\)-responsive soluble adenylyl cyclase as a putative metabolic sensor. Trends Endocrinol. Metab. 12, 366–370
12. Buck, J., Sinclair, M. L., Schapal, L., Cann, M. J., and Levin, L. R. (1999) Cytosolic adenylyl cyclase defines a unique signaling molecule in mammals. Proc. Natl. Acad. Sci. U.S.A. 96, 79–84
13. Merglen, A., Theander, S., Rubi, B., Chaffard, G., Wollheim, C. B., and Maechler, P. (2004) Glucose sensitivity and metabolism-secretion coupling studied during two-year continuous culture in INS-1E insulinoma cells. Endocrinology 145, 667–678
14. Antinozzi, P. A., Ishihara, H., Newgard, C. B., and Wollheim, C. B. (2002) Mitochondrial metabolism sets the maximal limit of fuel-stimulated insulin secretion in a model pancreatic beta cell. A survey of four fuel secretagogues. J. Biol. Chem. 277, 11746–11755
15. Herbert, V., Lau, K. S., Gottlieb, C. W., and Bleicher, S. J. (1965) Coated charcoal immun assay of insulin. J. Clin. Endocrinol. Metab. 25, 1375–1384
16. Tinsley, F. C., Taicher, G. Z., and Heiman, M. L. (2004) Evaluation of a quantitative magnetic resonance method for mouse whole body composition analysis. Obes. Res. 12, 150–160
17. Braun, T. (1975) The effect of divalent cations on bovine spermatozoal adenyl cyclase activity. J. Cyclic Nucleotides Res. 1, 271–281
18. Traut, T. W. (1994) Physiological concentrations of purines and pyrimidines. Mol. Cell. Biochem. 140, 1–22
19. Esposito, G., Jaiswal, B. S., Xie, F., Krajnc-Franken, M. A., Robben, T. J., Strik, A. M., Kuli, C., Philipsen, R. L., van Duin, M., Conti, M., and Gossen, J. A. (2004) Mice deficient for soluble adenylyl cyclase are infertile because of a severe sperm-motility defect. Proc. Natl. Acad. Sci. U.S.A. 101, 2993–2998
20. Hess, K. C., Jones, B. H., Marquez, B., Chen, Y., Ord, T. S., Kamenetsky, M., Miyamoto, C., Zippin, J. H., Kopf, G. S., Suarez, S. S., Levin, L. R., Williams, C. J., Buck, J., and Moss, S. B. (2005) The “soluble” adenylyl cyclase in sperm mediates multiple signaling events required for fertilization. Dev. Cell 9, 249–259
21. Neer, E. J. (1978) Multiple forms of adenylate cyclase. Adv. Cyclic Nucleotide Res. 9, 69–83
22. Jaiswal, B. S., and Conti, M. (2001) Identification and functional analysis of splice variants of the germ cell soluble adenylyl cyclase. J. Biol. Chem. 276, 31698–31708
23. Acin-Perez, R., Salazar, E., Kamenetsky, M., Buck, J., Levin, L. R., and Manfredi, G. (2009) Cyclic AMP produced inside mitochondria regulates oxidative phosphorylation and prevention of cytochrome oxidase inhibition by ATP: coupled mechanisms of energy metabolism regulation. Cell Metab. 13, 712–719
24. Acin-Perez, R., Gatti, D. L., Bai, Y., and Manfredi, G. (2011) Protein phosphorylation and prevention of cytochrome oxidase inhibition by ATP: coupled mechanisms of energy metabolism regulation. Cell Metab. 9, 265–276
25. Di Benedetto, G., Scalzotto, E., Mongillo, M., and Pozzan, T. (2013) Mitochondrial Ca\(^{2+}\) uptake induces cyclic AMP generation in the matrix and modulates organelle ATP levels. Cell Metab. 17, 965–975
26. Lefkimiatti, K., Leronni, D., and Hofer, A. M. (2013) The inner and outer compartments of mitochondria are sites of distinct CAMP/PKA signaling dynamics. J. Cell Biol. 202, 453–462
27. Rutter, G. A. (2001) Nutrient-secretion coupling in the pancreatic islet β-cell: recent advances. Mol. Aspects Med. 22, 247–284
28. Dyachok, O., Isakov, Y., Sägetorp, J., and Tengholm, A. (2006) Oscillations of cyclic AMP in hormone-stimulated insulin-secreting β-cells. Nature 439, 349–352
29. Dyachok, O., Idevall-Hagren, O., Sägetorp, J., Tian, G., Wuttke, A., Arrieux, C., Aksamijärvi, G., Gyle, E., and Tengholm, A. (2008) Glucose-induced cyclic AMP oscillations regulate pulsatile insulin secretion. Cell Metab. 8, 26–37
30. Wu, K. Y., Zippin, J. H., Huron, D. R., Kamenetsky, M., Hengst, U., Buck, J., Levin, L. R., and Jaffrey, S. R. (2006) Soluble adenylyl cyclase is required for netrin-1 signaling in nerve growth cones. Nat. Neurosci. 9, 1257–1264
31. Tian, G., Sandler, S., Gyle, E., and Tengholm, A. (2011) Glucose- and hormone-induced CAMP oscillations in α- and β-cells within intact pancreatic islets. Diabetes 60, 1535–1543
32. Steegborn, C., Litvin, T. N., Hess, K. C., Capper, A. B., Taussig, R., Buck, J., Levin, L. R., and Wu, H. (2005) A novel mechanism for adenylyl cyclase
inhibition from the crystal structure of its complex with catechol estrogen. J. Biol. Chem. 280, 31754–31759
33. Lee, Y. S., Tresguerres, M., Hass, K., Marmostein, L. Y., Levin, L. R., Buck, J., and Marmostein, A. D. (2011) Regulation of anterior chamber drainage by bicarbonate-sensitive soluble adenylyl cyclase in the ciliary body. J. Biol. Chem. 286, 41335–41358
34. Choi, H. R., Gordon, G. R., Zhou, N., Tai, C., Rungta, R. L., Martinez, J., Milner, T. A., Ryu, J. K., McLarnon, J. G., Tresguerres, M., Levin, L. R., Buck, J., and MacVicar, B. A. (2012) Metabolic communication between astrocytes and neurons via bicarbonate-responsive soluble adenylyl cyclase. Neuron 75, 1094–1104
35. Straub, S. G., and Sharp, G. W. (2002) Glucose-stimulated signaling pathways in biphasic insulin secretion. Diabetes Metab. Res. Rev. 18, 451–463
36. Parkkila, A. K., Scarim, A. L., Parkkila, S., Waheed, A., Corbett, J. A., and McLarnon, J. G., Tresguerres, M., Levin, L. R., and Buck, J. (2013) Neuronal expression of soluble adenylyl cyclase in the mammalian brain. Brain Res. 1518, 1–8
37. Hardie, D. G. (2003) Mini-review: the AMP-activated protein kinase cascade: the key sensor of cellular energy status. Endocrinology 144, 5179–5183
38. Ruiz-Stewart, I., Tiyyagura, S. R., Lin, J. E., Kazeronian, S., Pitar, G. M., Schulz, S., Martín, E., Murad, F., and Waldman, S. A. (2004) Guanylyl cyclase is an ATP sensor coupling nitric oxide signaling to cell metabolism. Proc. Natl. Acad. Sci. U.S.A. 101, 37–42
39. Parkkila, A. K., Scarim, A. L., Parkkila, S., Waheed, A., Corbett, J. A., and Sly, W. S. (1998) Expression of carbonic anhydrase V in pancreatic beta cells suggests role for mitochondrial carbonic anhydrase in insulin secretion. J. Biol. Chem. 273, 24620–24623
40. Zippin, J. H., Farrell, J., Huron, D., Kamenetsky, M., Hess, K. C., Fischman, D. A., Levin, L. R., and Buck, J. (2004) Bicarbonate-responsive “soluble” adenylyl cyclase defines a nuclear cAMP microdomain. J. Cell Biol. 164, 527–534
41. Seino, S., Shibusaki, T., and Minami, K. (2011) Dynamics of insulin secretion and the clinical implications for obesity and diabetes. J. Clin. Invest. 121, 2118–2125
42. Charles, M. A., Fanska, R., Schmid, F. G., Forsham, P. H., and Grodsky, G. M. (1973) Adenosine 3′,5′-monophosphate in pancreatic islets: glucose-induced insulin release. Science 179, 569–571
43. Charles, M. A., Lawecki, J., Pictet, R., and Grodsky, G. M. (1975) Insulin secretion. Interrelationships of glucose, cyclic adenosine 3′,5′-monophosphate, and calcium. J. Biol. Chem. 250, 6134–6140
44. Landa, L. R., Jr., Harbeck, M., Kihara, K., Chepurny, O., Kitiphongsapattana, K., Graf, O., Nikolaev, V. O., Lohse, M. J., Holz, G. G., and Roe, M. W. (2005) Interplay of Ca2+ and cAMP signaling in the insulin-secreting MIN6 β-cell line. J. Biol. Chem. 280, 31294–31302
45. Pastor-Soler, N., Beaulieu, V., Litvin, T. N., Da Silva, N., Chen, Y., Brown, D., Buck, J., Levin, L. R., and Breton, S. (2003) Bicarbonate-regulated adenylyl cyclase (sAC) is a sensor that regulates pH-dependent V-ATPase recycling. J. Biol. Chem. 278, 49523–49529
46. Păunescu, T. G., Da Silva, N., Russo, L. M., McKee, M., Lu, H. A., Breton, S., and Brown, D. (2008) Association of soluble adenylyl cyclase with the V-ATPase in renal epithelial cells. Am. J. Physiol. Renal Physiol. 294, F130–F138
47. Păunescu, T. G., Ljuboevic, M., Russo, L. M., Winter, C., McLaughlin, M. W., Wagner, C. A., Breton, S., and Brown, D. (2010) cAMP stimulates apical V-ATPase accumulation, microvillar elongation, and proton extrusion in kidney collecting duct A-intercalated cells. Am. J. Physiol. Renal Physiol. 298, F643–F654
48. Ammála, C., Ashcroft, F. M., and Rorsman, P. (1993) Calcium-independent potentiation of insulin release by cyclic AMP in single β-cells. Nature 363, 356–358
49. Kang, G., Joseph, J. W., Chepurny, O. G., Monaco, M., Wheeler, M. B., Bos, J. L., Schwede, F., Genieser, H. G., and Holz, G. G. (2003) Epac-selective cAMP analog 8-pCPT-2′-O-Me-cAMP as a stimulus for Ca2+-induced Ca2+ release and exocytosis in pancreatic β-cells. J. Biol. Chem. 278, 8279–8285
50. Renström, E., Eliasson, L., and Rorsman, P. (1997) Protein kinase A-dependent and -independent stimulation of exocytosis by cAMP in mouse pancreatic β-cells. J. Physiol. 502, 105–118
51. Holz, G. G. (2004) Epac: a new cAMP-binding protein in support of glucagon-like peptide-1 receptor-mediated signal transduction in the pancreatic β-cell. Diabetes 53, 5–13
52. Ozaki, N., Shibasaki, T., Kashima, Y., Miki, T., Takahashi, K., Ueno, H., Sunaga, Y., Yano, H., Matsuura, Y., Iwanaga, T., Takai, Y., and Seino, S. (2000) cAMP-GEFII is a direct target of cAMP in regulated exocytosis. Nat. Cell Biol. 2, 805–811
53. Shibasaki, T., Sunaga, Y., Fujimoto, K., Kashima, Y., and Seino, S. (2004) Interaction of ATP sensor, cAMP sensor, Ca2+ sensor, and voltage-dependent Ca2+ channel in insulin granule exocytosis. J. Biol. Chem. 279, 7956–7961
54. Fujimoto, K., Shibasaki, T., Yokoi, N., Kashima, Y., Matsumoto, M., Sasaki, T., Tajima, N., Iwanaga, T., and Seino, S. (2002) Piccolo, a Ca2+ sensor in pancreatic β-cells. Involvement of cAMP-GEFII-Rim2-Piccolo complex in cAMP-dependent exocytosis. J. Biol. Chem. 277, 50497–50502
55. Bitterman, J., Ramos-Espiritu, L. S., Diaz, A., Levin, L. R., and Buck, I. (2013) Pharmacological distinction between soluble and transmembrane adenylyl cyclases. J. Pharmacol. Exp. Ther. 10.1124/jpet.113.208496