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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 on the plasma membrane. They differ in their tissue distributions and regulation by other second messengers and kinases (1). In contrast, sAC lacks transmembrane domains and localizes 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_i, sAC functions as a physiological $CO_2/HCO_3^-/pH_i$ 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; sAC_t, 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 (CO_2, ATP, and Ca^{2+}) 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 Ca^{2+} 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_t 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_t 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 $MgCl_2$, and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) with or without 25 mM $NaHCO_3$ 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 HCO_3^- (see Fig. 1*B*), cells are grown in HEPES-buffered DMEM under ambient CO_2 for 1 h prior to HCO_3^- addition (and growth in 5% CO_2).

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 (Me_2SO 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 antisera 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 $Sacy^{tm1Lex}$ allele into the C57BL/6 genetic background and analyzed mutant mice following the 10th generation of backcrossing; we refer to these mice (*i.e.* $Sacy^{tm1Lex}/Sacy^{tm1Lex}$ 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 CO_2 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 $NaHCO_3$, 4.8 mmol/liter KCl, 1.2 mmol/liter KH_2PO_4, 2.5 mmol/liter $CaCl_2$, 1.2 mmol/liter $MgSO_4$, 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 mmol/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 perifusate 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 ($Sacy^{tm1Lex}/Sacy^{tm1Lex}$) and heterozygous littermates ($Sacy^{tm1Lex}/Sacy^{+}$) 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 necroscopy 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 adenylyl cyclase activity has examined its *in vitro* activity using either native sAC protein purified or immunoprecipitated from testis (3, 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

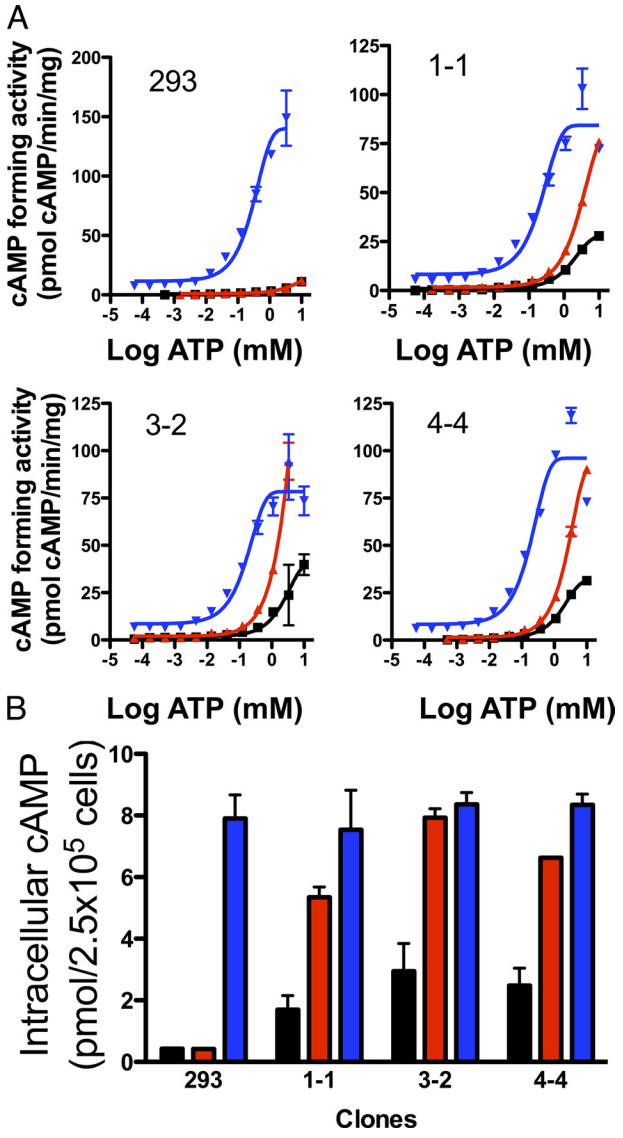

FIGURE 1. **Characterization of sAC_t-overexpressing stable cell lines.** *A*, adenylyl cyclase activities as a function of increasing substrate (ATP) in whole cell extracts (50 μg of protein) from parental HEK293 cells or the independent stably sAC_t-transfected clones 1-1, 3-2, and 4-4 measured in the presence of no activators (*black*), 25 mM $NaHCO_3$ (*red*), or 50 μM FSK (*blue*). Values are specific activities (picomoles of cAMP formed per min/mg of total protein). *B*, cAMP accumulated over 10 min in the presence of 0.5 mM IBMX in parental HEK293 cells or three independent stably sAC_t-transfected clones measured in the presence of no activators (*black*), 25 mM $NaHCO_3$ (*red*), or 50 μM FSK (*blue*).

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 (19–21), but these sources are impractical for cell biological studies. Therefore, to specifically study sAC-dependent cAMP synthesis in a cellular context, we generated three independent stable cell lines overexpressing the sAC_t isoform (12, 22). We overexpressed sAC in HEK293 cells, which are not known to generate cAMP in response to changes in ATP. Overexpression of sAC protein in stable clones was demonstrated by Western blotting (data not shown) and by cyclase assay (Fig. 1*A*). The basal *in vitro* cAMP-producing activity in whole cell extracts of the parental HEK293 cells was insensitive to HCO_3^- addition but was stimulated by the tmAC-specific activator FSK (Fig. 1*A*). This FSK-stimulated activity was detectable at MgATP concentrations starting at 10 μM, commensurate with the known affinities of tmACs for substrate (their K_m values for MgATP are ~100 μM) (1). In whole cell extracts of the three stable sAC_t-overexpressing clones, the basal activities were elevated relative to those in parental HEK293 cells and were potently stimulated by the addition of HCO_3^-. The basal and HCO_3^--stimulated activities in each clone were first observed as ATP levels reached 100 μM and increased with ATP concentrations up to 10 mM (the highest value used) without reaching a plateau. Thus, the contribution of sAC to cAMP generation, reflected in the elevated basal and bicarbonate-stimulated activities in sAC-overexpressing stables, required at least 10-fold higher ATP concentrations compared with tmACs, consistent with the relatively high K_m of sAC for ATP (4). Of note, when assayed in whole cell lysates, the stably overexpressed sAC_t isoform did not show inhibition at high ATP concentrations (in excess of 5 mM) as observed with purified sAC_t (4).

Cellular sAC Activity Is Dependent on Intracellular ATP Levels—The cellular levels of cAMP are determined by the balance between synthesizing adenylyl cyclases and catabolizing phosphodiesterases. Therefore, to measure cyclase activity in intact cells, we measured cAMP accumulation over 10 min in the presence of the broad specificity phosphodiesterase inhibitor IBMX. The parental HEK293 cells accumulated <1 pmol of cAMP/2.5 × 10^5 cells/10 min, and this activity was insensitive to the addition of HCO_3^- (Fig. 1*B*). In agreement with the *in vitro* cyclase assay results, under the same conditions, the cAMP accumulation in each of the sAC-overexpressing cell lines was 2–5 pmol of cAMP/2.5 × 10^5 cells/10 min, and this activity was stimulated by the addition of HCO_3^-. The tmAC-specific activator FSK stimulated the cAMP accumulation in HEK293 cells to ~6 pmol of cAMP/2.5 × 10^5 cells/10 min. Thus, in each of the three independent sAC-stable cell lines, cAMP accumulation reflects predominantly the contribution of sAC, whereas the FSK-stimulated cAMP accumulation in the parental HEK293 cells reflects almost exclusively tmAC-generated cAMP.

To study the effect of intracellular ATP concentration fluctuations on the activity of the different forms of adenylyl 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. 2*A*). Although FSK-stimulated cAMP accumulation in parental cells (which reflects tmAC activity) was not appreciably affected (Fig. 2*B*, *black line*), cAMP accumulation in the sAC-stable clones (which reflects predominantly cellular sAC activity) decreased (Fig. 2*B*, *red lines*) in concert with the decrease in intracellular ATP (Fig. 2*A*).

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,

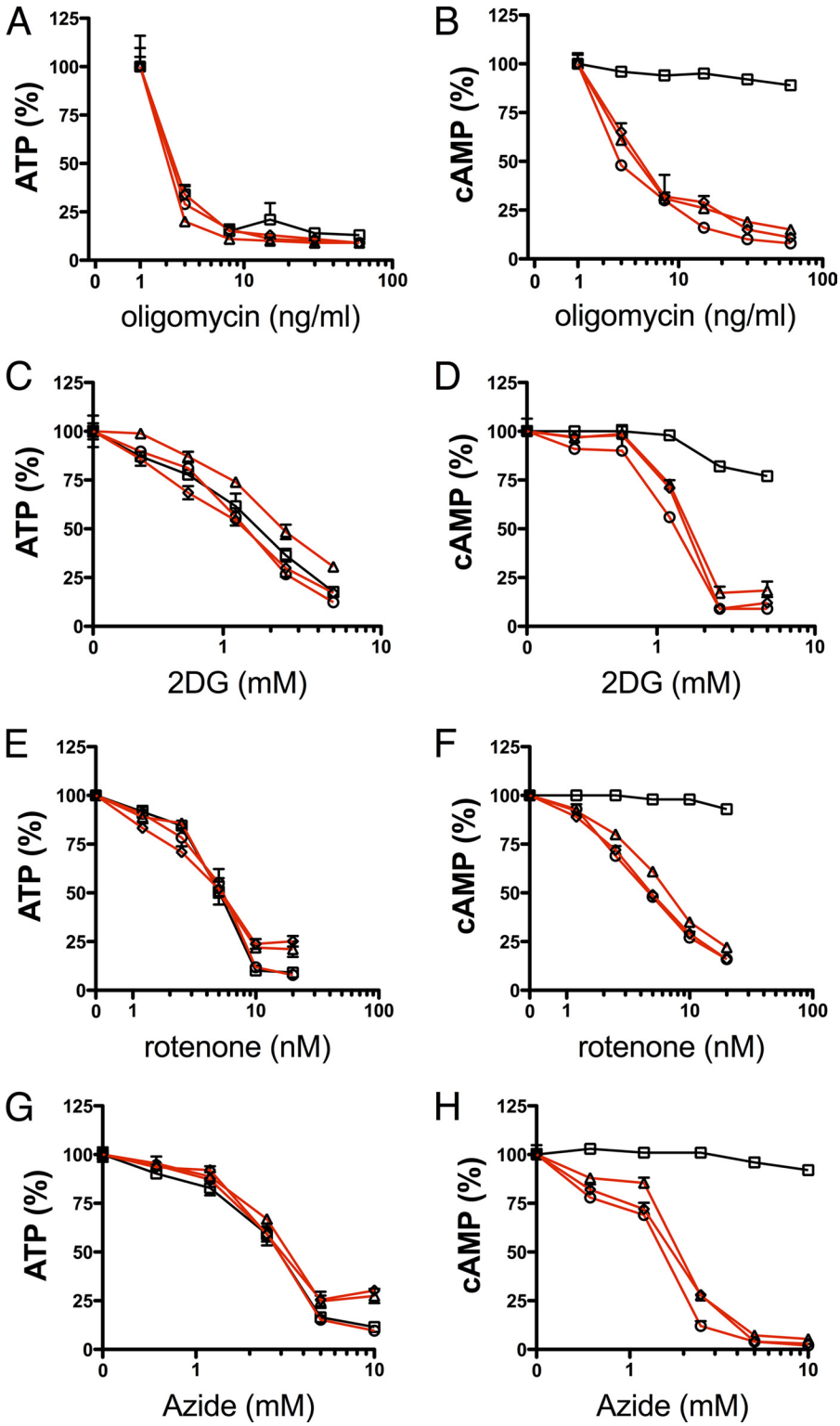

FIGURE 2. **Cellular sAC activity is dependent on intracellular ATP levels.** Intracellular ATP (*A*, *C*, *E*, and *G*) and cAMP (*B*, *D*, *F*, and *H*) accumulated in parental HEK293 cells (□) and the sAC_t-stable clones 1-1 (△), 3-2 (○), and 4-4 (◇) were measured 10 min after incubation in 0.5 mM 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.

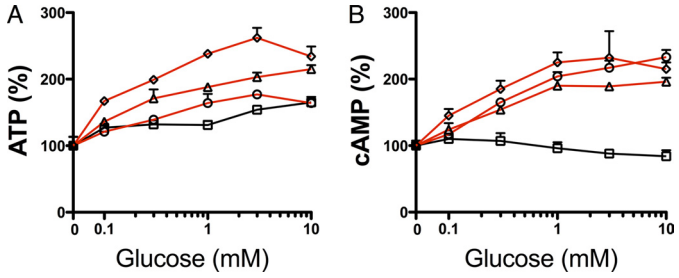

FIGURE 3. **Overexpression of sAC confers glucose responsiveness to HEK293 cells.** Intracellular ATP (*A*) and cAMP (*B*) accumulated in parental HEK293 cells (□) and sAC_t-stable clones 1-1 (△), 3-2 (○), and 4-4 (◇) were measured after a 20-min incubation in the presence of 0.5 mM IBMX and the indicated 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 3703 relative absorbance units; 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.

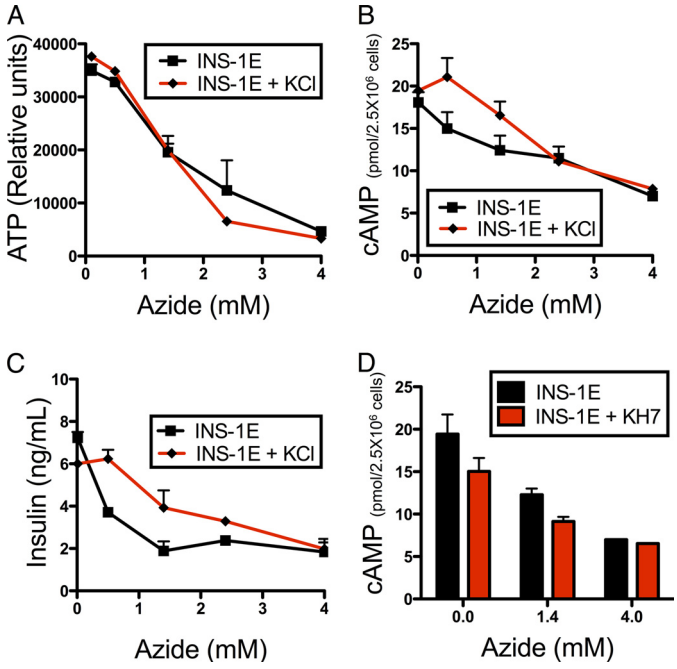

FIGURE 4. **cAMP levels in INS-1E cells are dependent on intracellular ATP levels.** *A–C*, intracellular ATP, accumulation of intracellular cAMP, and insulin release, respectively, in INS-1E cells in the presence of 16 mM glucose, 0.5 mM IBMX, and the indicated concentrations of azide in the presence (◆) or absence (■) of 28 mM 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.* tmAC-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. 3*A*). 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. 3*B*). 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. 3*A*); 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. 4*A*), and similar to the cAMP generated in sAC-overexpressing HEK293 cells, diminishing ATP levels with azide also blunted the cAMP accumulation (Fig. 4*B*) and insulin release (Fig. 4*C*) 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. 4*D*), 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. 4*B*) or insulin release (Fig. 4*C*) 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. 4*B*). 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-

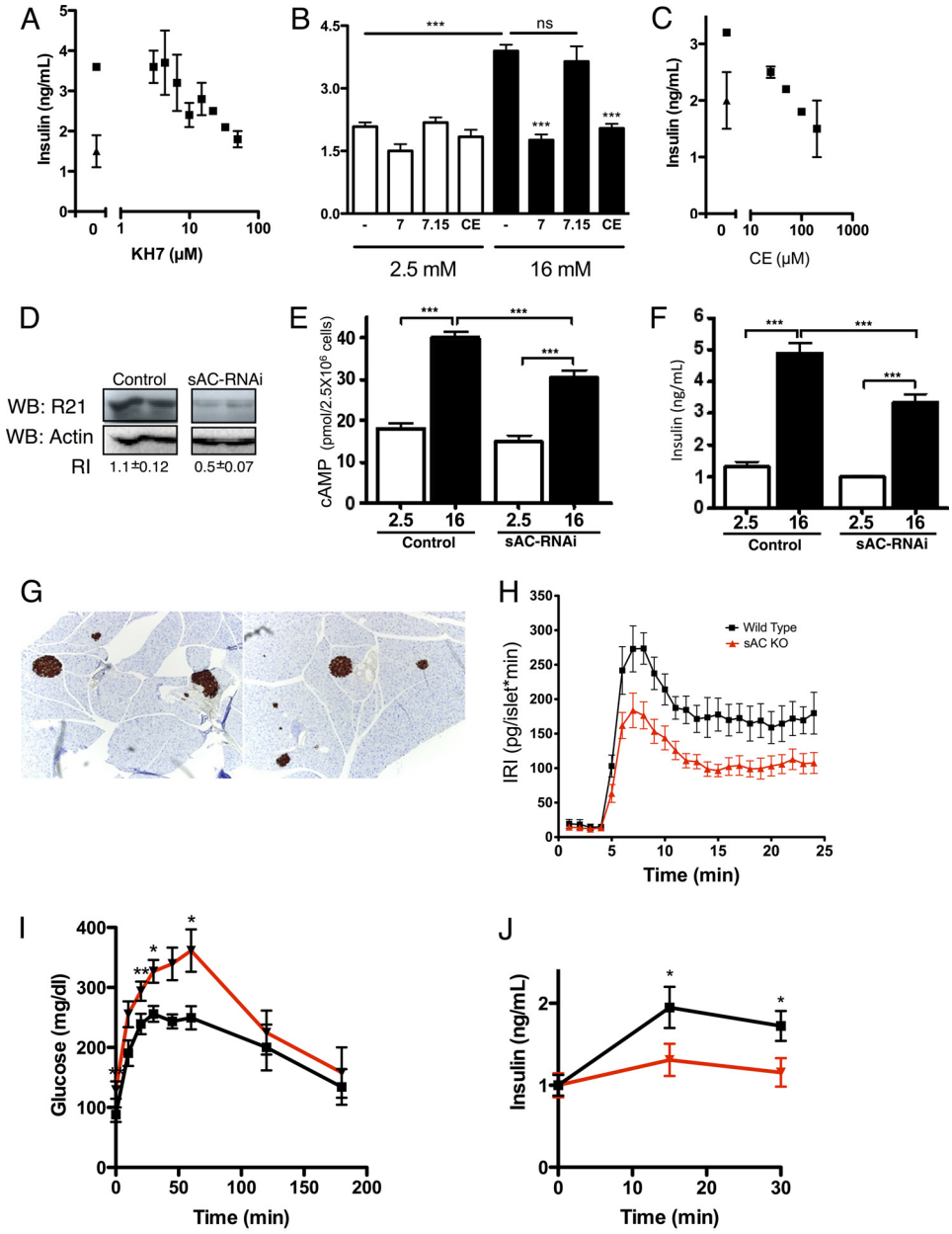

FIGURE 5. **sAC is necessary for normal GSIS.** *A* and *C*, insulin released from INS-1E cells over 30 min incubated in low glucose (2.5 mM; ▲) or 16 mM glucose (■) with the indicated concentrations of KH7 (*A*) or 2-hydroxyestradiol (catechol estrogen (*CE*); *C*). *B*, insulin released from INS-1E cells over 15 min in the presence of 2.5 (*white bars*) or 16 (*black bars*) mM glucose with vehicle alone (−; 0.6% Me_2SO), 30 μM KH7 (*7*), 30 μM KH7.15 (*7.15*), or 50 μM catechol estrogen plus 0.5 mM IBMX. Data in *A* and *C* are representative figures of experiments performed at least twice. Data in *B* represent mean values from a compilation of three separate experiments, each with duplicate determinations. *Error bars* represent S.E. Statistical relevance was determined by analysis of variance. *** = $p < 0.001$; *ns*, not significant. *D–F*, Western blot (*WB*), cAMP accumulation, and insulin released over 15 min, respectively, in INS-1E cells transfected with nonspecific RNAi oligonucleotides (*Control*) or sAC-specific RNAi oligonucleotides (*sAC-RNAi*) in the presence of low glucose (2.5 mM; *white bars*) or high glucose (16 mM; *black bars*). In *D*, *RI* refers to relative intensity. *G*, pancreatic sections from WT (*left panel*) and sAC-C1 KO (*right panel*) animals were immunostained for insulin (*brown*) and co-stained with hematoxylin (*blue*). Images are representative of multiple sections examined (>6) from at least two animals per genotype. *H*, GSIS responses of isolated pancreatic islets. The islets were perifused initially in Krebs-Ringer buffer/HEPES with 5 mM glucose and stimulated after 4 min with 30 mM glucose. WT (*black squares*) and sAC-C1 KO (*red triangles*) islets were treated with the vehicle control (Me_2SO) 30 min prior to glucose stimulation. Plotted values are insulin release/islet/min (*IRI*). *I*, glucose levels in sAC-C1 KO mice ($Sacy^{tm1Lex}/Sacy^{tm1Lex}$; ■) and heterozygous littermates ($Sacy^{tm1Lex}/Sacy^{+}$; ▼) at the indicated times following intraperitoneal glucose injection (1 g/kg) into animals following a 16-h fast. $n = 16$ for sAC-C1 KO; $n = 10$ for heterozygous littermates. *J*, insulin levels in sAC-C1 KO mice (*black squares*) and heterozygous littermates (*red inverted triangles*) at the indicated times following intraperitoneal glucose injection (1 g/kg) into animals following a 16-h fast. $n = 16$ for sAC-C1 KO; $n = 10$ for heterozygous littermates. For *I* and *J*, *error bars* represent S.E., and statistics were measured using repeated measures analysis of variance with a Student-Newman-Keuls post hoc test. **, $p < 0.01$; *, $p < 0.05$.

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. 5*A*) but not by KH7.15 (Fig. 5*B*), 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. 5*B*) in a concentration-de-

pendent manner (Fig. 5*C*), whereas sAC-specific RNAi, which resulted in ~50% knockdown of sAC protein (Fig. 5*D*), diminished glucose-induced cAMP generation (Fig. 5*E*) and insulin secretion (Fig. 5*F*) by ~50%.

We next examined the role of sAC in insulin release using sAC KO mice (*Sacy*^{tm1Lex}/*Sacy*^{tm1Lex}). sAC null mice were originally described to exhibit male-specific sterility (19, 20). More recently, after backcrossing the *Sacy*^{tm1Lex} 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 necroscopy 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. 5*G*). 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. 5*H*). 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.* *Sacy*^{tm1Lex}/*Sacy*$^{+}$), sAC-C1 KO mice ($n = 8$) displayed elevated maximum glucose and delayed glucose clearance (Fig. 5*I*) and diminished insulin release (Fig. 5*J*) compared with heterozygous littermates ($n = 8$). Thus, CO_2/HCO_3^-/pH-, Ca^{2+}-, 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 CO_2/HCO_3^-/pH-, Ca^{2+}-, 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 K_{ATP} channel in pancreatic β cells. Closure of K_{ATP} channels by high ATP depolarizes the β cell, eliciting a Ca^{2+} 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 K_m 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: CO_2/HCO_3^- (24, 34), Ca^{2+} (9, 25), and ATP. In the brain, sAC is most abundant in astrocytes (34, 36). Neuronal activity stimulates HCO_3^- transport in a nearby astrocyte, and the consequent CO_2/HCO_3^- 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 CO_2/HCO_3^- (24) and by cytoplasmic Ca^{2+} (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 (CO_2, ATP, and Ca^{2+}) that regulate sAC (3–5). We demonstrated previously in INS-1E cells that glucose-dependent activation of sAC requires elevation of intracellular Ca^{2+} via voltage-dependent Ca^{2+} channels (9). We have shown here that sAC also senses the glucose-elicited 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 CO_2, has been suggested to be important for insulin release (39), and we have shown that, inside mitochondria, metabolically generated CO_2 modulates sAC activity (24). However, it is difficult to discern whether CO_2 and HCO_3^- contribute to the regulation of sAC in INS-1E cells because CO_2 and Ca^{2+} are linked to ATP levels via sAC. Tricarboxylic acid cycle-derived CO_2 (24) and cytoplasmically derived Ca^{2+} (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

(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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