Insights into the Regulatory Properties of Human Adenylyl Cyclase Type 9

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ABSTRACT

Membrane-bound adenylyl cyclase (AC) isoforms have distinct regulatory mechanisms that contribute to their signaling specificity and physiologic roles. Although insight into the physiologic relevance of AC9 has progressed, the understanding of AC9 regulation is muddled with conflicting studies. Currently, modes of AC9 regulation include stimulation by $G_{\alpha s}$, protein kinase C (PKC) β II, or calcium-calmodulin kinase II (CaMKII) and inhibition by $G_{\alpha i/o}$, novel PKC isoforms, or calcium-calmodulin. Conversely, the original cloning of human AC9 reported that AC9 is insensitive to $G_{\alpha i}$ inhibition. The purpose of our study was to clarify which proposed regulators of AC9 act directly or indirectly, particularly with respect to $G_{\alpha i/o}$. The proposed regulators, including

G proteins ($G_{\alpha s}$, $G_{\alpha i}$, $G_{\alpha o}$, $G_{\beta\gamma}$), protein kinases (PKC β II, CaMKII), and forskolin, were systematically evaluated using classic in vitro AC assays and cell-based cAMP accumulation assays in COS-7 cells. Our studies show that AC9 is directly regulated by $G_{\alpha s}$ with weak conditional activation by forskolin; other modes of proposed regulation either occur indirectly or possibly require additional scaffolding proteins to facilitate regulation. We also show that AC9 contributes to basal cAMP production; knockdown or knockout of endogenous AC9 reduces basal AC activity in COS-7 cells and splenocytes. Importantly, although AC9 is not directly inhibited by $G_{\alpha i/o}$, it can heterodimerize with $G_{\alpha i/o}$ -regulated isoforms, AC5 and AC6.

Introduction

Adenylyl cyclase (AC) synthesizes the second messenger cAMP, initializing a variety of cell signaling cascades. The AC family is composed of nine membrane-bound isoforms (AC1–AC9) and one soluble isoform. Each isoform has a distinct regulatory mechanism contributing to its signaling specificity and physiologic roles. Regulators of the membrane-bound AC isoforms include heterotrimeric G proteins, protein kinases, phosphatases, calcium, and the plant-derived diterpene forskolin. All of the membrane-bound isoforms can be stimulated by $G_{\alpha s}$. From there, the membrane AC isoforms are separated into four groups based on regulatory patterns: group I, Ca^{2+} -stimulated AC1, AC3, and AC8; group II, $G_{\beta\gamma}$ -stimulated AC2, AC4, and AC7; group III, $G_{\alpha i}$ - and Ca^{2+} -inhibited AC5 and AC6; and group IV, forskolin-insensitive AC9 (Sadana and Dessauer, 2009).

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Of the AC isoforms, AC9 is the most divergent in sequence and the most understudied. Expression analysis shows that AC9 is widely expressed in the central nervous system, heart, and other tissues (Premont et al., 1996; Paterson et al., 2000; Sosunov et al., 2001; Berndt et al., 2013; Li et al., 2017). Although AC9 knockout mice were initially reported to be embryonically lethal (Antoni, 2006), mice lacking AC9 protein expression are viable despite a preweaning subviable homozygous phenotype with incomplete penetrance (Li et al., 2017). Knockdown and gene-trapping studies provide clear in vivo roles for AC9 in neutrophil chemotaxis (Liu et al., 2010, 2014) and cardiac function (Li et al., 2017). In addition, polymorphism of the ADCY9 gene is associated with risk for asthma and mood and body weight disorders (Small et al., 2003; Berndt et al., 2013). The association of AC9 with the A-kinase anchoring protein Yotiao facilitates protein kinase A (PKA) phosphorylation of potassium voltage-gated channel subfamily Q member 1 (KCNQ1) and likely serves to regulate I_{Ks} channel activity and cardiac repolarization (Piggott et al., 2008; Li et al., 2012, 2017).

Although the regulatory mechanisms of many AC isoforms are defined (reviewed extensively elsewhere; Dessauer et al., 2017),

ABBREVIATIONS: AC, adenylyl cyclase; β -gal, β -galactosidase; BiFC, bimolecular fluorescence complementation; BSA, bovine serum albumin; CaM, calmodulin; CaMKII, calcium-calmodulin kinase II; CaN, calcineurin; DAMGO, [D-Ala², N-MePhe⁴, Gly-ol]-enkephalin; DAPI, 4',6-diamidino-2-phenylindole; DTT, dithiothreitol; [³⁵S]GTP γ S, guanosine 5'-O-(3-[³⁵S]thio)triphosphate; HA, hemagglutinin; HEK293, human embryonic kidney 293; IBMX, 3-isobutyl-1-methylxanthine; MACS, magnetic-activated cell sorting; MBP, myelin basic protein; μ OR, μ opioid receptor; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PKA, protein kinase A; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PP1, protein phosphatase 1; shRNA, short hairpin RNA; siRNA, small interfering RNA; VC, C-terminal half of Venus; VN, N-terminal half of Venus; YFP, yellow fluorescent protein.

studies of AC9 regulation have yielded conflicting results. Currently, models of AC9 regulation include stimulation by *Gas*, protein kinase C (PKC) β II (Liu et al., 2014), or calcium-calmodulin kinase II (CaMKII) (Cumbay and Watts, 2005) and inhibition by *Gai/o* (Cumbay and Watts, 2004), novel PKC isoforms (Cumbay and Watts, 2004), or calcium-calmodulin kinase II (CaN) (Antoni et al., 1998). Conversely, the original cloning of human AC9 characterized it as insensitive to inhibition by *Gai* and CaN (Hacker et al., 1998).

The conflicting reports on AC9 regulation dampen a broader understanding of how this enzyme is controlled in vivo. The purpose of our study was to clarify which of the previously identified regulators of AC9 act directly or indirectly. Thus, we systematically evaluated proposed regulators, including G proteins (*Gas*, *Gai*, *Gao*, $G\beta\gamma$), protein kinases (PKC β II, CaMKII), and forskolin, using classic in vitro AC assays. Results of *Gai/o* biochemical assays were paired with COS-7 cell-based assays. We show that AC9 is not directly regulated by most G proteins or protein kinases, except *Gas*, in vitro. Although AC9 is forskolin insensitive in the absence of other regulators, it is weakly activated by forskolin in the presence of *Gas*. Biochemical and whole-cell assays in COS-7 cells confirm that AC9 is not directly regulated by *Gai/o*. Alterations in AC9 expression levels affect basal cAMP in multiple cell types, including primary splenocytes. Finally, AC9 shows homodimerization and modest heterodimerization with *Gai*-sensitive AC5 and AC6, suggesting a possible mechanism for the confusion surrounding the regulation of this AC isoform.

Materials and Methods

Materials and Antibodies. Drugs used included forskolin (Sigma-Aldrich, St. Louis, MO), isoproterenol hydrochloride (Calbiochem, Darmstadt, Germany), [D-Ala², N-MePhe⁴, Gly-ol]-enkephalin (DAMGO) (Bachem, Bubendorf, Switzerland), 4',6-diamidino-2-phenylindole phorbol (DAPI), 12-myristate 13-acetate (PMA), and 3-isobutyl-1-methylxanthine (IBMX) (Sigma-Aldrich).

Antibodies used for immunoprecipitation and Western blotting included the following: mouse anti-FLAG M2 agarose affinity gel (Sigma-Aldrich), mouse anti-DYKDDDDK tag (Cell Signaling Technologies, Danvers, MA), goat anti-AC9 and rabbit anti-AC5/6 (N18 and C-17, respectively; Santa Cruz Biotechnology, Dallas, TX), mouse anti-AC5 (previously described; Bavencoffe et al., 2016), mouse anti-A.V. monoclonal antibody for green fluorescent protein (JL-8; Takara Bio, Kusatsu, Japan), rabbit anti-sodium potassium ATPase (EP1845Y; Abcam, Cambridge, MA), and anti-mouse hemagglutinin (HA) (12CA5; Roche, Basel, Switzerland). Rabbit polyclonal anti-AC9 was generated against human AC9 peptide KINPKQLSSNSHPKHPC conjugated to keyhole limpet hemocyanin. Affinity-purified antibody showed high selectivity for AC9 over AC3, AC5, and AC6 (data not shown). Antibodies used for Western blots were diluted in Tris-buffered saline/Tween 20 (1:1000), except anti-DYKDDDDK tag (1:1500) and anti-sodium potassium ATPase (1:10,000). For green fluorescent protein immunoprecipitation, JL-8 was used (1:100).

Plasmids and Viruses. Human AC6, rat AC1, and rat AC2 baculoviruses were amplified and expressed in Sf9 cells as previously described (Tang and Gilman, 1991; Tang et al., 1991; Chen-Goodspeed et al., 2005). Human Flag-tagged AC9 (amino acids 1–1252; Hacker et al., 1998) was cloned into the SalI and NotI restriction sites of pFastBacDual to generate a baculovirus according to the manufacturer's specifications (Invitrogen, Carlsbad, CA). A baculovirus expressing β -galactosidase (β -gal) was used as a control. Eukaryotic expression vectors for human Flag-AC9

(full-length, AJ133123; Paterson et al., 2000), AC5, Flag-AC5, AC6, and yellow fluorescent protein (YFP)-AC6 in pcDNA3.1 were previously described (Kapiloff et al., 2009; Sadana et al., 2009; Li et al., 2012; Brand et al., 2015). A truncated eukaryotic expression vector for human Flag-AC9 (amino acids 1–1252, AF036927; Hacker et al., 1998) was also generated to compare the two clones; the truncated form has comparable *Gas*-stimulated activity to the full-length form when expressed in human embryonic kidney 293 (HEK293) cells (data not shown). The HA-tagged μ opioid receptor (μ OR) was a generous gift from Dr. Heather Carr (University of Texas Health Science Center, Houston, TX). The bimolecular fluorescence complementation (BiFC) expression construct for YN-AC5 was previously described (Brand et al., 2015). Two types of constructs were designed for AC9. For Myc-AC9-VN and HA-AC9-VC, the N-terminal half of Venus (VN) and the C-terminal half of Venus (VC) were cloned using polymerase chain reaction (PCR) in frame with the C terminus of full-length AC9, separated by a sequence encoding a 12- and 19-amino-acid linker, respectively. Additional AC9-VN and AC9-VC constructs that lacked an N-terminal tag were generated and consisted of a C-terminal 7-amino-acid linker (AAAGGGS) followed by VN or VC tags. All AC9-VN/VC constructs behaved similarly in assays. Human AC5-VN and AC5-VC were cloned by PCR, replacing YFP using the KpnI/BamHI restriction sites in AC5-YFP (Efendiev et al., 2010). To generate AC6 BiFC clones, the stop codon was deleted and a KpnI site was inserted at the end of the coding region. VN and VC were then cloned in frame by PCR to the C terminus of human AC6, separated by a sequence encoding a 10- and 9-amino-acid linker, respectively. All clones were verified by DNA sequencing.

Proteins and Sf9 Membranes. Kinases used included CaMKII α , a generous gift from Dr. M. Neil Waxham (University of Texas Health Science Center), and active PKC β II and protein phosphatase 1 α (PP1 α) (SignalChem, Richmond, BC, Canada). Myelin basic protein (MBP) for control kinase assays was from Invitrogen. Purified calmodulin (CaM) was a gift from Dr. John Putkey (University of Texas Health Science Center). Myristoylated *Gao* expressed in *Escherichia coli* was a generous gift from Dr. Greg G. Tall (University of Michigan, Ann Arbor, MI). *Gao* was activated with guanosine 5'-O-(3-[³⁵S]thio)triphosphate ([³⁵S]GTP γ S) in the absence of resistance to inhibitors of cholinesterase 8A (Ric-8A) (Tall et al., 2003). *Gas*-H₆ and myristoylated *Gai* were expressed in *E. coli*, purified by nickel-nitrilotriacetic acid and ion exchange chromatography, and activated with [³⁵S]GTP γ S (Dessauer et al., 1998). $G\beta_1$, $G\gamma_2$, and H₆-tagged *Gai* were used for expression and purification of nontagged $G\beta_1\gamma_2$ from Sf9 cells (Kozasa and Gilman, 1995). To purify nontagged *Gai*₁ and *Gai*₃ from Sf9 cells, proteins were coexpressed with H₆-tagged $G\beta_1$ and $G\gamma_2$ and purified by nickel-nitrilotriacetic acid chromatography (Kozasa and Gilman, 1995). All G proteins were activated by [³⁵S]GTP γ S; free GTP γ S was subsequently removed by size-exclusion chromatography (Dessauer et al., 1998). Baculoviral expression of AC isoforms in Sf9 cells and subsequent plasma membrane preparation was performed as described previously (Chen-Goodspeed et al., 2005).

Cell Culture, Transfection, Lysate, and Membrane Preparation. COS-7 and HEK293 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum at 37°C with 5% CO₂; cell lines were authenticated by short tandem repeat profiling (HEK293) or mitochondrial cytochrome c oxidase I DNA barcodes (COS-7) by American Type Culture Collection (Manassas, VA). The day before transfection, the cells were seeded at 2.5×10^6 cells or 1.5×10^6 cells per 10-cm dish for HEK293 and COS-7 cells respectively. The cells were transfected with appropriate plasmids (10 μ g total DNA per 10-cm plate) using Lipofectamine 2000 (Invitrogen). The transfected cells were incubated for 4–6 hours before the medium was replaced. HEK293 and COS-7 cells were harvested 40–48 hours after transfection for use in immunoprecipitation (Li et al., 2012; Brand et al., 2015) or preparation of cell membranes (Brand et al., 2013). To prepare lysates for Western blotting or

immunoprecipitation, transfected cells were rinsed in cold phosphate-buffered saline (PBS), lysed with buffer (50 mM HEPES, pH 7.4, 1 mM EDTA, 1 mM MgCl_2 , 150 mM NaCl, 0.5% C_{12}E_9 , and protease inhibitors), and homogenized with a 23-gauge syringe. Homogenate was cleared of cellular debris by centrifugation. An aliquot of total lysate was saved for AC assay and/or Western blot prior to immunoprecipitation at 4°C for 1.5 hours with antibody, followed by an additional 1.5 hours with protein A or G Sepharose. Immunoprecipitation with FLAG-agarose was rotated at 4°C for 3 hours. Samples were washed twice in lysis buffer (0.05% C_{12}E_9) and then resuspended in lysis buffer (0 mM NaCl, 0.04% C_{12}E_9). To prepare cell membranes, cells were rinsed and harvested in cold PBS, pelleted, and resuspended in a buffered medium containing 20 mM HEPES, pH 7.4, 1 mM EDTA, 2 mM MgCl_2 , 1 mM dithiothreitol (DTT), 250 mM sucrose, and protease inhibitors. Cells were Dounce homogenized and subjected to centrifugation at 500g to pellet nuclei, followed by centrifugation at 100,000g; membranes were resuspended in buffer without protease inhibitors. The resulting samples were immediately used for AC assays or frozen for future use.

AC9 Short Hairpin RNA and Small Interfering RNA Knockdown. AC9 short hairpin RNA (shRNA) and control shRNA constructs were a generous gift from Dr. Carole Parent (University of Michigan Medical School) and were described previously (Liu et al., 2010). AC9 (SASI_Hs01_00098729 and SASI_Hs01_00098727) and control small interfering RNAs (siRNAs) (SIC001 siRNA universal negative control 1) were purchased from Sigma-Aldrich. Control and AC9 shRNA plasmids were packaged into lentiviruses using HEK293 T cells. COS-7 cells were then transduced with shRNA-encoding lentivirus and stable knockdowns were generated by selection with 2 $\mu\text{g}/\text{ml}$ puromycin (Liu et al., 2010). For siRNA knockdown, COS-7 cells were transiently transfected with control siRNA (600 pmol) or a mixture of two AC9 siRNAs (300 pmol of each) using Lipofectamine 2000, replacing the medium 4–6 hours after transfection. Cells were harvested for membrane assays 60 hours after transfection.

AC Membrane Assays. Membrane assays were performed as described previously (Dessauer, 2002). Briefly, membrane (Sf9, HEK293, COS-7, or mouse spleen) preparations were incubated for 10 minutes at 30°C with an AC mix containing 5 mM MgCl_2 , 200 μM Mg-ATP, [α - ^{32}P]ATP, and purified proteins or drugs. Reactions were terminated with a mix of 2.5% SDS, 50 mM ATP, and 1.75 mM cAMP. Each reaction was subjected to column chromatography to separate nucleotides and isolate [^{32}P]cAMP produced during the reaction; [^3H]cAMP was used to monitor column recovery rates by scintillation counting.

For kinase-AC assays, kinase reactions preceded measurement of cAMP production. Proteins and membranes were diluted as follows: CaMKII (100 ng) was diluted in kinase buffer, which comprised 10 mM HEPES, pH 7.4, 200 mM KCl, 0.1% Tween-20, and 1 mg/ml bovine serum albumin (BSA); Sf9 membranes were diluted in 20 mM HEPES, pH 7.4, and 2 mM DTT; and CaM was diluted in 5 mM 4-morpholinepropanesulfonic acid, pH 7.0, and 0.01 mg/ml BSA. In a 25 μl reaction, the kinase assay was initiated by the addition of 100 ng CaMKII to 30 μg Sf9 membranes, 1 μM CaM, and 10 $\times$ reaction mix (final concentration: 25 mM HEPES, pH 7.4, 10 mM MgCl_2 , 50 mM KCl, 2 mM CaCl_2 , 400 μM DTT, and 100 μM ATP). The reaction was incubated on ice for 10 minutes and then transferred to 30°C for 5 minutes. The assay of AC activity was initiated with 25 μl AC mix (containing 100 μM Mg-ATP, [α - ^{32}P]ATP $\pm$ 100 nM G α s) and incubated for an additional 10 minutes at 30°C. PKC β II reactions were similar in design. PKC β II and Sf9 membranes were diluted in 20 mM HEPES, pH 7.4, and 2 mM DTT. In a 60 μl reaction, PKC β II (5 or 20 nM) was added to Sf9 membranes (15 μg AC2, 30 μg AC9 or β -gal), 100 μM CaCl_2 , and 1 μM phorbol PMA; reactions were initiated with activation buffer (final concentration: 20 mM HEPES, pH 7.4, 5 mM MgCl_2 , and 100 μM ATP) and placed at 30°C for 7 minutes. The subsequent AC assay was initiated with 40 μl AC mix (containing 5 mM MgCl_2 , 100 μM Mg-ATP, and

[α - ^{32}P]ATP $\pm$ 100 nM G α s) and incubated for an additional 7 minutes at 30°C. PKC β II and CaMKII α activity were confirmed by measuring [γ - ^{32}P] incorporation into MBP; control kinase assays substituted [γ - ^{32}P]ATP for [α - ^{32}P]ATP.

The catalytic subunit of PP1 α was diluted in 10 mM imidazole, 0.02% beta mercaptoethanol, and 12 ng/ μl BSA. Phosphatase treatments of AC membranes were performed as follows: 30 μg AC9 Sf9 membranes (diluted in 20 mM HEPES, pH 7.4, plus 2 mM DTT) were incubated with 75 ng PP1 α (or dilution buffer) in a reaction mix (containing 20 mM HEPES, pH 7.4, 20 mM MgCl_2 , and 1 mM DTT) for 5 minutes at 30°C. Pretreated membranes were then used for PKC β II-AC assays as described above. β -glycerophosphate was added to the PKC activation buffer (25 mM final) to prevent PP1 from reversing effects of PKC phosphorylation.

Throughout this article, AC activity is primarily displayed as specific activity (in nanomoles per minute per milligram) unless otherwise indicated. Otherwise, results are generally displayed after subtracting control background or as fold over basal, as noted in the figures.

Live-Cell cAMP Accumulation Monitoring (cAMP Difference Detector In Situ and GloSensor). COS-7 cells were transiently transfected with an empty vector or μOR , as well as AC9 or AC6; the medium was changed 4 hours after transfection. After 24 hours, cells (0.05×10^6 cells/well) were resuspended in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum and 6 mM valproic acid, replated on a black, poly(L-lysine)-coated 96-well plate with a clear bottom, and incubated with 30 μl BacMam sensor (red upward cAMP difference detector in situ; Montana Molecular, Bozeman, MT), according to the manufacturer's guidelines. Fluorescent experiments were conducted with a Tecan Infinite 200 Pro plate reader (Tecan, Männedorf, Switzerland) 24 hours after the addition of the sensor. Red fluorescence was excited at 560 nm, and emitted light was collected at 605 nm. Prior to fluorescent reads, the cell medium was replaced with PBS for 20–30 minutes to acclimate. A baseline read was measured for 7.5 minutes prior to the addition of drug, and fluorescence was then monitored for an additional 15 minutes. Iso-proterenol and DAMGO were prepared and diluted in AT buffer (0.1 mM ascorbic acid and 1 mM thiourea) for cell treatments. The average of the 7.5-minute baseline read for each well was subtracted from each point to account for variability between assays. Background subtracted fluorescence was averaged over 5–7 minutes after the addition of drug (12–14 minutes after the start of the assay).

For measurement of basal cAMP accumulation, COS-7 cells were transiently transfected with 5 μg pGloSensor-20F plasmid (Promega, Madison, WI) and with AC isoforms or control vectors on a 10-cm plate. Thirty-six hours after transfection, cells were replated at a density of 0.05×10^6 cells per well on white, poly(L-lysine)-coated 96-well plates and assayed 4 hours later according to the manufacturer's protocol. Cells were pretreated with 1 mM IBMX for 10 minutes, followed by a 5-minute baseline read. Baseline reads were averaged over the 5 minutes.

Preparation of Spleen Membranes and Splenocytes. All protocols utilizing animals were approved by the Institutional Animal Care and Use Committee at the University of Texas Health Science Center in Houston in accordance with the Animal Welfare Act and National Institutes of Health guidelines. Isolation of membranes from wild-type and AC9 knockout mice was performed as previously described (Piggott et al., 2008; Efendiev et al., 2010). Briefly, fresh or frozen spleens were washed in ice-cold PBS and quartered. Tissue was resuspended and homogenized with a polytron homogenizer followed by Dounce homogenization in a buffered medium containing 10 mM HEPES, 5 mM EDTA, 300 mM sucrose, and protease inhibitors. The homogenate was centrifuged at 2000g to remove cell nuclei; the supernatant from the first spin was then centrifuged at 100,000g to collect membranes. Collected membranes were resuspended in a buffer containing 50 mM HEPES, 1 mM EDTA, and 300 mM sucrose. The resulting samples were immediately used for AC assays.

For preparation of splenocytes, spleens were removed from wild-type and AC9 knockout mice and placed on ice in PBS. Tissue was homogenized in magnetic-activated cell sorting (MACS) buffer (0.5% BSA and 2 mM EDTA in PBS) and filtered through a 40- μ m strainer, and cells were collected by centrifugation (300g for 5 minutes). Cells were resuspended in Hybri-Max buffer (Sigma-Aldrich) for 4 minutes to lyse red blood cells. The reaction was neutralized with excess MACS buffer, centrifuged, and resuspended in MACS buffer for counting. Collected splenocytes were then centrifuged and resuspended in RPMI 1640 medium with 25 mM HEPES, pH 7.4, and immediately used for cAMP accumulation assays. Splenocytes were treated with 1 mM IBMX at 37°C for 20 minutes before the addition of vehicle or 1 μ M isoproterenol. Reactions were stopped by 2-fold dilution with 0.2 N HCl. Cell particulates were removed by centrifugation and cAMP in the supernatant was detected by enzyme immunoassay (catalog no. ADI-900-066; Enzo Life Sciences, Farmingdale, NY).

Genotyping and Real-Time PCR. Primers used to genotype AC9^{-/-} mice and for real-time PCR of mouse AC isoforms were described previously (Li et al., 2017). Real-time PCR was performed and analyzed as described (Bavencoffe et al., 2016), using glyceraldehyde-3-phosphate dehydrogenase as a control template.

BiFC. COS-7 cells were transiently transfected as indicated in 12-well plates with VN- or VC-tagged AC9, AC5, or AC6. Approximately 40–48 hours after transfection, cells were stained with DAPI (10 mg/ml) for 1 hour at 37°C. Cells were then scrapped in PBS and transferred to a black 96-well plate with a clear, flat bottom (Corning Inc., Corning, NY). Venus and DAPI signals were measured with a multiwell plate reader (Tecan Infinite 200 Pro) at room temperature. Venus intensity was measured at an excitation wavelength of 506 and emission wavelengths of 536–542 nm (2-nm step measurements). DAPI intensity was measured at wavelengths of 358 nm (excitation) and 461 nm (emission). Peak signals from 540 to 542 nm emissions were averaged and normalized to the DAPI signal to account for

differences in cell number. Background fluorescence from control samples expressing pcDNA3 and/or only VN- or VC-tagged protein was subtracted.

Statistical Analysis. Individual experiments were performed in duplicate or triplicate and repeated a minimum of three times. Experiments are expressed as means $\pm$ S.D. Statistical analyses of sample differences are discussed in the figure legends. Analysis was performed with SigmaPlot (Systat Software, San Jose, CA) or GraphPad Prism (GraphPad Inc., La Jolla, CA) statistical analysis software.

Results

To investigate direct regulatory properties of AC9, Flag-AC9 was expressed in Sf9 cells and membranes were purified; expression was confirmed via Western blotting against the FLAG tag and AC9 (Fig. 1A). To ensure that the N-terminal FLAG tag did not alter AC9 activity, membranes from HEK293 cells expressing pcDNA3, nontagged AC9, and Flag-AC9 were assayed with 300 nM G α s. No difference in the activity of Flag-AC9 was observed; the fold change in AC activity over the pcDNA3 control was 7.7 ± 2.0 and 7.7 ± 0.8 for AC9 and Flag-AC9, respectively.

AC9 Is Conditionally Stimulated by Forskolin. AC9 is the only member of the AC isoform group IV, characterized by forskolin insensitivity (Paterson et al., 1995; Hacker et al., 1998). Although AC9 is insensitive to forskolin stimulation alone, conditional stimulation has not been tested. In vitro AC assays were performed with membranes from Sf9 or HEK293 cells expressing Flag-AC9. AC9 activity was examined in the presence of increasing amounts of activated G α s with

Fig. 1. AC9 is conditionally activated by forskolin. (A) Western blot using α -FLAG (top) and goat α -AC9 (bottom) for membranes from Sf9 cells expressing vector control (β -gal) or human AC9. (B and C) Dose-response curves of G α s stimulation of AC9 and β -gal Sf9 membranes in the presence or absence of 50 μ M forskolin (Fsk) (B) and membranes from HEK293 cells expressing AC9 in the presence or absence of 100 μ M forskolin (C) [activity from control membranes (D) was subtracted]. The inset in (B) shows individual replicates for 3 μ M G α s with or without forskolin. (D) AC activity of membranes isolated from HEK293 cells expressing pcDNA3 (background AC activity). Data are shown as means $\pm$ S.D. Statistical analyses in (B) through (D) were performed with two-way ANOVA followed by the Sidak multiple-comparisons test of experimental means comparing vehicle and forskolin-stimulated groups at each concentration indicated. $n = 3$ to 4 with experiments performed in duplicate. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. ns, not significant.

vehicle (dimethylsulfoxide) or forskolin. β -gal- or pcDNA3-expressing membranes served as negative controls for Sf9 and HEK293 membranes, respectively. AC9-containing Sf9 membranes treated with forskolin in the presence of high concentrations of $G_{\alpha s}$ were weakly activated compared with membranes treated with $G_{\alpha s}$ alone (Fig. 1B). The same weak conditional activation was observed in HEK293 membranes at high concentrations of $G_{\alpha s}$ (Fig. 1C). Background AC activity in HEK293 membranes is shown in Fig. 1D.

AC9 Regulation by $G_{\alpha s}$ and $G\beta\gamma$ Subunits. It was often noted previously that greater concentrations of $G_{\alpha s}$ were required to activate AC9 in immunoprecipitation-AC assays than other AC isoforms (Piggott et al., 2008; Efendiev et al., 2010). To gain further insight into $G_{\alpha s}$ stimulation of AC9 activity compared with other well characterized isoforms, $G_{\alpha s}$ dose-response curves (1 nM–1 μ M) were generated for membranes from Sf9 cells expressing AC9, AC6, and β -gal. Although dose-response curves for $G_{\alpha s}$ activation of AC6 were comparable to previously published results ($EC_{50} = 100$ nM, $E_{max} = 5.3$ nM; Chen-Goodspeed et al., 2005), the $G_{\alpha s}$ dose-response curve for AC9 appears right shifted ($EC_{50} > 300$ nM; Fig. 2A). For β -gal membranes, the EC_{50} is 175 nM and the E_{max} is 0.9 nM.

Fig. 2. AC9 is less sensitive to $G_{\alpha s}$ compared with AC6 and is insensitive to $G\beta\gamma$. (A) Dose-response curves of $G_{\alpha s}$ stimulation of membranes from Sf9 cells expressing AC9, AC6, or β -gal. (B) Dose-response curves of $G_{\alpha s}$ stimulation of Sf9 membranes expressing AC9 or β -gal in the presence and absence of 100 nM $G\beta_1\gamma_2$. Data are shown as means $\pm$ S.D. Statistical analyses in (B) were performed with two-way ANOVA followed by a Sidak multiple-comparisons test comparing experimental means of vehicle- and $G_{\alpha s}$ - or $G\beta_1\gamma_2$ -stimulated groups at each concentration indicated. $n = 3$. No statistical difference was found in the presence of $G\beta\gamma$.

$G\beta\gamma$ either inhibits (AC1, AC3, AC8) or conditionally stimulates (AC2, AC4–7) all other membrane-bound AC isoforms (Gao and Gilman, 1991; Tang and Gilman, 1991; Yoshimura et al., 1996; Diel et al., 2006; Steiner et al., 2006; Gao et al., 2007). To determine whether AC9 was sensitive to $G\beta\gamma$, $G_{\alpha s}$ dose-response curves were performed in the presence or absence of 100 nM $G\beta_1\gamma_2$ (Fig. 2B). $G\beta_1\gamma_2$ had no direct effect on AC9 activity at any $G_{\alpha s}$ concentration.

AC9 Is Insensitive to Direct Regulation by $G_{ai/o}$ In Vitro. AC9 inhibition by dopamine (D2L)-coupled $G_{ai/o}$ (Cumbay and Watts, 2004) and AC9 insensitivity to somatostatin-coupled $G_{ai/o}$ (Hacker et al., 1998) have been reported. Sequence alignments of the G_{ai} binding site for AC5 and AC6 with AC9 reveal no homology of key G_{ai} binding residues (Dessauer et al., 1998) (Fig. 3A). The degree of inhibition of AC5 and AC6 by G_{ai} is dependent on $G_{\alpha s}$ concentrations (Chen-Goodspeed et al., 2005); therefore, inhibition of AC9 by myristoylated G_{ai} (purified from *E. coli*) was tested in the presence of a fixed concentration of G_{ai} (300 nM) and varying concentrations of $G_{\alpha s}$ (Fig. 3B) or a fixed concentration of activated $G_{\alpha s}$ (300 nM) in the presence of increasing G_{ai} (Fig. 3C). Regardless of $G_{\alpha s}$ or G_{ai} concentrations, no inhibition of AC9 was observed, despite inhibition of AC6 activity by G_{ai} in the same assays (Fig. 3D).

G_{ai} is dually modified in mammalian cells by myristoylation of the N-terminal glycine and palmitoylation of the neighboring cysteine residue. Coexpression of *N*-myristoyl transferase with G_{ai} in *E. coli* results in myristoylated G_{ai} that lacks palmitoylation (Linder et al., 1991), whereas Sf9 expression gives rise to both myristoylation and palmitoylation of G_{ai} (Linder et al., 1993). To rule out the requirement for palmitoylation of G_{ai} and/or differences in $G_{i/o}$ family members, G_{ai1} (*E. coli* or Sf9), G_{ai3} (Sf9), and G_{ao} (*E. coli*) were also tested. No inhibition of AC9 activity was observed (Fig. 3D) when incubated with G_{ai1} or G_{ai3} , despite observed inhibition to AC6 activity (Fig. 3E). G_{ao} likewise did not alter basal or $G_{\alpha s}$ -stimulated AC9 activity, although AC1 activity was inhibited by G_{ao} , as previously shown (Fig. 3, F and G; Taussig et al., 1994).

AC9 Is Insensitive to CaMKII and PKC β II In Vitro. CaMKII and PKC β II have previously been reported to regulate AC9 in cellular assays (Cumbay and Watts, 2005; Liu et al., 2014). To assess whether these kinases act directly on AC9, kinase-AC assays were performed. AC9 activity was measured in the presence of Ca^{2+} /CaM, non-stimulated CaMKII, or Ca^{2+} /CaM-activated CaMKII (Fig. 4A). CaMKII activity was confirmed by evaluating phosphorylation of MBP (Fig. 4A, inset). Despite observed CaMKII phosphorylation of MBP in the assays and inhibition of AC3 as previously reported (Fig. 4B) (Wei et al., 1996, 1998), no changes in AC9 activity were observed. PKC β II assays were performed in a similar manner to CaMKII assays. $G_{\alpha s}$ -stimulated AC9, AC2, and control membranes were assayed in the presence of the phorbol ester, PMA, and activated PKC β II (PMA plus PKC β II). PKC β II activity was confirmed by an in vitro kinase assay measuring MBP phosphorylation (Fig. 4C, inset). Although PKC β II did not activate AC9, it stimulated AC2, as previously reported (Fig. 4C; Zimmermann and Taussig, 1996).

It is possible that endogenous Sf9 phosphorylation of AC9 prevented our ability to observe PKC β II regulation of AC9. To assess this, AC9 membranes were pretreated with the catalytic subunit of PP1 α prior to PKC-AC assays. Pretreatment of

Fig. 3. AC9 is not directly regulated by *Gai/o* in vitro. (A) Structure-based alignment of amino acids that correspond to the *Gai* binding site in AC5. (B) Gas dose-response curves for AC9 and β -gal Sf9 membranes in the presence and absence of 300 nM *Gai*. (C) *Gai* dose-response curves for AC9 and β -gal Sf9 membranes in the presence of 300 nM Gas. (D) *E. coli* vs. Sf9 purified *Gai* isoforms. AC9 Sf9 membranes were stimulated with 300 nM Gas in the presence or absence of 300 nM *Gai*₁ (*E. coli* or Sf9) or *Gai*₃. (E) AC6 membranes with 50 nM Gas $\pm$ 300 nM *Gai* proteins served as a positive control. (F) *Gao* regulation of AC9 Sf9 membranes stimulated with 300 nM Gas. (G) AC1 membranes stimulated with 100 μ M Ca^{2+} /300 nM CaM with 1 μ M *Gao*

Fig. 4. Neither CaMKII nor PKCβII directly regulates AC9. (A) AC9 membranes were stimulated with 100 nM *Gαs* in the presence or absence of 200 μM Ca^{2+} /1 μM calmodulin (Ca^{2+} /CaM), 100 ng CaMKII, or Ca^{2+} /CaM plus CaMKII. The inset shows ^{32}P labeling of MBP by CaMKII, performed in duplicate. As a positive control, AC3 was stimulated with 100 nM *Gαs* in the presence of 200 μM Ca^{2+} /1 μM CaM in the presence or absence of 100 ng CaMKII. (B) AC2, AC9, and β-gal membranes were stimulated with 100 nM *Gαs* in the presence or absence of 1 μM PMA or PMA plus purified PKCβII. The inset shows ^{32}P labeling of MBP by PKCβII, performed in duplicate. Data are shown as means $\pm$ S.D. Statistical analyses were performed as follows: (A), two-way ANOVA followed by a Sidak multiple-comparisons test; (B), a paired *t* test comparing the control and the kinase group; and (C), one-way ANOVA followed by a Tukey multiple-comparisons test. For all experiments, *n* = 3, performed in duplicate. **P* < 0.05; ***P* < 0.01. ns, not significant.

AC9 membranes with vehicle or PP1α did not alter *Gαs*-stimulated AC9-specific activity (0.76 ± 0.06 vs. 0.69 ± 0.04 nmol/min per milligram; *n* = 3, *P* > 0.05). In addition, pretreatment of AC9 membranes with vehicle or PP1α did not impart PKCβII regulation of *Gαs*-stimulated AC9 (0.84 ± 0.04 vs. 0.79 ± 0.11 nmol/min per milligram; *n* = 3, *P* > 0.05). Statistical differences were assessed with a one-way ANOVA followed by a Tukey multiple-comparisons test.

AC9 Is Not Inhibited by *Gai* in COS-7 Cells. Previous work examining AC9 regulation was performed using whole-cell assays in HEK293 cells. However, these cells express endogenous *Gi/o*-inhibitable AC activity that complicates analysis (Lefkimmiatis et al., 2009). To test whether *Gai/o* inhibition of AC9 could be observed in other cell lines, cAMP accumulation assays were performed using the cAMP sensor, cAMP difference detector in situ (Tewson et al., 2016), in COS-7 cells. Live-cell cAMP accumulation was monitored in COS-7 cells transiently transfected with the *Gi/o*-coupled μOR , in the absence or presence of AC6 or AC9. Cells were treated with isoproterenol with or without the μOR agonist, DAMGO (Fig. 5, A and B). Compared with isoproterenol treatment, the addition of DAMGO did not alter cAMP levels in COS-7 cells expressing the μOR alone, consistent with previous reports that COS-7 cells express largely AC9 (Fig. 5, C and D) and AC7 (Premont, 1994). As expected, cells expressing AC6 showed a decrease in isoproterenol-stimulated cAMP accumulation when treated with DAMGO. DAMGO had no effect on isoproterenol-stimulated cAMP accumulation in cells expressing AC9 (Fig. 5, A and B). This was not due to a loss of μOR (Fig. 5C); rather, both endogenous and overexpressed AC9 were insensitive to μOR -coupled *Gi/o*.

AC9 Expression Alters Basal Cellular Levels of cAMP. To examine endogenous AC9 basal activity, we used shRNA and siRNA knockdown of AC9 in COS-7 cells. Knockdown of AC9 was confirmed by Western blot and AC activity assays in membranes isolated from cells expressing a control or AC9 shRNA/siRNA. Knockdown of AC9 resulted in a $39\% \pm 3\%$ reduction in AC9 protein (Fig. 5D) and a reduction in *Gαs* ($39\% \pm 13\%$) and forskolin ($37\% \pm 15\%$) stimulated AC activity (Fig. 6A). Overall changes in low basal AC activity are difficult to detect in membrane preparations; therefore, the cAMP GloSensor was used to measure basal cAMP levels. The GloSensor plasmid was transiently transfected into COS-7 cells expressing control shRNA, AC9 shRNA, or AC9 overexpression plasmid. Knockdown of AC9 reduced basal cAMP levels, whereas overexpression of AC9 enhanced basal cAMP levels compared with control shRNA (Fig. 6B).

AC9 is generally expressed at low levels in most tissues (Hacker et al., 1998). For example, AC9 represents less than 3% of total AC activity in the heart (Li et al., 2017). In addition, deletion of AC9 does not alter RNA expression of other AC isoforms in the heart (Li et al., 2017) or spleen (Fig. 6C). Surprisingly, knockout of AC9 reduced AC activity in spleen membranes by $54\% \pm 4\%$ and $47\% \pm 9\%$ for *Gαs* and forskolin stimulation, respectively, compared with wild-type mice (Fig. 6D). The considerable contribution of AC9 to total spleen AC activity presented a unique opportunity to examine endogenous AC9 regulation by *Gai*. However, wild-type spleen membranes show only modest inhibition by 300 nM *Gai* ($19\% \pm 2\%$); the degree of *Gai* inhibition was not altered in spleen membranes from AC9^{-/-} mice. This was not due to alterations in the levels of *Gai*-regulated AC isoforms, in which the fold change ($2^{\Delta\Delta\text{Ct}}$) for RNA expression

served as a positive control. Data are shown as means $\pm$ S.D. Statistical analyses were performed as follows: (B) and (D), two-way ANOVA followed by a Sidak multiple-comparisons test comparing the vehicle and *Gai/o* groups at each concentration indicated; (C), (E), and (F), one-way ANOVA followed by a Tukey multiple-comparisons test comparing experimental means; and (G), *t* test comparing Ca^{2+} /CaM with or without *Gao*. For all experiments, *n* = 3 to 4, performed in duplicate or triplicate. **P* < 0.05; ***P* < 0.01; ****P* < 0.001. No statistical difference was found for *Gai/o* under any condition with AC9 (B–D and F). ns, not significant.

Fig. 5. Regulation of endogenous and overexpressed AC9 in COS-7 cells. (A) Representative traces of cells expressing μ OR with or without AC6 or AC9 treated with 1 μ M isoproterenol (ISO; black square) $\pm$ 10 μ M DAMGO. Arrows indicate the addition of drugs (ISO/DAMGO). (B) Quantitation of individual cell traces. Data points were averaged (avg) from the 12- to 14-minute timepoints of ISO (1 μ M) and ISO plus 10 μ M DAMGO. (C) Western blot of COS-7 whole-cell lysates, confirming expression of endogenous or exogenous AC9 (top: rabbit anti-AC9) and AC6 (bottom). (D) Confirmation of endogenous AC9 protein by Western blot of membranes isolated from COS-7 cells, or cells expressing an AC9 siRNA or control siRNA. The Na-K ATPase served as a loading control for COS-7 membranes. Data are shown as means $\pm$ S.D. Statistical analyses in (B) were performed with two-way ANOVA followed by a Sidak multiple-comparisons test comparing ISO and ISO plus DAMGO. $n = 3$ –5 with experiments performed in duplicate. ** $P < 0.01$. au, arbitrary unit; KD, knockdown; ns, not significant.

of AC5 (0.96 ± 0.12) or AC6 (1.01 ± 0.071) was unchanged in AC9^{-/-} compared with the wild type (Fig. 6C).

To further reveal contributions from AC9, membranes were treated with the P-site inhibitor Ara-A, which shows preferential inhibition of AC1, AC3, AC4, AC5, and AC6 versus AC9 (Brand et al., 2013). In membranes treated with 300 nM Ara-A, Gas-stimulated AC activity in AC9^{-/-} membranes was reduced by $70\% \pm 9\%$ compared with the wild type, whereas forskolin-stimulated activity was only reduced by $41\% \pm 7\%$ (Fig. 6D). Although differences in basal activity between wild-type and AC9^{-/-} spleen membranes did not reach significance, basal whole-cell cAMP accumulation in splenocytes isolated from AC9^{-/-} mice compared with the wild type was reduced in cells treated with IBMX (Fig. 6E). Similarly, cAMP accumulation was also reduced in AC9^{-/-} splenocytes treated with IBMX and isoproterenol (Fig. 6F), supporting a noteworthy contribution from AC9 in basal and Gas-stimulated AC activity in the spleen.

AC9 Can Homo- and Heterodimerize. To explore why some cell lines (but not others) show inhibition of overexpressed AC9 by Gi/o-coupled receptors, we examined whether AC9 could form heterodimers with other AC isoforms, particularly AC5 and AC6, which show strong G_{ai} inhibition. Homo- and heterodimerization of AC9 was examined in COS-7 cells using BiFC with AC isoforms tagged with VN and VC. No fluorescence was detected upon expression of the individual

AC9-VN or AC9-VC constructs. Coexpression of AC9-VN and AC9-VC resulted in strong reconstitution of fluorescence, indicating an interaction and homodimerization between AC9 proteins (Ejendal et al., 2013). However, only modest homodimerization was detected for AC5 and no BiFC interaction between similarly tagged AC6 proteins was observed (Fig. 7A).

The ability of AC9 to heterodimerize with AC5 and AC6 was also examined by BiFC. AC9 interaction with AC5 and AC6 was observed in a configuration-dependent manner, as not all tested configurations resulted in interaction (Fig. 7B). Statistically significant heterodimerization was detected with AC5/6-VN and AC9-VC constructs and when the Venus tags were reversed. However, no fluorescence signals were obtained for AC9-VN and N-terminal tagged AC5. AC5 and AC6 heterodimerization was not observed in any configuration (Fig. 7B). AC9-AC5 and AC9-AC6 heterodimerization was confirmed by coimmunoprecipitation in COS-7 cells (Fig. 7, C and D). Flag-tagged AC9 with or without nontagged AC5 or AC6 were coexpressed and complexes were isolated using FLAG agarose. Immunoprecipitates of Flag-AC5 and YFP-AC6 were used as positive controls (Fig. 7, C and D, respectively). Neither AC5 nor AC6 was pulled down in the absence of Flag-AC9 expression, whereas both AC5 and AC6 were detected in immunoprecipitates of Flag-AC9.

Fig. 6. Basal AC activity is dependent on AC9 in COS-7 cells and mouse splenocytes. (A) AC assay of membranes from COS-7 cells stably expressing control or AC9 shRNA/siRNA stimulated with 400 nM $G\alpha_s$ or 50 μ M forskolin. (B) Whole-cell basal cAMP accumulation was measured in COS-7 cells expressing control shRNA, AC9 shRNA, or overexpressing (OVE) AC9 using a GloSensor bioluminescent cAMP reporter. Cells were pretreated with 1 mM IBMX for 10 minutes. (C) Quantitative real-time PCR of AC isoforms present in spleen from AC9^{-/-} mice, normalized to wild-type (WT) expression levels ($n = 3$). (D) AC assay of WT and AC9 knockout (KO) spleen membranes stimulated with 400 nM $G\alpha_s$ or 50 μ M forskolin in the presence or absence of the P-site inhibitor, Ara-A (300 nM). (E and F) Whole-cell cAMP accumulation of mouse splenocytes isolated from WT and AC9KO mice treated with 1 mM IBMX $\pm$ 1 μ M ISO. Data are shown as means $\pm$ S.D. Statistical analysis was performed as follows: (A) and (D), two-way ANOVA followed by a Sidak multiple-comparison test [comparisons within Ara-A treatments in (D) were made separately]; (B), (E), and (F), an unpaired t test of means comparing the indicated groups; and (C), one-way ANOVA followed by a Tukey multiple-comparisons test. For all, experiments were performed in duplicate. $n = 3$ to 4. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. KO, knockout; ns, not significant.

Discussion

We investigated the mechanism of AC9 regulation by G proteins and kinases, concluding that AC9 is directly regulated by $G\alpha_s$ with weak, conditional activation by forskolin; other modes of proposed regulation occur indirectly, or possibly require additional scaffolding proteins to facilitate regulation. We also show that AC9 contributes to basal cAMP production; knockdown or genetic elimination of endogenous AC9 reduces basal AC activity. Importantly, although AC9 is not directly inhibited by *Gai/o*, it can heterodimerize with *Gai/o*-regulated isoforms, AC5 and AC6.

AC9 Is Not Directly Regulated by Most G Proteins or Kinases. Like all transmembrane AC isoforms, AC9 is directly activated by $G\alpha_s$. However, the $G\alpha_s$ dose-response curve for AC9 is right shifted compared with other ACs (Chen-Goodspeed et al., 2005; Dessauer et al., 2017), possibly due to the shorter $\alpha 1'$ and $\alpha 2'$ loop, which directly contacts switch II of $G\alpha_s$ (Tesmer et al., 1997). $G\alpha_s$ stimulation of AC9 is sensitive to glycosylation (Cumbay and Watts, 2004); however, this does not explain the shift in sensitivity, as the requirement for increased $G\alpha_s$ has been observed in both Sf9 and HEK293 cells (Piggott et al., 2008; Efendiev et al., 2010).

Although no splice variants of AC9 are reported, proteolytic alterations of AC9 give rise to two main forms observed in rodent and human hearts (Pálvolgyi et al., 2018). The extended C terminus of the larger form (~170 kDa) is reported to autoinhibit AC9; this is lacking in the smaller form (~130 kDa) (Pálvolgyi et al., 2018). To complicate analysis, two human clones were previously used in studies of AC9 regulation. A truncated AC9, lacking the last 75 amino acids of C2b (amino acids 1–1252; predicted molecular mass of 144.2 kDa), was employed to examine *Gai/o*, CaMKII, and novel PKC regulation (Hacker et al., 1998; Cumbay and Watts, 2004, 2005), whereas others used full-length AC9 (amino acids 1–1327; predicted molecular mass of 147.7 kDa) for CaN and PKC β II regulation (Antoni et al., 1998; Paterson et al., 2000; Liu et al., 2014). Our study used both forms: the truncated version for Sf9 expression and the full-length version for mammalian cell expression; however, no difference in $G\alpha_s$ -stimulated activity was observed when expressed in HEK293 cells (data not shown).

AC9 is categorized as the only forskolin-insensitive membrane-bound AC (Hacker et al., 1998). Although forskolin cannot stimulate basal AC9 activity (Hacker et al., 1998), it

Fig. 7. AC9 homo- and heterodimers. Quantification of COS-7 cells expressing BiFC constructs for AC9, AC5, and AC6. One of two halves of a fluorescent protein: the N-terminal portion (VN) or the C-terminal portion (VC) is fused in frame to the C terminus of each AC isoform, except where noted. (A) The homodimers are expressing both AC-VN and AC-VC for the given isoform. AC9-VN alone is a negative control. (B) Heterodimer formation used AC-VN (top one listed) from one isoform with AC-VC from a second isoform. For example, AC5-AC9 corresponds to cotransfection of AC5-VN and AC9-VC. AC5* is N-terminally tagged with VC. (C and D) Cell extracts from cells transiently transfected with Flag-AC9 (fAC9) with or without AC5 (C) or Flag-AC9 with or without AC6 (D) were subjected to immunoprecipitation with FLAG-agarose and Western blotted for FLAG, AC5, or AC6. As positive controls, Flag-AC5 and YFP-AC6 were immunoprecipitated with FLAG-agarose or anti-GFP and protein G. The delta symbol indicates a nonspecific band; no endogenous AC6 is ever observed in pulldowns of Flag-AC9. Note, although the FLAG tag on the control Flag-AC5 can be used for immunoprecipitation, it is not readily detected by Western blotting. Data are shown as means $\pm$ S.D. Statistical analyses in (A) and (B) were performed with one-way ANOVA followed by the Dunnett multiple-comparisons test comparing AC9 VN to each condition indicated. $n = 3$ –5 with experiments performed in duplicate. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. GFP, green fluorescent protein; ns, not significant.

can weakly activate AC9 in the presence of *G α s*. Synergistic activation by *G α s*, observed with other isoforms, likely promotes forskolin binding, despite alterations in the forskolin binding pocket of AC9 (Yan et al., 1998). This highlights the importance of the cell system, as endogenous AC isoforms can contribute to or mask regulation, even when an AC isoform of interest is overexpressed.

G $\beta\gamma$ either conditionally stimulates (AC2, AC4–6, AC7) or inhibits (AC1, AC3, AC8) all other AC isoforms (reviewed in Sadana and Dessauer, 2009; Dessauer et al., 2017). Several studies, including this study, have shown that *G $\beta\gamma$* does not directly regulate AC9 (Premont et al., 1996; Hacker et al., 1998), although it may indirectly activate AC9 in neutrophils (Liu et al., 2010). Despite not directly regulating AC9, *G $\beta\gamma$* binds to the N terminus of AC9 (Brand et al., 2015), suggesting

that the N termini of AC9 may serve as a scaffolding site for heterotrimeric G proteins (Sadana et al., 2009).

The lack of direct regulation by *G α i/o*, although contradictory with some whole-cell studies, is not entirely surprising. AC9 has little homology with the AC5 *G α i* binding site; only one of six residues required for AC5 inhibition is conserved in AC9 (Dessauer et al., 1998). Moreover, *G α i/o*-coupled chemokine receptors in neutrophils promote *G $\beta\gamma$* activation of the mammalian target of rapamycin and subsequently PKC β II, ultimately stimulating cAMP production by AC9 (Mahadeo et al., 2007). Direct inhibition of AC9 by *G α i* in this system would be counterproductive to the indirect stimulation of AC9 by *G $\beta\gamma$* (Liu et al., 2014; Surve et al., 2016). Rather, *G α i* in neutrophils appears to act on other effectors in a cAMP-independent manner,

targeting Ras-related protein 1 GTPase-activating protein II (Mochizuki et al., 1999) to regulate neutrophil adhesion (To and Smrcka, 2018).

AC9 was originally cloned as a CaN-inhibited isoform (Paterson et al., 2000), implying that phosphorylation is an important aspect of AC9 regulation. AC9 contains two predicted PKC sites (T309 and S374) (Scansite 4.0; Massachusetts Institute of Technology, Boston, MA), located at the base of transmembrane domain TM6 or within an 18-amino-acid insert unique to AC9, prior to the catalytic core. Although our *in vitro* results do not support a direct role for PKC β II or CaMKII regulation of AC9, it is possible that a scaffolding protein, absent from our *in vitro* studies, is required to facilitate phosphorylation (Dessauer, 2009). Alternatively, PKC β II and CaMKII may phosphorylate an unidentified upstream regulator of AC9.

Regulation of Basal cAMP Levels. Examining basal levels of cAMP is difficult; not only is basal activity frequently used to normalize data but it can also be error prone, occurring at lower limits of detection. Reports have noted differences in AC basal activities, specifically AC1 and AC3 in olfaction (Bakalyar and Reed, 1990) and AC2 and AC6 (Pieroni et al., 1995), suggesting that basal activity is an important aspect of their physiologic roles. Supporting this concept, the most notable effects of AC9 knockout on cardiac function occurred at baseline, where mice displayed bradycardia, diastolic dysfunction, and reduced phosphorylation of the small heat shock protein 20 (Li et al., 2017). AC9 contributes to considerable basal AC activity in COS-7 cells and splenocytes; a similar effect was recently reported in HEK293 cells (Pálvölgyi et al., 2018).

Whole-Cell Versus *In Vitro* Biochemical Assessment of AC9 Regulation. Surprisingly, our data support an indirect mechanism for most previously proposed AC9 regulators. One dilemma is how to study AC regulation. For years, the gold standard was AC expression in Sf9 cells combined with *in vitro* biochemical assays using purified regulators. However, requirements for scaffolding proteins or modifications of AC are missed by this approach. For example, PKA phosphorylation and inhibition of AC5 and AC6 requires a large excess of kinase in membranes or with detergent-solubilized preparations of AC; however, regulation of AC5/AC6 by PKA is readily apparent when in complex with A-kinase anchoring protein AKAP79-PKA (Iwami et al., 1995; Chen et al., 1997; Bauman et al., 2006). Generally, scaffolding proteins simply make these reactions more efficient; thus, we expected to detect direct PKC regulation of AC9 *in vitro* using an excess of kinase sufficient to activate AC2.

To further evaluate differences between our biochemical results and previous whole-cell experiments, a live-cell cAMP assay was employed in COS-7 cells. HEK293 cells are widely used for AC studies but they endogenously express multiple AC isoforms, such as AC1, AC2, AC3, AC5, AC6, AC7 (weakly), AC9, and soluble AC (Lefkimiatis et al., 2009). COS-7 cells provided the advantage of a mammalian cell line, lacking expression of *Gai*-inhibitable ACs while endogenously expressing AC7 (Premont, 1994) and AC9 (representing ~40% of *Gas*-stimulated activity). Agonist stimulation of μ OR-coupled *Gi/o* receptors inhibited overexpressed AC6 in COS-7 cells, demonstrating that these cells have all of the necessary components for *Gai/o* inhibition.

The fact that we observed no inhibition of endogenous AC activity or AC9 overexpressed activity in whole cells by μ ORs further supports the lack of direct regulation of AC9 by *Gai/o*.

AC Homo- and Heterodimerization. Numerous studies have suggested that AC isoforms form homodimers based on mutational complementation (AC1), cooperative activation (AC5), or coimmunoprecipitation and fluorescence resonance energy transfer (AC6 and AC8) (Tang et al., 1995; Gu et al., 2002; Chen-Goodspeed et al., 2005). Weak heteromeric formation was reported for AC8 with AC6 (Gu et al., 2002). More recently, AC5 was shown to interact through its transmembrane helices with adenosine (A_2 AR) and dopamine (D_2 R) receptors; the functional precoupling of this complex was necessary to promote canonical *Gs*-*Gi* regulation of AC5 (Navarro et al., 2018). We show that AC9 not only forms homodimers, but it can also form heteromers with *Gai*-regulated AC5 and AC6. The ability of AC9 to form heteromers may explain why studying AC9 regulation in cell lines that express multiple AC isoforms is so difficult. It is tempting to speculate that dimerization of AC9 with AC5/AC6 could potentially result in *Gai/o* inhibition of the complex or even AC9. Heteromer formation may also explain why a reduction of both *G α s*- and forskolin-regulated activity is observed upon knockdown or knockout of AC9; given the relative forskolin insensitivity of AC9, we would have expected minimal loss of forskolin-stimulated activity.

In conclusion, our findings support several physiologic roles for AC9. Basal AC9 activity likely regulates baseline heat shock protein 20 phosphorylation and cardiac stress responses, whereas indirect regulation of AC9 by *G $\beta\gamma$* mediates neutrophil chemotaxis. In the heart, AC9-containing complexes contribute to sympathetic activation of the I_{Ks} channel. Decreased sensitivity of AC9 to *G α s* emphasizes the importance of local control and requirements for Yotiao-dependent scaffolding to facilitate both activation of AC9 and PKA phosphorylation of I_{Ks} . Interestingly I_{Ks} is not regulated by *Gai/o*-coupled muscarinic receptors; rather, acetylcholine appears to directly inhibit the channel independent of cAMP (Freeman and Kass, 1995), consistent with a lack of direct regulation of AC9 by *Gai/o*. Finally, homo- and heterodimerization of AC9 may present another mechanism to facilitate crosstalk between signaling pathways.

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Authorship Contributions

Participated in research design: Baldwin, Li, Brand, Dessauer.
Conducted experiments: Baldwin, Li, Brand, Watts.
Contributed new reagents or analytic tools: Li, Watts, Dessauer.
Performed data analysis: Baldwin, Dessauer.
Wrote or contributed to the writing of the manuscript: Baldwin, Dessauer.

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