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Pharmacological Distinction between Soluble and Transmembrane Adenylyl Cyclases

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ABSTRACT

The second messenger cAMP is involved in a number of cellular signaling pathways. In mammals, cAMP is produced by either the hormonally responsive, G protein–regulated transmembrane adenylyl cyclases (tmACs) or by the bicarbonate- and calcium-regulated soluble adenylyl cyclase (sAC). To develop tools to differentiate tmAC and sAC signaling, we determined the specificity and potency of commercially available adenylyl cyclase inhibitors. In cellular systems, two inhibitors, KH7 and catechol estrogens, proved specific for sAC, and 2′,5′-dideoxyadenosine proved specific for tmACs. These tools provide a means to define the specific contributions of the different families of adenylyl cyclases in cells and tissues, which will further our understanding of cell signaling.

Introduction

Since its discovery in 1958 (Rall and Sutherland, 1958; Sutherland and Rall, 1958), cAMP has been implicated in a variety of cellular signaling pathways in virtually every mammalian cell type and organ system. The second messenger is produced by adenylyl cyclases (ACs) and is broken down by phosphodiesterases (PDEs), and its cellular effects are mediated by its known effectors: protein kinase A (PKA), exchange proteins activated by cAMP (EPACs), and cyclic nucleotide regulated ion channels.

As cAMP signaling pathways were discovered, it became evident that multiple cAMP cascades were present within individual cells (Hayes et al., 1980; Hayes and Brunton, 1982; Buxton and Brunton, 1983). This appreciation demanded revisions of the traditional model, in which cAMP is produced at the plasma membrane and diffused to its effector proteins distributed throughout the cell, into modern models, which posit that cAMP signaling occurs in independently regulated, intracellular compartments or microdomains (Dessauer, 2009; Houslay, 2010; Zaccolo, 2011). Within a compartment, cAMP can be produced locally, by a dedicated adenylyl cyclase (Bundey and Insel, 2004; Acin-Perez et al., 2009; Wachten et al., 2010; Willoughby et al., 2010; Zippin et al., 2010; Sample et al., 2012; Di Benedetto et al., 2013; Lefkimmiatis et al., 2013), and it can modulate the activity of a local effector, such as PKA tethered by an A kinase-anchoring protein (Dessauer, 2009). The sanctity of individual microdomains would be maintained by physical or enzymatic barriers, such as membranes (Rich et al., 2000; Acin-Perez et al., 2009; Di Benedetto et al., 2013) or PDEs (Houslay, 2010), which limit diffusion of the second messenger, as well as by the anchoring properties of A kinase-anchoring proteins (Dessauer, 2009), which keep the cyclase and effector proteins within the desired microdomains. This model allows distinct cyclase isoforms to respond to different input signals and transduce independent messages into unique effects (Zippin et al., 2001; Bundey and Insel, 2004).

In mammalian cells, there are two distinct families of adenylyl cyclases; nine genes encode a family of transmembrane adenylyl cyclases (tmACs), and a single, alternatively spliced gene encodes a family of soluble adenylyl cyclase (sAC) isoforms. Although sAC and tmACs share similar active sites and mechanisms of action (reviewed in Kamenetsky et al., 2006), they differ in both their subcellular localization and their regulation. TmACs, which possess two domains comprising six membrane spanning segments each, signal at the plasma membrane and during internalization (Calebiro et al., 2009; Ferrandon et al., 2009) in response to extracellular stimuli. They are regulated by G-protein-coupled receptors (GPCRs) and heterotrimeric G proteins. In contrast, sAC lacks predicted membrane spanning domains and is found throughout the cytoplasm (Zippin et al., 2004), as well as inside mitochondria

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ABBREVIATIONS: 2-CE, 2-hydroxyestradiol; 4-CE, 4-hydroxyestradiol; 9-CP, 9-cyclopentyladenine; ddAdo, 2′,5′-dideoxyadenosine; ddATP, 2′,5′-dideoxyadenosine 3′-triphosphate; DMSO, dimethylsulfoxide; EPAC, exchange proteins activated by cAMP; FBS, fetal bovine serum; GPCR, G-protein-coupled receptor; HEK293, human embryonic kidney cell line 293; KH7, 2-(1*H*-benzo[*d*]imidazol-2-ylthio)-*N*′-(5-bromo-2-hydroxybenzylidene) propanehydrazide; MDL-12,330a, *cis*-*N*-(2-phenylcyclopentyl)azacyclotridec-1-en-2-amine hydrochloride; MEF, mouse embryo fibroblast; NKY80, 2-amino-7-(2-furanyl)-7,8-dihydro-5(6*H*)-quinazoline; PACAP, pituitary adenylate cyclase–activating peptide; PDE, phosphodiesterase; PACAP, pituitary adenylate–cyclase activating peptide; PKA, protein kinase A; sAC, soluble adenylyl cyclase; sAC KO MEFs, sAC knockout mouse embryo fibroblasts; SQ22,536, 9-(tetrahydrofuryl)-adenine; tmAC, transmembrane adenylyl cyclase.

(Zippin et al., 2003; Acin-Perez et al., 2009) and nuclei (Zippin et al., 2003, 2004, 2010). Also distinct from tmACs, sAC activity is modulated by bicarbonate (Chen et al., 2000) and calcium ions (Jaiswal and Conti, 2003; Litvin et al., 2003), and it is sensitive to variations in intracellular ATP concentrations (Litvin et al., 2003; Zippin et al., 2013). Through its bicarbonate regulation, sAC has been shown to function as a physiologic $CO_2/HCO_3^-/pH_i$ sensor (Tresguerres et al., 2010).

Both tmACs and sAC are widely expressed in mammalian cells and tissues (Chen et al., 2000, 2013; Geng et al., 2005; Kamenetsky et al., 2006), with many cells shown to express both sources of cAMP (Stessin et al., 2006; Wu et al., 2006; Ramos et al., 2008; Dunn et al., 2009; Strazzabosco et al., 2009; Halm et al., 2010; Hollenhorst et al., 2012). Therefore, to understand fully the regulation of cAMP signaling pathways, it is essential to be able to discern the relative contributions of sAC versus tmACs. Genetic methods, including sAC knockout (KO) mice (Esposito et al., 2004; Hess et al., 2005; Lee et al., 2011; Choi et al., 2012; Chen et al., 2013) and knockdown using sAC-specific siRNA (Stessin et al., 2006; Wu et al., 2006; Ramos et al., 2008), have been informative for identifying a number of sAC functions (reviewed in Tresguerres et al., 2011). Similarly, individual tmAC knockout (Wu et al., 1995; Abdel-Majid et al., 1998; Storm et al., 1998; Patel et al., 2001; Yan et al., 2007; Sadana and Dessauer, 2009; Chien et al., 2010) and overexpressing transgenic mice (Sadana and Dessauer, 2009) have been useful for identifying roles for specific tmAC isoforms. However, because of the number of tmAC genes, genetic studies are limited in their utility for differentiating the more general question of whether an identified cAMP signaling cascade is dependent on tmAC-generated cAMP or sAC-generated second messenger. For such questions, it would be beneficial to have a pharmacologic strategy for selectively inhibiting sAC or the family of tmACs.

Considerable effort has been made toward developing pharmacological inhibitors that distinguish between the nine tmAC isoforms (Johnson et al., 1997; Onda et al., 2001; Seifert et al., 2012), but much of this toolbox has not been tested against sAC. In this study, we explored the potency and specificity of a number of commercially available AC inhibitors in in vitro cyclase assays and in cAMP accumulation in intact cells using cell lines where the cAMP generated was predominantly sAC-dependent or exclusively tmAC-dependent. Based on their specificities, we recommend a strategy for exploring whether sAC or tmACs produce the cAMP contributing to an individual intracellular signaling pathway.

Materials and Methods

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. KH7 was synthesized by the Milstein Synthetic Chemistry Core Facility at Weill Cornell Medical College. 2-Hydroxyestradiol (2-CE) and 4-hydroxyestradiol (4-CE) were purchased from Steraloids (Newport, RI); 2′,5′-dideoxyadenosine (ddAdo) was purchased from Enzo Life Sciences (Farmingdale, NY). SQ22,536 and MDL-12,330a were purchased from Santa Cruz Biosciences (Dallas, TX). ^{32}P-labeled ATP and ^{3}H-labeled cAMP were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). KH7 was used in the presence of protein carrier; all other inhibitors were dissolved fresh for each experiment.

Cell Cultures

To create sAC stably transfected 4-4 cells, human embryonic kidney cells (HEK293) were transfected with plasmid containing the sAC_t cDNA (Buck et al., 1999) and placed under selection pressure with gentamicin. Resistant cells were selected and diluted to individual cells, and single clones were established; 4-4 cells represent one such clone. Once single clones were grown for multiple generations, gentamicin was removed from the media. Overexpression of sAC_t was periodically confirmed by Western blot or activity assay.

Immortalized sAC KO mouse embryo fibroblasts (MEFs) were generated by the 3T3 method involving serial passage of primary mouse embryo fibroblasts. In brief, 16- to 19-day-old embryos were minced and trypsinized (0.5% trypsin-EDTA) overnight at 4°C. After removal of tissue clumps and trypsin inactivation, remaining cells were dispersed in DMEM supplemented with 10% fetal bovine serum (FBS) and passaged every 3 days until passage ~20–25.

sAC Purification

Rat sAC_t protein was cloned into baculovirus expression vector (Life Technologies, Carlsbad, CA) containing a C-terminal His tag. Hi5 insect cells were infected for 53 hours followed by centrifugation at 5000*g* for 15 minutes. Cells were frozen in liquid nitrogen and stored overnight at −80°C. Cells were thawed and resuspended in lysis buffer (50 mM Tris, pH 7.5, 300 mM NaCl, 10 mM imidazole, 10 μg/ml aprotinin/leupeptin, 1 mM phenylmethylsulfonyl fluoride) and sonicated 4 × 1 minute with 1 minute of rest in between. The lysate was clarified by a spin at 15,000*g* for 30 minutes at 4°C, and sAC_t was purified from the supernatant by Sephadex G25 and nickel-NTA columns. Eluates were tested for cyclase activity, and the highest activity fractions were combined and stored at −20°C in 50% glycerol.

Cyclase Assays

Using Purified Protein. Assays for sAC activity were performed in 100-μl reactions containing 10 mM $MnCl_2$, 2.5 mM ATP, and 50 mM Tris, pH 7.5. Each reaction contained ~1,000,000 counts of α-^{32}P-labeled ATP. Generated cAMP was purified using sequential Dowex and Alumina chromatography as previously described (Salomon, 1979). For SQ 22,536, MDL-12,330a, NKY80, and KH7, the assays contained 5% (v/v) dimethylsulfoxide (DMSO). Assays for vidarabine contained 1% (v/v) DMSO. Assays for ddATP and ddAdo contained no DMSO. In control experiments, DMSO alone did not alter the cyclase activities of sAC or the extracts from HEK293 or 4-4 cells.

Using Whole-Cell Extracts. HEK293T and 4-4 cells were grown to 80% confluence in DMEM with 10% FBS, 1% penicillin/streptomycin, and 1% glutamine. Cells were removed by trypsinization, collected, and resuspended in 500 μl of lysis buffer (150 mM NaCl, 50 mM Tris pH 7.5, 1 mM aprotinin/leupeption, 1 mM phenylmethyl sulfonyl fluoride, 1 mM benzamidine, 1 mM DTT). Cells were sonicated, and the sonicated lysate was used for cyclase reactions. Each reaction was performed in 100 μl containing 10 μl of the lysate (~40 μg total protein), 50 mM Tris, pH 7.5, 2.5 mM ATP (containing 1,000,000 counts α-^{32}P labeled ATP), 10 mM $MnCl_2$, 500 μM 3-isobutyl-1-methylxanthine (IBMX), 1 mM DTT, 20 mM creatine phosphate, 5 U of creatine phosphate kinase, and the indicated concentrations of inhibitors. Assays of 293T cell lysates included 50 μM forskolin to stimulate tmAC activity. For SQ22,536, MDL-12,330a, NKY80, and KH7, the assays contained 5% (v/v) DMSO. Assays for vidarabine contained 1% (v/v) DMSO. Assays for ddATP and ddAdo contained no DMSO. cAMP was purified using the two-column method described already herein. Each dose response was performed alongside a no-drug control and a time-zero control, and all values were normalized against these values. Dose-response curves were generated by nonlinear regression using GraphPad Prism (GraphPad Prism, Inc. San Diego, CA). For 4-4 cell lysates, the specific activity was 1.0 ± 0.3 pmol cAMP min^{-1} μg^{-1} lysate, and for HEK293 cells plus forskolin, the specific activity was 0.20 pmol cAMP min^{-1} μg^{-1} lysate.

Cellular cAMP Accumulation

For in vivo cAMP accumulation assays, 2.5×10^5 cells were plated in each well of a 24-well plate in high-glucose DMEM supplemented

with 10% FBS. The next day, compounds (i.e., KH7, ddAdo, 2-CE, and 4-CE) diluted in culture media were added to the cells. Cells were pretreated with the drugs for 10 minutes, followed by the addition of culture media with 500 μM IBMX and 50 μM dipyridamole and where appropriate (i.e., in sAC KO MEFs) 50 μM forskolin. Cells were incubated for another 5 minutes at 37°C. Media were aspirated, and the cells were lysed with 200 μl of 0.1 M hydrochloric acid (HCl) per well. Assays contained 0.2% (v/v) DMSO. Intracellular cAMP content was determined using Correlate-EIA cAMP Direct Assay (Enzo Life Sciences, Farmingdale, NY). Specific activity was as follows: for unstimulated 4-4 cells, 1.3 ± 0.3 pmol cAMP 10^6 cells^{-1} min^{-1}; for unstimulated KO MEFS, 0.025 ± 0.003 pmol cAMP 10^6 cells^{-1} min^{-1}; for forskolin-stimulated HEK293 cells, 5.0 ± 1.4 pmol cAMP 10^6 cells^{-1} min^{-1}; and for forskolin-stimulated KO MEFS, 2.2 ± 0.2 pmol cAMP 10^6 cells^{-1} min^{-1}.

Results

Numerous commercially available compounds have been described as adenylyl cyclase inhibitors; these include KH7, catechol derivatives of estrogen, such as 2-CE or 4-CE, ddATP, ddAdo, MDL-12,330a, SQ22,536, 9-CP, NKY80, and vidarabine (Fig. 1). To our knowledge, only ddATP (Gille et al., 2004) and the CEs (Steegborn et al., 2005) have been directly compared against purified sAC and individual tmAC isoforms, and these compounds inhibited both sAC and the tmACs. We showed that purified sAC protein and tmAC isoforms I, II, and V, as well as a solubilized version of tmAC type VII, were inhibited by catechol estrogens with IC_{50} values in the low micromolar range (Steegborn et al., 2005). These data led us to conclude that CEs were general AC inhibitors. Gille et al. (2004) found that ddATP inhibited sAC and tmACs type I, II, and V with submicromolar IC_{50} values, although sAC was 3- to 20-fold less sensitive. Their experiments were performed under suboptimal conditions for measuring sAC activity; they assessed the ddATP inhibitory profile in the presence of 100 μM ATP, which is commensurate with the K_m for ATP of the tmACs, but it is 10-fold below sAC's K_m (Litvin et al., 2003). Therefore, we re-examined the inhibitory potential of ddATP, along with the commercially available AC inhibitors, against purified sAC_t protein (Chen et al., 2000; Litvin et al., 2003) in its most potently activated state (i.e., in the presence of the divalent cation Mn^{2+} with 2.5 mM ATP as substrate) (Braun and Dods, 1975; Litvin et al., 2003). Under these conditions, ddATP inhibited purified sAC with an IC_{50} of ~138 μM (Fig. 2A). KH7 is the only compound advertised as an sAC inhibitor, and it inhibited Mn^{2+}-dependent sAC activity with an IC^{50} of 2.7 ± 1.3 μM, similar to its previously reported potency (Hess et al., 2005). KH7 inhibited physiologically stimulated sAC (i.e., assayed in the presence of Mg^{2+}-ATP and the activators calcium and bicarbonate) (Litvin et al., 2003) with similar potency (IC_{50} of 8.0 ± 1.1 μM), consistent with it being a noncompetitive inhibitor (Hess et al., 2005). Among the other commercially available AC inhibitors, only MDL-12,330a and ddAdo affected the maximally stimulated (i.e., using Mn^{2+}-ATP as substrate) in vitro activity of pure sAC; MDL-12,330a inhibited with an IC_{50} of ~240 μM, and ddAdo inhibited sAC at concentrations greater than 100 μM (Fig. 2B). The remaining compounds tested, SQ22,536 (Fig. 2C), 9-CP (Fig. 2D), NKY80 (Fig. 2E), and vidarabine (Fig. 2F), did not inhibit pure sAC protein at concentrations up to 500 μM.

Previous studies exploring adenylyl cyclase inhibitor selectivity have focused on identifying compounds that can differentiate between the nine tmAC isoforms. For those experiments, inhibitor profiles were measured on heterologously expressed and purified individual tmAC isoforms (Onda et al., 2001; Gille et al., 2004; Iwatsubo et al., 2004; Seifert et al., 2012). We wanted to address a different question; we asked whether inhibitors can distinguish between sAC-mediated processes and processes mediated by any member of the family of tmACs. Therefore, we wanted to assess the inhibitory potential against a physiologically relevant mixture of tmACs. Historically, this would be accomplished by assaying the particulate fraction from any of a variety of tissues known to have abundant tmAC activities (i.e., brain, liver). Unfortunately, in most cells and

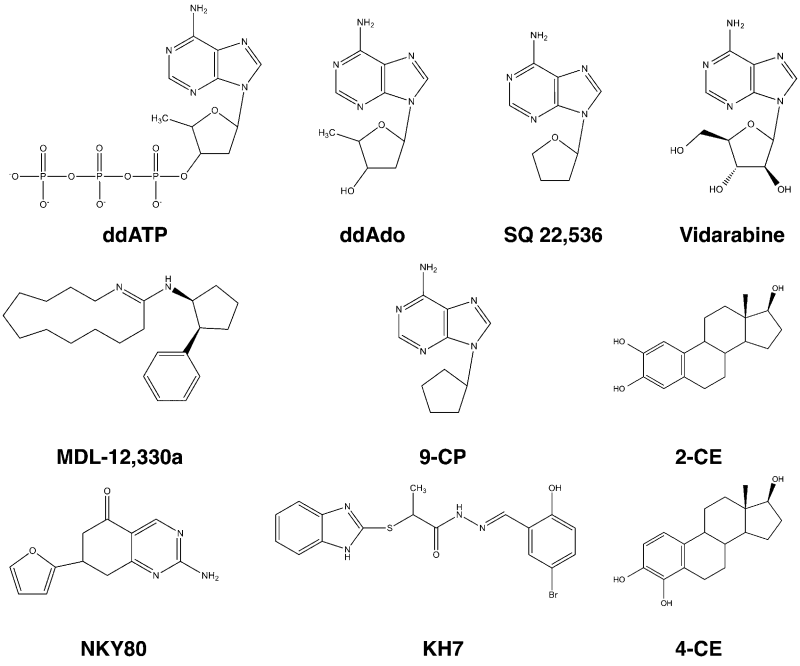

Fig. 1. Structures of compounds used in this study. Dideoxyadenosine 3′-triphosphate (ddATP), 2′,5′-Dideoxyadenosine (ddAdo), 9-(tetrahydrofuryl)-adenine (SQ 22,536), 9-β-D-arabinosyladenine (vidarabine), 9-cyclopentyladenine (9-CP), *cis-N*-(2-phenylcyclopentyl)azacyclotridec-1-en-2-amine hydrochloride (MDL-12,330a), 2-amino-7-(2-furanyl)-7,8-dihydro-5(6*H*)-quinazoline (NKY80), 2-(1*H*-benzo[*d*]imidazol-2-ylthio)-*N*′-(5-bromo-2-hydroxybenzylidene) propanehydrazide (KH7), and catechol derivatives of estrogen, such as 2-hydroxyestradiol (2-CE) or 4-hydroxyestradiol (4-CE).

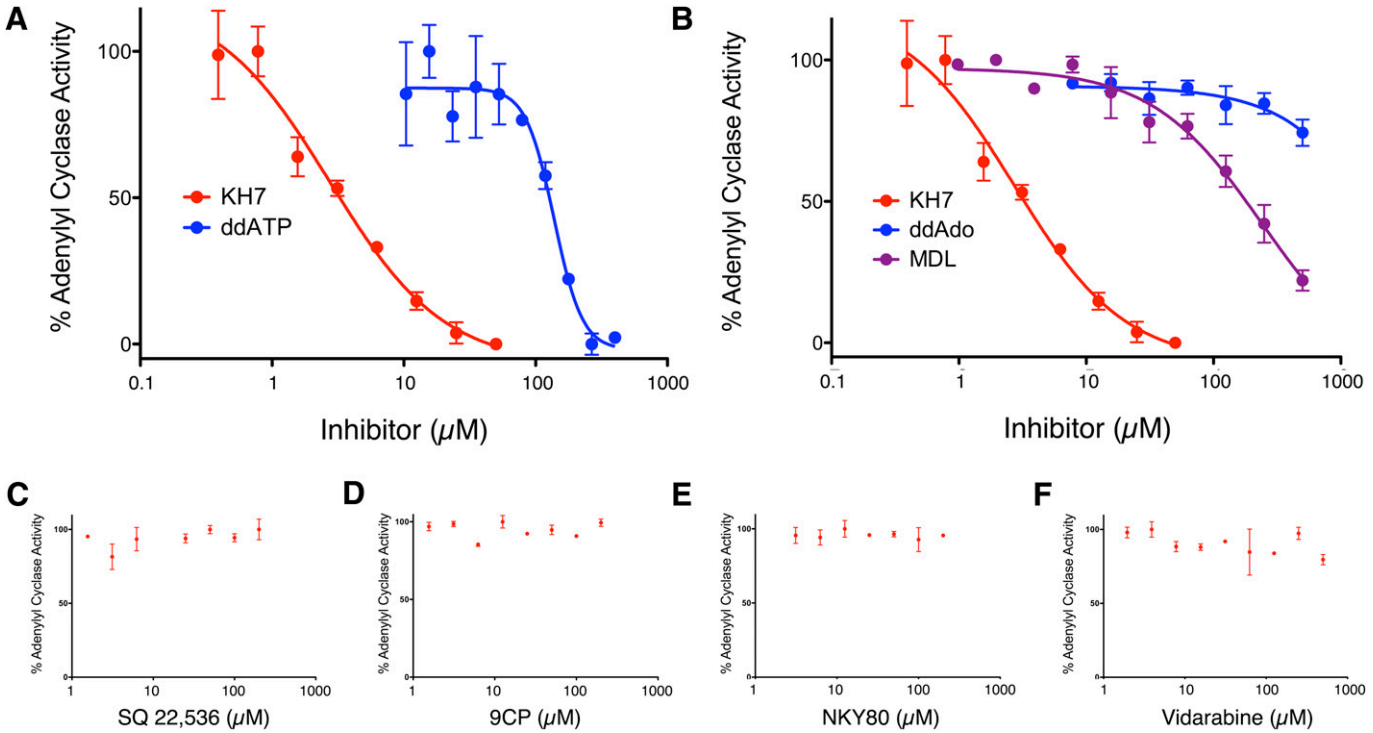

Fig. 2. Inhibition of purified rat sAC_t activity. (A) Rat sAC_t in vitro adenylyl cyclase activity in the presence of 2.5 mM ATP and 10 mM $MnCl_2$ and the indicated concentrations of KH7 (red; IC_{50} = 3 μM) or ddATP (blue; IC_{50} = 400 μM); (B) ddAdo (blue) or MDL-12,330a (purple; IC_{50} = 240 μM) (shown with KH7 for comparison); (C) SQ 22,536; (D) 9-CP; (E) NKY80; or (F) vidarabine. Graphs are representative assays repeated at least twice; values represent activity normalized to sAC_t activity in the absence of any inhibitor. Curves are nonlinear fits generated by Prism.

tissues, sAC resides in the particulate fraction with tmACs, and discerning the relative contributions of sAC and tmACs to total adenylyl cyclase activity is problematic. In addition, we need to compare the inhibitory profiles of these compounds against sAC in parallel with a mixture of tmACs. The only tissue or cell type where total adenylyl cyclase activity reflects predominantly sAC is testis cytosol, and comparisons between membrane (or detergent rich) fractions and cytosol would include undesirable biases.

Therefore, to compare directly sAC-dependent versus tmAC-dependent cAMP synthesis in a whole-cell context, we generated a stable cell line overexpressing the sAC_t isoform (Buck et al., 1999; Jaiswal and Conti, 2001). Clone 4-4 cells stably overexpress sAC_t via the cytomegalovirus promoter in human embryonic kidney (HEK293) cells (Fig. 3A). Relative to the parental HEK293 cells, whole-cell extracts from 4-4 cells have elevated basal adenylyl cyclase activity, which is due primarily to the overexpressed sAC_t protein (Zippin et al., 2013). To complement this sAC-dependent activity, we used forskolin-stimulated whole-cell extracts from the parental HEK293 cells as our source of tmAC-dependent cyclase activity. Kidney cells express tmAC isoforms IV, V, VI, VII, and IX (Defer et al., 2000). Of particular relevance to these studies, the parental HEK293 cell line has low levels of endogenous sAC activity; its basal activity is unaffected by the addition of the sAC-specific activator bicarbonate (Zippin et al., 2013). Plus, forskolin does not stimulate sAC (Buck et al., 1999); therefore, forskolin-stimulated HEK293 activity is due almost exclusively to its mixture of endogenous tmACs.

As expected, KH7 dose-dependently inhibited 4-4 whole-cell lysate activity, assayed in its maximally stimulated state (i.e., in the presence of Mn^{2+}-ATP as substrate), whereas it had no significant effect on forskolin-stimulated HEK293 activity (Fig. 3A). Its IC_{50} in the 4-4 whole-cell lysate was significantly higher (IC_{50} = 21 ± 7 μM) than on purified protein (IC_{50} = 2.7 ± 1.3 μM), presumably because of the presence of proteins and lipids, which will decrease the concentration of free KH7 relative to the nominal concentration (Rusinova et al., 2011) (N. B. Ramsey and O. S. Andersen, manuscript in preparation). The P-site inhibitors ddATP and ddAdo showed potent inhibition of tmAC activity with IC_{50} values of 0.8 ± 0.4 μM and 8.5 ± 3.5 μM, respectively (Fig. 3, B and C), and similar to their effect on purified sAC protein, these P-site ligands inhibited sAC activity in 4-4 lysate. Both P-site inhibitors were more potent against sAC in the lysate assay than they were against purified protein; ddATP inhibited 4-4 lysates with an IC_{50} of 12.8 ± 2.0 μM, and ddAdo inhibited with an IC_{50} of ~500 μM. Thus, ddATP seemed nearly as potent against sAC as it is against tmACs, but ddAdo inhibition profiles reveal an effective window, between 1 and 50 μM, where tmACs are preferentially inhibited relative to sAC. MDL-12,330a showed similar, but slightly less potent, inhibition of sAC in lysates compared with pure protein (Fig. 3D). Its relatively low affinity for sAC suggested that it might also prove to be a selective inhibitor of tmACs relative to sAC. However, in line with a recent report (Emery et al., 2013), MDL-12,330a failed to inhibit fully tmAC-dependent cAMP generation at concentrations up to 500 μM. In fact, its predicted IC_{50} of 375 μM was actually greater than the observed IC_{50} for inhibiting purified sAC protein.

Finally, the inhibitors that did not affect purified sAC protein also proved inert against sAC activity in the 4-4 cell

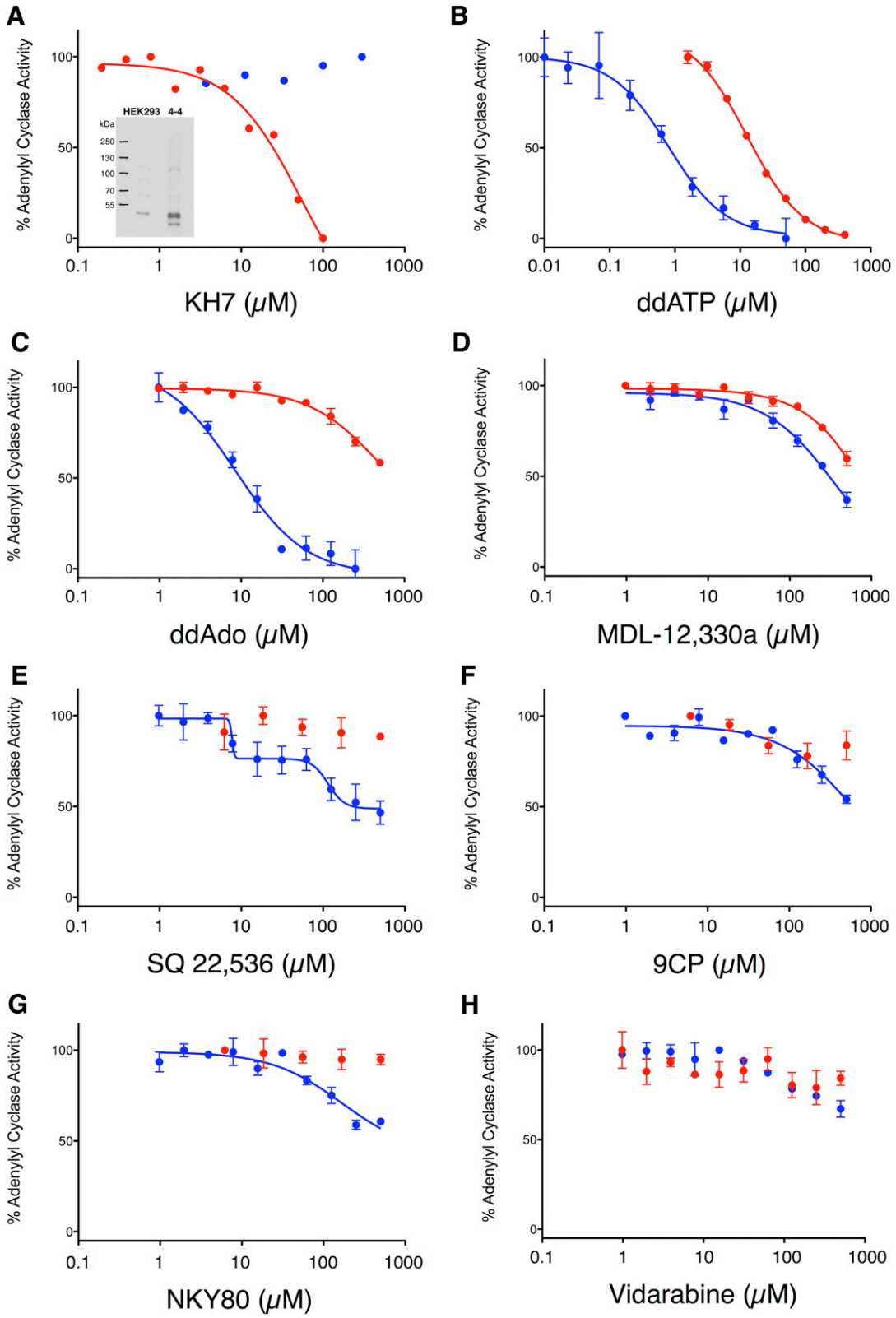

Fig. 3. Inhibition of sAC and tmACs in cellular lysates. (A, inset) Western blot of sAC in whole-cell lysates (10 μg) from parental HEK293 cells and 4-4 cells using the anti-sAC monoclonal antibody R21 (Zippin et al., 2003). (A–H) Adenylyl cyclase activities in whole-cell lysates from 4-4 cells (red) assayed in the presence of 2.5 mM ATP, 10 mM $MnCl_2$, and 500 μM IBMX or from 293 cells (blue) assayed in the presence of 2.5 mM ATP, 10 mM $MnCl_2$, 500 μM IBMX, and 50 μM forskolin in the presence of the indicated concentrations of (A) KH7, (B) ddATP, (C) ddAdo, (D) MDL-12,330a, (E) SQ 22,536, (F) 9-CP, (G) NKY80, or (H) vidarabine. Graphs are representative assays repeated at least twice; values represent activity normalized to activity in each whole-cell lysate in the absence of any inhibitor. Curves are nonlinear fits generated by Prism.

lysate (Fig. 3, E–H). Unfortunately, these inhibitors were not very potent against the mixture of tmAC activities present in whole-cell lysates of HEK293 cells. None fully inhibited the forskolin-stimulated cyclase activity at the maximum tested concentration (500 μM). SQ22,536 showed a two-step inhibition curve exhibiting IC_{50} values of ~8 and ~115 μM. However, it still achieved only ~45% inhibition by 500 μM drug added. Because SQ22,536 has previously been shown to have IC_{50} values ranging from 118 to 665 μM on different tmAC isoforms (Johnson et al., 1997), these effects may be due to differential inhibition of the tmAC isoforms present in this lysate. Similarly, 9-cyclopentyladenine, NKY80, and vidarabine showed only partial inhibition; 9-CP had not reached 50% inhibition by a 500 μM concentration of drug added to the assay, and NKY80 inhibition leveled off by 500 μM with an IC_{50} for this effect of 166 μM. Vidarabine showed a similar weak degree of inhibition and leveling off by the 500 μM dose. The leveling off and partial inhibition using NKY80 and vidarabine are likely due to the isoform selectivity of these compounds, as they are reported to be tmAC V selective in vitro (Onda et al., 2001; Iwatsubo et al., 2003, 2004; Seifert et al., 2012), and tmAC V is only one of the many tmAC isoforms expressed in HEK293 cells. Similarly, the shallow inhibition curves for several of the compounds are likely due to the complex mixture of tmAC isoforms constituting the cell lysate. Our data suggest that since NKY80 and vidarabine proved to be inert against purified sAC protein (Fig. 2G) and sAC activity in a cellular extract (Fig. 3H), they could retain their utility for probing isoform-specific questions about tmAC V, but they are not useful for the more general question distinguishing sAC from all tmACs. However, a recent report questions their utility as a tmAC V-selective reagent when querying cyclase activity in vivo (Braeunig et al., 2013). In a similar vein, cAMP-dependent processes identified based on inhibition by SQ22,536 or 9-CP may be due to tmACs, but these tools are not optimal for distinguishing between sAC and tmACs in general.

We next explored the utility of the subset of cyclase inhibitors that may be useful for distinguishing between sAC and tmACs on intact cells. We focused on KH7 as a putative sAC-selective inhibitor and on ddAdo as a putative tmAC-selective inhibitor; ddATP is not membrane-permeable and therefore cannot be used in cell-based assays. For these experiments, we measured the quantity of cAMP, which accumulates in a cell in the presence of a cocktail of phosphodiesterase (PDE) inhibitors. By inhibiting all known PDEs, the cAMP accumulation reflects only the amount of second messenger generated by the resident adenylyl cyclases. In 4-4 cells, the cAMP that accumulates is due almost exclusively to the overexpressed sAC_t (Zippin et al., 2013). For cells expressing a mixture of tmAC isoforms devoid of sAC, we measured forskolin-stimulated cAMP accumulation in immortalized mouse embryo fibroblasts (MEFs) generated from sAC-C1 KO mice. These sAC KO MEFs express tmAC types I, III, IV, VI, VII, VIII, and IX, as determined by RNA-seq (fragments per kilobase of exon per million fragments mapped >40th percentile); therefore, they provide an overlapping, but distinct, complement to 4-4 cells relative to the in vitro extract studies.

Consistent with its effects in whole-cell extracts, KH7 decreased the cAMP production in 4-4 cells with an IC_{50} of 27 ± 6 μM, and it had no effect on the tmAC-dependent cAMP accumulated in forskolin-stimulated sAC KO MEFs (Fig. 4A). In contrast to KH7, ddAdo inhibited forskolin-stimulated cAMP production in sAC KO MEFs but had no effect on the sAC-dependent cAMP accumulation in 4-4 cells (Fig. 4B). Thus, KH7 and ddAdo define a tool kit for probing the source of cAMP in cells; KH7 effectively inhibits sAC-dependent cAMP accumulation without affecting tmACs, whereas ddAdo inhibits tmAC-generated cAMP accumulation without affecting sAC.

As with all pharmacological reagents, there is always the danger of "off-target" activity. In fact, KH7 was recently described to exhibit sAC-independent (i.e., "off-target") effects on cellular metabolism (Tian et al., 2011; Di Benedetto et al., 2013). Therefore, we sought to identify a structurally unrelated sAC-selective inhibitor that can be used to complement KH7 and ddAdo. The catechol estrogens 2-CE and 4-CE inhibit pure sAC protein with low micromolar EC_{50}s (Steegborn et al., 2005), and although they were shown to inhibit specific tmAC isoforms in vitro, they have been used as sAC-selective inhibitors in a number of physiologic systems (Pastor-Soler et al., 2003; Wu et al., 2006; Choi et al., 2012; Corredor et al., 2012; Di Benedetto et al., 2013). We used our two cell lines, sAC-overexpressing 4-4 cells and sAC KO MEFs, to definitively explore their utility in cell-based assays. As seen in Fig. 4, C and D, 2-CE and 4-CE can be used to distinguish sAC-generated from tmAC-generated cAMP in cellular cAMP accumulation assays. Both 2-CE and 4-CE blocked cAMP accumulation in 4-4 cells but were inert in the sAC KO MEFs.

Discussion

Pharmacological tools provide the means for studying the acute effects of modulating a signaling pathway. Addition of a pharmacological inhibitor rapidly, and usually reversibly, turns off its target and the corresponding pathway. In contrast, genetic methods, including mouse knockouts, RNAi, and gene overexpression, although more specific, provide a means for studying chronic effects of altering a pathway. When asking the question of whether a cAMP signaling pathway is initiated by sAC or one of the family of tmACs, inhibitors become even more important; with current methods, it is not feasible to knock out or knock down all nine tmAC isoforms at once.

To clarify which pharmacological inhibitors are exploitable in this context, we used pure sAC protein, as well as cellular systems whose cAMP is derived almost exclusively from either sAC or tmACs, to determine the potency and specificity of a number of commercially available compounds advertised as adenylyl cyclase inhibitors (summarized in Table 1). Their efficacy compared with sAC- and tmAC-dependent activities suggests a strategy for using these compounds to distinguish sAC from tmAC as a source of cAMP governing a particular physiologic process. The sAC inhibitor discovered in our laboratory, KH7, showed potent inhibition of sAC activity with no discernible inhibition of tmAC activity both in vitro and in cellular assays. Both CEs tested, 2-CE and 4-CE, also proved to be sAC-selective inhibitors in cellular systems. To complement KH7 and CEs, ddAdo can be used to inhibit tmAC-dependent cAMP generation. In cellular systems, ddAdo appears to be tmAC specific up to 500 μM, but if used in an in vitro assay, we recommend using ddAdo at concentrations ranging from 30 to 50 μM, where it will fully inhibit tmAC activity while having little to no effect on sAC activity. The other inhibitors studied (ddATP, MDL-12,330a, SQ22,536, 9-CP, NKY80, and vidarabine) had

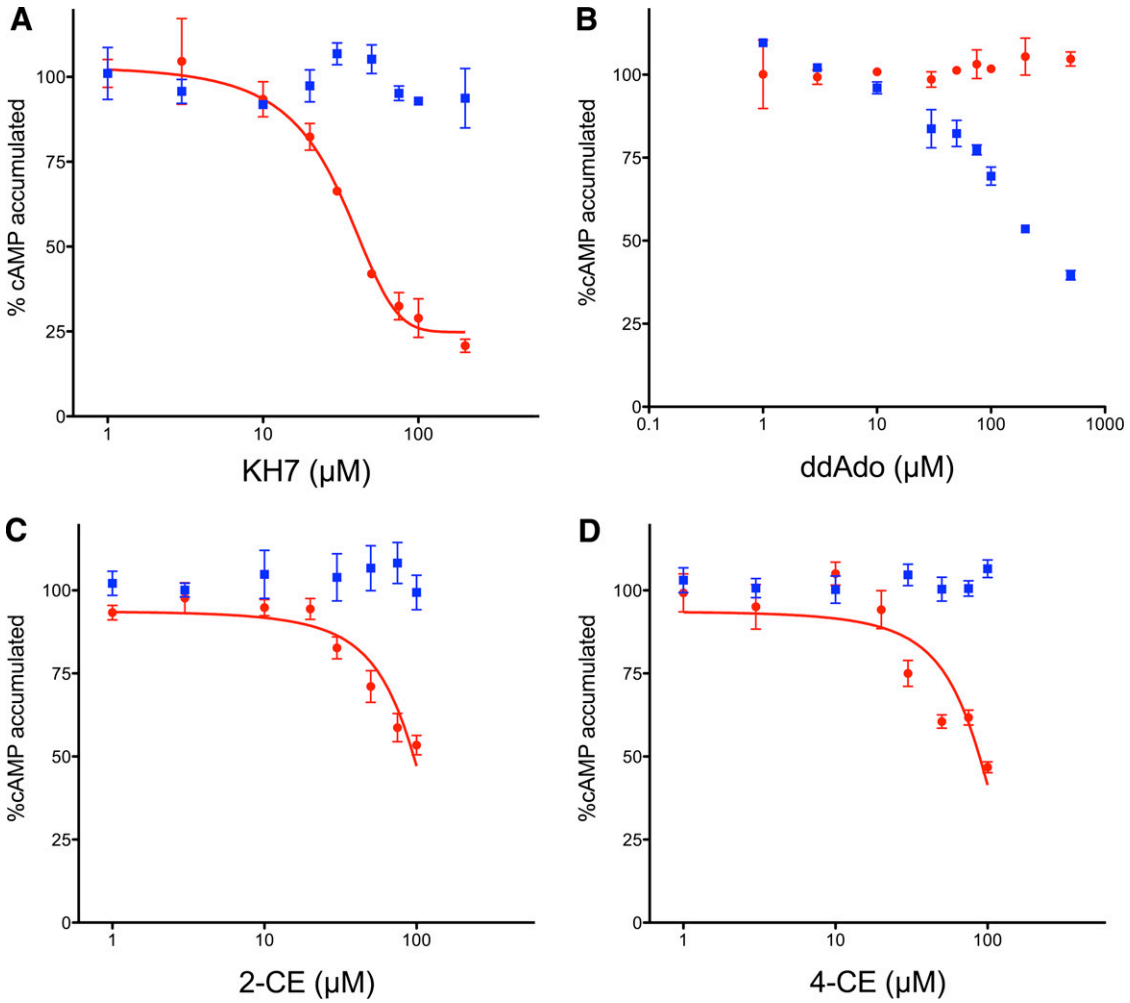

Fig. 4. Inhibition of sAC and tmACs in intact cells. Cellular cAMP accumulation in 4-4 cells (red) or in forskolin-stimulated (50 μM) sAC KO MEFs (blue) in the presence of 500 μM IBMX and 50 μM dipyridamole and the indicated concentrations of (A) KH7, (B) ddAdo, (C) 2-CE, or (D) 4-CE. Graphs are representative assays repeated at least twice; values represent activity normalized to cAMP accumulation in each cell line in the absence of any inhibitor. Curves are nonlinear fits generated by Prism.

little utility for probing the general question of whether a source of cAMP is sAC or one of the many tmAC isoforms. Because they seemed to have little effect on sAC activity, they may remain useful for probing tmAC isoform specific functions. In fact, the low efficacies, including shallow inhibition curves and incomplete inhibition, for SQ22,536, 9-CP, NKY80, and vidarabine are likely due to their tmAC isoform selectivity and the complex mixture of tmACs in HEK293 cell extracts.

A number of studies have exploited KH7, CEs, or ddAdo to discern whether sAC or tmACs represent the physiologically

TABLE 1
Summary of inhibitor data

Inhibitor	Purified sAC		sAC Lysate		tmAC Lysate		Cellular cAMP Accumulation	
	IC_{50}	Inhibition	IC_{50}	Inhibition	IC_{50}	Inhibition	sAC IC_{50}	tmAC IC_{50}
	μM	*%@500 μM*		*μM*		*%@500 μM*	*μM*	
KH7	3	>95	21	>95	–	0	27	–
ddAdo	–	25	500	40	8	>95	–	~100
ddATP	130	>95	13	>95	0.8	>95		
MDL - 12,330a	240	80	–	40	400	65		
SQ - 22,536	–	0	–	10	8;115	55		
9-CP	–	0	–	15	600	45		
Vidarabine	–	20	–	26	160	33		
NKY80	–	0	–	0	170	40		
2CE	~2[1]				~100[1]		~100	–
4CE	~10[1]				~100[1]		~100	–

From Steegborn et al. (2005).

relevant source of cAMP. In some systems, it proved essential to have inhibitors able to distinguish between sAC and tmACs because both sAC-generated and tmAC-generated cAMP elicited similar cellular effects, but they responded to distinct extracellular signals. Axonal outgrowth in response to the neuronal guidance cue netrin-1 was demonstrated to be sAC-dependent because it was blocked by KH7 and CEs and insensitive to ddAdo (Wu et al., 2006). Conversely, in the same cultures, axonal outgrowth in response to the hormone (PACAP) was confirmed to act via tmACs. PACAP signals via a Gs-coupled GPCR; therefore, it had been presumed to signal via tmACs. Consistently, its activity was inhibited by ddAdo and insensitive to KH7. Similarly, in PC12 cells, the PACAP-induced, cAMP-dependent activation of Rap1 was blocked exclusively by ddAdo, whereas nerve growth factor-stimulated Rap activation was concluded to be mediated via sAC because it was blocked by KH7 but not ddAdo (Stessin et al., 2006). In the pancreatic β cell–like cell line INS1-E, the cAMP rise in response to the Gs-coupled hormone GLP-1 was ascribed to tmACs because it was blocked by ddAdo, but not by KH7, and the glucose-dependent rise in cAMP was concluded to be due to sAC because it was blocked by KH7 but not by ddAdo (Ramos et al., 2008). Interestingly, nicotine stimulation of ion transport in mouse tracheal epithelium was diminished by both KH7 and ddAdo, leading the authors to conclude that both sAC and tmACs play a role (Hollenhorst et al., 2012).

Given their similar catalytic centers and mechanisms of action (Kamenetsky et al., 2006), it is somewhat surprising that there does not appear to be a pan mammalian cyclase inhibitor usable in cell-based assays. The P-site inhibitor ddATP inhibits both sAC- and tmAC-dependent activities with about a one-log selectivity for tmACs. In fact, ddATP is among the most potent of the P-site inhibitors (Desaubry et al., 1996a,b), but because it is not cell permeant, it is not usable in cell-based assays. The macrocycle-based compound MDL-12,330a also inhibited both tmAC and sAC activities, but it is less potent and did not fully inhibit either enzyme. Interestingly, CEs appear to be pan mammalian cyclase inhibitors in vitro (Steegborn et al., 2005), but they proved to be sAC selective in cells (Fig. 4). CEs inhibit a bacterial ortholog of sAC, *Spirulina platensis CyaC,* by binding to a conserved hydrophobic patch near the ATP-binding cleft in the catalytic center (Steegborn et al., 2005). Catechol binding chelates one of the two essential catalytic divalent cations, which distorts the active site. Because the CE binding hydrophobic pocket and the requirement for two divalent metals is conserved between mammalian sAC and the tmACs, it is not surprising that CEs inhibit both families of adenylyl cyclase in vitro. We do not yet understand why CEs are sAC-selective in cell-based systems.

P-site inhibitors, such as ddAdo (and ddATP), were initially described to be uncompetitive or noncompetitive with ATP; subsequent studies demonstrated that they bind as product analogs and serve as dead-end inhibitors (Dessauer and Gilman, 1997; Tesmer et al., 2000). Inhibition usually requires the presence of product inorganic pyrophosphate. It has been hypothesized that P-site selectivity for tmACs relative to sAC may be related to differences in the hydrophobic pocket of the P-site binding site within sAC (Gille et al., 2004). However, a true explanation for sAC's insensitivity to ddAdo, as well as an understanding of the mechanism of sAC inhibition by KH7, awaits crystallographic studies.

A common caveat with using pharmacological tools is their potential for "off-target" effects. Among adenylyl cyclase inhibitors, the original report identifying MDL-12,330a described an adenylyl cyclase–independent effect on the Na^+, K^+-ATPase (Grupp et al., 1980), and a more recent study identified an off-target effect on glycine transport (Gadea et al., 1999). As these inhibitors are used more widely, nonspecific effects become appreciated; for example, a recent report found that SQ22,536 has an effect on ERK activation unrelated to cAMP signaling (Emery et al., 2013), and two recent reports described sAC-independent effects of KH7 (Tian et al., 2011; Di Benedetto et al., 2013). In mitochondria, where KH7 has been used to demonstrate sAC's role regulating ATP synthesis (Acin-Perez et al., 2009), KH7 also has an uncoupling effect, unrelated to its effects on sAC (Di Benedetto et al., 2013), which may be responsible for its reported cAMP-independent effect on metabolism (Tian et al., 2011). Fortunately, CEs did not suffer from this side effect (Di Benedetto et al., 2013). KH7 also suffers from detergent sensitivity and intrinsic fluorescence, which limits its utility for live-cell imaging experiments. We do not yet know of off-target effects from NKY80, CEs, or ddAdo; however, the low potency of several of the tmAC compounds makes it likely that off-target effects will occur at the high doses needed for complete inhibition.

In general, nonspecific effects from pharmacological inhibitors can be "controlled" by complementing inhibitor studies with genetic knockout studies. Although this has been done for sAC, sAC's role in both male fertility (Hess et al., 2005) and intraocular pressure regulation in the eye (Lee et al., 2011) has been demonstrated both pharmacologically and in knockout studies, but it is currently unrealistic with tmACs. With adenylyl cyclases, under certain circumstances, it may be possible to control for off-target effects by rescuing pharmacological inhibition with the enzyme's product (i.e., by the addition of membrane-permeable cAMP analogs). However, it should be appreciated that because of cAMP's organization into microdomains, where proper spatial and temporal generation of the second messenger may be important, cAMP rescue may not always be straightforward.

There remains the need for development of pharmacological tools modulating adenylyl cyclase activities. For tmACs, inhibitors believed to be isoform specific are proving to be nonspecific in cellular assays (Braeunig et al., 2013), and their relatively low potency reveals a need for additional tmAC-selective inhibitors. For sAC, CEs are likely to have as yet undiscovered side effects, but these will presumably differ from KH7's, making KH7 and CEs useful in concert to identify sAC-dependent processes. However, there are no known pharmacological activators of sAC. The tmAC activator forskolin, which has been useful for studying cAMP signaling, is inert on sAC (Forte et al., 1983; Buck et al., 1999). The cellular systems used in this study (i.e., sAC overexpressing 4-4 cells along with sAC KO MEFs) can be taken advantage of to screen, and counterscreen, for sAC activators, as well as additional, structurally distinct sAC-specific inhibitors.

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Performed data analysis: Bitterman, Ramos-Espiritu, Levin, Buck.

Contributed to the writing of the manuscript: Bitterman, Ramos-Espiritu, Levin, Buck.

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