5-2015

Isoform Selective Regulation of Adenylyl Cyclase by Small Molecule Inhibitors and Gbetagamma Protein

Cameron S. Brand

ISOFORM SELECTIVE REGULATION OF ADENYLYL CYCLASE BY SMALL MOLECULE INHIBITORS AND Gβγ PROTEIN

by

Cameron S. Brand, M.S.

APPROVED:

Carmen W. Dessauer, PhD
Supervisory Professor

Jeffrey A. Frost, PhD

Richard B. Clark, PhD

Alemayehu A. Gorfe, PhD

John S. McMurray, PhD

APPROVED:

Dean, The University of Texas
Graduate School of Biomedical Sciences at Houston

ISOFORM SELECTIVE REGULATION OF ADENYLYL CYCLASE BY SMALL MOLECULE INHIBITORS AND Gβγ PROTEIN

A

DISSERTATION

Presented to the Faculty of

The University of Texas

Health Science Center at Houston

and

The University of Texas

MD Anderson Cancer Center

Graduate School of Biomedical Sciences

in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

by

Cameron Servetus Brand, M.S.

Houston, Texas

May 2015

Acknowledgements

I would like to thank Dr. Carmen Dessauer for her mentorship. She provided considerable training and support during my entry to graduate school research at a young age. She consistently provided useful guidance, especially for describing research precisely, and made sure I kept taking forward steps with my development. I cannot express enough gratitude for the expert advice and direction that has been given.

I also would like to thank the faculty who served on my advisory committees. Dr. Terry Walters, Dr. Zhizhong Pan, Dr. Andy Bean, Dr. Alex Gorfe, and Dr. John McMurray are all greatly appreciated for their critiques and advice. Particularly with regards to "seeing the big picture", Dr. Richard Clark and Dr. Jeff Frost deserve special mention for, along with Dr. Dessauer, sitting as members on my committees as well as giving feedback on my projects during inter-lab meetings and spur-of-the-moment conversations.

Fellow members of the Dessauer Lab both past and present are also appreciated for their support. These include Kathryn Hassell, Dr. Riad Efendi, Dr. Yong Li, Yan Wang, and Dr. Alexis Bavencoffe, and fellow graduate student Tanya Baldwin. A combination of expertise and enthusiasm, you all made life in the laboratory that much easier and satisfying.

There are too many Cell and Regulatory Biology members of both past and present to list here, but they and the program in general have my profound thanks. My fellow CRB students have been a fantastic support system, and I simply could not have completed this thesis without all of them.

Lastly, I would like to thank my parents, Anita and Gary Brand. For 27 years, they have raised, taught, and encouraged me, brought me back to Earth when necessary, and have served as a constant source of love and support.

ISOFORM SELECTIVE REGULATION OF ADENYLYL CYCLASE BY SMALL MOLECULE INHIBITORS AND Gβγ PROTEIN

Cameron Servetus Brand

Supervisory Professor: Carmen W. Dessauer, Ph.D.

The nine membrane-bound isoforms of adenylyl cyclase (AC), via synthesis of the signaling molecule cyclic AMP (cAMP), are involved in many isoform specific physiological functions. All nine isoforms share a similar structural organization; thus, AC isoform differences in physiological function are due to different regulatory profiles. A physiological example is Gβγ, which can conditionally enhance stimulation of ACs 2, 4, 5, 6, and 7, but inhibit ACs 1, 3, and 8. There is also pharmacological control of AC isoforms through small molecule inhibitors.

Isoform specific AC functions could be explained by regulatory differences as subtle as single amino acid changes. For both pharmacological targeting and known physiological regulators, differences between isoforms are not well understood. Two approaches were taken to explore AC5/6 isoform selectivity. The first approach was to more completely characterize allegedly AC5 selective small molecule AC inhibitors. The other approach was to examine AC isoform regulation by Gβγ.

The AC inhibitors SQ22,536 and Ara-A, rather than being AC5 selective as previously described, are AC5/6 inhibitors. Another AC inhibitor, NB001, showed AC1-dependent

decreases in cAMP within cells without directly inhibiting AC1. These results highlighted the importance of AC inhibitor characterizations for AC specificity and isoform selectivity.

Gβγ regulation of AC5/6 was also explored. We showed that Gβγ binds to many AC N-termini (NT), but only AC5 NT binding did not require the Gβγ "hotspot", a surface involved in many Gβγ regulatory events. Mutations of the hotspot showed it was necessary for AC5 stimulation but not AC5 NT binding. In contrast, the hotspot was required for both AC6 NT binding and AC6 stimulation. Peptide competition to disrupt Gβγ hotspot dependent binding events affected regulation of AC1/2/6 by Gβγ, but not that of AC5. Gβγ also interacted with both the AC5/6 C1/C2 catalytic domains, with similar hotspot dependency. This work suggested that Gβγ stimulation of AC5/6 occurs in a similar hotspot-dependent binding event, but that Gβγ/AC5NT binding is an isoform specific G protein anchoring event.

Lay Abstract

Adenylyl cyclase (AC) is a protein that allows signals from outside the cell to be transferred into the cell. There are nine types of AC that, despite being structurally similar, have differences in their functions. For example, just a single one of the nine AC types is involved in aspects of heart function, pain sensation, and dependency/withdrawal behaviors from the use of opioids such as morphine. The other AC types have additional, and sometimes overlapping, functions. This makes it important to know how each type of AC is controlled, both for explaining specific biological effects from AC signaling and for selective targeting with drug treatments while limiting side effects.

Since the nine AC types are controlled differently despite similar structures, selective control of AC activity is explained by subtle differences. For both drugs and proteins that control AC, many such differences are not well understood. Some AC-targeting drugs which were possible treatments to prevent heart failure have been incorrectly described previously as able to selectively decrease the activity of a single AC type. My studies indicated that these drugs also affect another AC type which causes increased risk of heart failure. I also studied the effects of a signaling protein on two closely related AC types. This signaling protein was able to increase activity of both AC types; however, only one had a unique spot for this signaling protein to remain anchored nearby. Overall, my studies clarified differential targeting of AC types by drugs, and the potential importance of a site that is unique for how one AC type is controlled.

Table of Contents

List of Figures

Chapter 1

Chapter 3

Chapter 4

Chapter 5

List of Tables

Chapter 1

Chapter 3

Chapter 5

Abbreviations

AC	Adenylyl Cyclase
AKAP	A-Kinase Anchoring Protein
Ara-A	Adenine 9-β-D-Arabinofuranoside
ATP	Adenosine Triphosphate
β-AR	Beta-Adrenergic Receptor
BLAST	Basic Local Alignment Search Tool
BRET	Bioluminescent Resonance Energy Transfer
C1	Cytosolic Domain 1
C2	Cytosolic Domain 2
cAMP	Cyclic Adenosine Monophosphate
CaM	Calmodulin
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
EPAC	Exchange Protein directly Activated by cAMP
FBS	Fetal Bovine Serum
FRET	Fluorescent Resonance Energy Transfer
Fsk	Forskolin
Gαi	Heterotrimeric G Protein Inhibitory Alpha Subunit
Gαs	Heterotrimeric G Protein Stimulatory Alpha Subunit
Gβγ	Heterotrimeric G Protein Beta-Gamma Subunit

GDP	Guanosine Diphosphate
GEF	Guanine nucleotide Exchange Factor
GIRK	G Protein Coupled Inwardly Rectifying Potassium Channel
GPCR	G Protein Coupled Receptor
GRK	G Protein Coupled Receptor Kinase
GTP	Guanosine Triphosphate
HEK	Human Embryonic Kidney Cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic Acid
MANT	Methylanthraniloyl
NB001	5-[[2-(6-Amino-9H-purin-9-yl)ethyl]amino]-1-pentanol
NKY80	2-Amino-7-(furanyl)-7,8-dihydro-5(6H)-quinazolinone
NT	Amino Terminus
PBS	Phosphate Buffered Saline
PDB	Protein Data Bank
PI3K	Phosphoinositide 3-Kinase
PLC	Phospholipase C
PKA	Protein Kinase A
PKC	Protein Kinase C
sAC	Soluble Adenylyl Cyclase
SQ22,536	9-(Tetrahydro-2-furanyl)-9*H*-purin-6-amine
SDS	Sodium Dodecyl Sulfate
Sf9	*Spodoptera frugiperda* (clonal isolate)
VMD	Visual Molecular Dynamics

Chapter 1

Introduction

1.1 Adenylyl Cyclase/Cyclic AMP Signaling

Adenylyl cyclase (AC) is an enzyme that converts adenosine triphosphate (ATP) into cyclic adenosine monophosphate (cAMP) and pyrophosphate (3). Within cells, cAMP produced by AC is a 2nd messenger that activates downstream signaling partners such as PKA and EPAC (4, 5), resulting in a variety of cellular signaling functions (6). Such cAMP signaling can be attenuated via degradation of the produced cAMP by phosphodiesterases (7, 8).

AC is a bottleneck enzyme for cAMP signaling pathways; cardiomyocytes have about 4.7×10^6 Gαs within the cell, but can only form 6×10^5 Gαs/AC signaling complexes upon G protein activation (9). Thus, AC activity is an important control node in signal transduction via pathways that use cAMP as a 2nd messenger. In mammals, there are nine membrane-bound AC isoforms (AC1-9), and a soluble isoform (sAC) that is more closely related to bacterial cyclases (10). The number of isoforms, or subtypes of the AC enzyme with the same cAMP-producing capability, varies by species.

1.2 AC Structure

In addition to catalyzing production of cAMP, AC1-9 also share a common overall structure (Figure 1A). This structure involves three cytosolic domains separated by a pair of 6-transmembrane helical domains; an N-terminus (NT), and two cytosolic domains (C1 and C2)

that comprise the catalytic site where ATP conversion to cAMP takes place (11). These cytosolic domains are also where Gαs and Gαi bind to stimulate or inhibit AC activity. The C1/C2 domains have roughly 40% sequence homology, and are pseudosymmetric. Crystal structures indicate that the catalytic site of AC is located at the C1/C2 domain interface, with each domain forming one half of a shell-like structure that can be accessed from the cytoplasm (12). There is also similarity between the C1/C2 domains of adenylyl cyclase and the guanylyl cyclase catalytic domains (11). Whereas the C1/C2 domains have 40% homology across AC1-9, the NT domain differs much more among isoforms in both amino acid sequence and length.

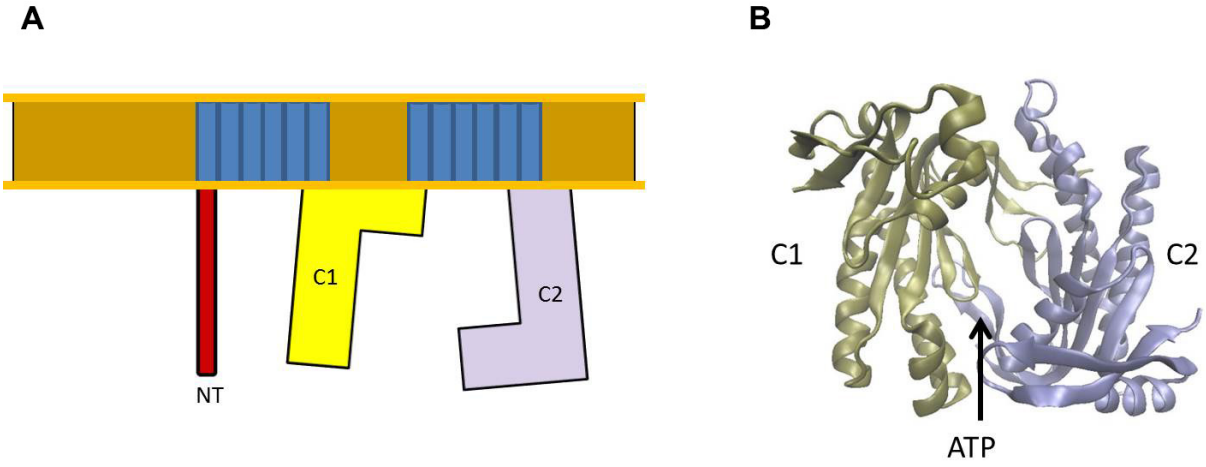

Figure 1: Adenylyl Cyclase Structure. A, Overall structural organization of transmembrane adenylyl cyclase. Three cytoplasmic domains (NT, C1, C2) are separated by a pair of 6-transmembrane domains. **B,** Crystal structure (from PDB 1CJT) of the C1/C2 domains from the cytoplasmic view, with the ATP-binding site indicated. Rendered with VMD.

1.3 AC Regulation

Various regulatory proteins interact directly with AC in order to increase or decrease AC activity, and thus control AC/cAMP signaling. AC isoforms have been categorized based on shared regulators as Group I (AC1/3/8), Group II (AC 2/4/7), Group III (AC5/6), and Group IV (AC9). These groups can also be formed based on phylogenic comparisons of the isoforms

(13). Group I isoforms share stimulation by calcium-activated calmodulin (14-17). Group II isoforms have considerable stimulation by Gβγ (18-21). Group III isoforms have sensitive, sub-micromolar level inhibition by free calcium (22), as well as inhibition by Gαi (23, 24). AC9 is set aside solely as Group IV, with a unique regulatory profile including less sequence homology relative to AC1-8 and insensitivity to the AC activator forskolin (25). Regulators of AC bind to the C1/C2 domains to directly modify AC activity, or alternatively bind at the NT for regulatory effect.

The most thoroughly studied physiological regulators of AC activity are the heterotrimeric G proteins. The heterotrimer consists of a guanine nucleotide binding α subunit, and an obligate βγ subunit heterodimer. Under classic G protein signaling, these subunits are held in an inactive heterotrimer by GDP-bound Gα. Following upstream Gα activation via exchange of GDP for GTP by ligand-bound G protein coupled receptors (GPCRs), these subunits dissociate, allowing for regulation of downstream targets such as AC (26). In some cases, these subunits may rearrange to expose regulatory sites rather than full dissociation taking place (27). This has been shown in FRET-based studies where GPCR activation resulted in rearrangement, but not dissociation, of Gαi and Gβγ (28, 29). Differential models for Gαs stimulation of AC1/2/6 suggest that the mechanisms of AC stimulation by a heterotrimeric G protein subunit may vary among AC isoforms, even when the regulatory effect on AC activity is the same (30). Characterization of AC5/6 regulation by Gα subunits has resulted in similar conclusions (23). Gαs stimulates all AC isoforms, while Gαi inhibits AC1/5/6 (23, 24, 31). These Gα subunit types were named “s” and “i” due to their respective stimulatory or inhibitory effects on AC activity. Gαi inhibits AC isoforms via binding to C1 in a site pseudosymmetrical

to that on C2 for Gαs (32). Furthermore, Gαz inhibits AC1/5/6 (33), while Gαo inhibits AC1 (34).

Gβγ subunits have more differential, AC isoform dependent effects. Gβγ can conditionally stimulate AC2/4/5/6/7 in the presence of Gαs or forskolin (18-21, 35), but inhibits the activity of AC1/3/8 (18, 36, 37). Thus, heterotrimeric G protein activation has the potential to either stimulate or inhibit AC activity, depending on the particular Gα subunit and the resulting target of the freed Gβγ subunits.

Heterotrimeric G proteins are not the only significant AC regulators. For example, differential effects on AC isoforms by kinase phosphorylation have been described. Although PKA is activated by cAMP, feedback inhibition from PKA has also been observed for AC5/6 and AC8, phosphorylating the C1 domain in a region required for Gαs stimulation of AC6 (38, 39). PKC has both stimulatory and inhibitory regulations on AC that are specific to particular combinations of both AC isoform and PKC isoform (40). There is also stimulation of AC1/3/8 by calmodulin and inhibition of AC5/6 by free calcium as mentioned previously (14-17).

Free calcium ions, in addition to inhibiting AC5/6 via a high-affinity binding site with a K_i of 0.2 μM, are capable of inhibiting all other AC isoforms at higher concentrations via a low-affinity binding site with a K_i of 100 μM (22, 41). In both cases, calcium inhibition of AC is via competition with Mg^{2+} at the catalytic site. This is because AC catalysis requires Mg^{2+} or Mn^{2+} to facilitate phosphate transfer; Zn^{2+} can also inhibit AC in a mechanism similar to that of Ca^{2+}, emphasizing the various metal ions with regulatory effects on AC activity (42-44).

Of note is the AC stimulator forskolin, a diterpene derived from the root of the plant *Coleus forskohlii* (45). It is able to clearly stimulate activity of all the membrane-bound AC isoforms except AC9 (25, 46), and is used frequently in research studying cAMP signaling

pathways for this purpose. A single forskolin molecule binds at the interface of the C1 and C2 domains, and its affinity increases with Gαs stimulation of AC (47). Due to the pseudosymmetrical structure of C1/C2, the forskolin binding pocket is structurally related to the ATP-binding active site. However, the evolutionary reason for this molecule's evolved binding site being so conserved is unclear. Although AC9 is considered to be forskolin-insensitive, this may just be due to a large negative shift in forskolin affinity; mutation of a key residue within the forskolin-binding pocket can elevate AC9's forskolin sensitivity to the level exhibited by the other AC isoforms (48). Thus, AC isoforms have many potential regulatory stimuli; notable regulators of AC isoforms are summarized in Table 1. This wide variety of potential regulators results in a complex picture for how AC activity is tightly controlled.

Table 1: Regulatory Properties of Adenylyl Cyclase Isoforms

Regulator	Regulatory Effect								
	Group I			Group II			Group III		Group IV
	AC1	AC3	AC8	AC2	AC4	AC7	AC5	AC6	AC9
Gαs	↑	↑	↑	↑	↑	↑	↑	↑	↑
Gαi	↓						↓	↓	
Gαo	↓								
Gαz	↓						↓	↓	
Gβγ	↓	↓	↓	↑	↑	↑	↑	↑	
PKA			↓				↓	↓	
PKC	↑ (α)	↑ (α)		↑ (α)	↓ (α)	↑ (α)	↑ (α, ζ)	↓ (δ, ε)	
CaM	↑	↑	↑						
Ca^{2+}							↓	↓	
CaMK	↓ (CaMK IV)	↓ (CaMK II)							
CaN									↓
Forskolin	↑	↑	↑	↑	↑	↑	↑	↑	

↑ = stimulation, ↓ = inhibition. PKC, calmodulin kinase (CaMK) isoforms are as indicated.

1.4 Gβγ Activation and Regulation of Effectors

There are five Gβ and 12 Gγ subunit isoforms. Gβ1-4 share over 80% sequence similarity, and along with the 12 Gγ isoforms are considered to have similar effects on

regulatory targets. Studies on the Gβγ isoform combinations, and whether they have differential signaling effects, have shown that various combinations of β and γ subunits are typically similar in how they interact with effector targets (35). For example, β1γ2, β1γ3, β2γ2, and β2γ3 all show inhibition of AC1, stimulation of AC2, and similar rates of associated Gαo ADP-ribosylation, with slight differences in their rates of associated Gαi ADP-ribosylation (35). A notable exception is Gβ1γ1, known as the signaling Gβγ involved in the retina. Complexes containing γ1 are consistently expressed in the retina for signaling with the Gα transducin, and are less potent at regulating AC and PLC isoforms (35, 49). When different Gβγ dimer combinations were tested in Gαi-coupled GPCR mediated inhibition of AC8, the effect was AC8 inhibition, although potency of this inhibitory effect varied between Gβγ isoforms (50). Thus, based on currently published work, the regulatory effect of Gβγ on AC is dependent on the AC isoform but not the Gβγ isoforms, as long as the Gβ subunit is 1-4.

As mentioned above, Gβγ subunits are an interesting case of isoform specific AC regulation, stimulating AC2/4/5/6/7 with Gαs (18-21, 35), but inhibiting AC1/3/8 (18, 36, 37). For AC/Gβγ binding, there appears to be a lack of conserved interaction sites, even between closely related AC isoforms. Using a combination of peptide probes and molecular modeling, it was identified that aa 956-982 in the AC2 C2 domain bound to the Gβγ hotspot (51-53). Four other Gβγ hotspot binding sites have also been identified on the C1/C2 domains of AC2, suggesting binding between Gβγ and AC involves multiple interactions and/or different binding sites for Gβγ regulatory function (36, 54, 55).

In addition to AC, Gβγ has a significant number of effectors it can regulate. Most notable is Gβγ regulation that stimulates opening of GIRK channels (56-58). Regulation by Gαi and Gβγ and their mechanisms of differential GIRK channel regulation have been extensively

studied (59-69). Gβγ can also bind to GRK2 and GRK3, recruit them to the plasma membrane, and facilitate GRK phosphorylation, β-arrestin recruitment, and internalization of β-adrenergic receptors (70, 71). Gβγ is also capable of binding to and directly activating PLCβ1-3; for all isoforms, this is a Gα-independent regulatory capability of Gβγ (72-76). Direct opening of calcium channels can also be regulated by Gβγ independently of Gα (despite Gα binding sites), such as N-type, P-type, and Q-type voltage gated calcium channels (77-80). Although sensitivity is dependent on adapter proteins, Gβγ can also bind and activate PI3K isoforms (81, 82). Gβγ is able to negatively regulate SNARE protein mediated vesicle formation via its interaction with SNAP25, most notably to inhibit presynaptic neurotransmitter release (83, 84). Gβγ is also able to stimulate GEFs that function downstream of heterotrimeric G protein signaling, such as P-Rex1 (85). In summary, Gβγ is like AC in that it has an endless list of proteins it regulates through indirect pathways or downstream effects. Thus, Gβγ regulation of AC is not only important for modulating cAMP production, but AC may also compete with other potential effector targets of Gβγ.

While GPCR signaling tends to activate Gα subunits through catalyzing GDP-to-GTP exchange, it appears that Gβγ is required for the plasma membrane association that is needed for Gα/GPCR coupling (86). Gβγ can bind to GPCRs, but mutation of the Gβγ binding site on M3 muscarinic receptor prevented receptor desensitization and not downstream signaling; thus, it is possible that Gβγ/GPCR direct binding events are involved in recruitment of desensitization signal proteins such as GRK more so than GPCR/G protein/AC/cAMP signaling events (87).

Lacking any enzymatic activation through nucleotide exchange like its Gα counterparts, Gβγ activation occurs through the dissociation and/or rearrangement caused by activation of its

associated Gα subunit. Crystal structures indicate minimal structural changes to the Gβγ subunit in the absence versus presence of Gαi (88, 89). Switch II of Gαi covers a particular surface of Gβγ in the heterotrimer conformation, namely the intersection of the Gβ structural blades (88). This so called Gβ "hotspot" is covered by Gα (Figure 2), and when exposed is capable of interacting with a variety of effectors; alanine substitution mutants have shown previously that interference with individual hotspot residues have differential yet overlapping effects on Gβγ regulation of a wide variety of downstream effector targets, including AC (90, 91). However, while hiding the hotspot appears to determine activation of Gβγ from an inactive heterotrimer, there are other surfaces down the blades of the Gβ propeller type structure that are also associated in Gβγ/effector interactions. This has most clearly been shown for Gβγ regulation of PLCβ (92).

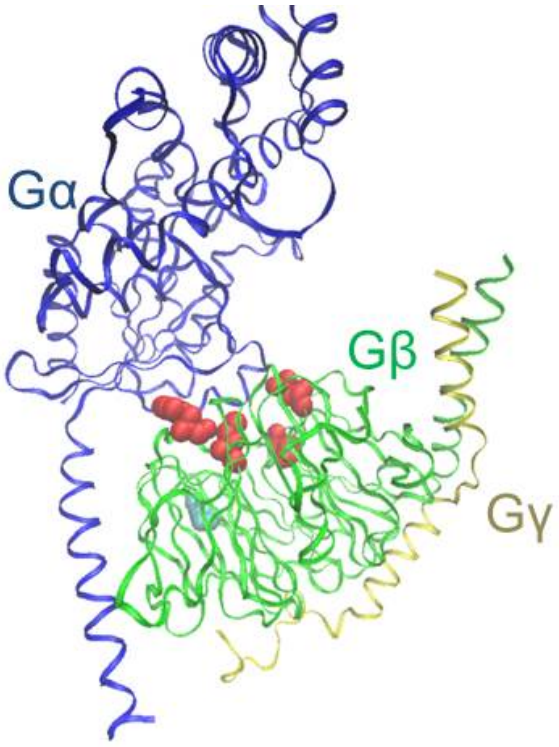

Figure 2: Gβ Hotspot Covered by Inactive G Protein Heterotrimer. From crystal structure of inactive Gαi/β1/γ2 protein heterotrimer (PDB 1GP2). Hotspot residues mutated in shown results (see Chapter 4) are indicated in red. Gα is blue, Gβ is green, and Gγ is yellow as are indicated.

1.5 NT Roles in AC Regulation

The AC NT has known regulatory roles, many of which are isoform-specific. NT domains of AC 5, 6, and 8 bind Gβγ (93, 94). A binding site for inactive G protein heterotrimer on the AC 5NT was previously identified at residues 66-137, with synergistic binding enhanced by the presence of both subunits (95). The 6NT binds Gβγ at residues 77-151 (93). A large number of PKC isoforms have been identified to have AC NT binding sites, including a possible PKC inhibition site at Ser10 on 6NT (96). The 6NT binds with Snapin, a SNARE complex associated protein that also was capable of blocking PKC inhibition of AC6 when bound (97). The 5NT associates with the guanine nucleotide exchange factor Ric8a (98). 8NT binds the phosphatase PP2A (94). Some AC NT have also had identified or proposed

mechanisms of regulatory action on the activity of their particular AC. 5NT takes part in RGS2 regulation of AC5 (99), 6NT is involved in Gαi inhibition of AC6 (100), and 8NT facilitates AC8 stimulation by calmodulin (101).

The NT of AC isoforms also mediate larger AC/cAMP signaling complexes. Typically, this has been done through interactions with the large A-Kinase Anchoring Proteins (AKAPs) that facilitate control of AC activity. This is primarily by providing a link between AC and PKA, but other kinases, phosphatases, and phosphodiesterases that can potentially modulate AC activity or cAMP accumulation can also be associated with an AC isoform via an AKAP complex. The AC2 NT can bind to the AKAP Yotiao; in the brain, this AC2/Yotiao interaction decreased cAMP production (102). The AC5/6/9 NT can interact with AKAP79, including mediating PKA feedback inhibition of AC5/6 (103, 104). Notably, a peptide blocking AC5/AKAP79 interactions but not disrupting other AC/AKAP complexes can specifically disrupt PKA feedback inhibition of AC5 (104). The AC5/AKAP79 complex is also capable of mediating AC/cAMP signaling that locally activates the TRPV1 channel, which can direct a sensitized response to thermal pain signaling (105-108). The AC9 NT interacts with Yotiao, linking AC9 and the KCNQ1 channel in a complex associated with cardiac repolarization (109-111). AC5 NT interacts with mAKAPβ as part of a hypertrophic signaling complex at the nuclear membrane of cardiomyocytes; similar to AC5/AKAP79 complexes, specific disruption of the AC5/mAKAPβ complex blocks hypertrophic effects (112-114). Thus, via AKAP interactions, the AC NT is involved in the constitution of isoform specific signal complexes.

1.6 AC5 and AC6 Isoforms

The Group III AC isoforms, AC5/6, are the most closely related isoforms with many shared regulators, yet also display differences in their regulation and physiological function. AC5/6 are both stimulated by Gαs and Gβγ (93). They are also both directly inhibited by Gαi (24), Ca^{2+} (22, 41), and PKA(38). However, while Gαs stimulated activity of either isoform is inhibited by Gαi, only basal activity of AC5 is also inhibited by Gαi (23). AC5/6 are both indirectly inhibited by Gαo (115). There is also diversity in PKC isoform effects between AC5 and AC6. Specifically, AC5 is directly stimulated by PKC-α and –ζ (40, 116), while AC6 is directly inhibited by PKC-δ and –ε (117, 118). Indirect regulatory effects on AC via Gαq signaling can enhance AC6 activity, but not that of AC5, within intact cells by a calcium/calmodulin dependent mechanism (119).

The regulation of AC5 and AC6 by Gβγ differs in several respects to that of AC2. In response to Gαs-coupled receptor activation, both heterotrimeric G protein subunits are needed to observe a full stimulatory AC6 response (93), while Gβγ stimulation of AC 2/4/7 is thought to occur via activation of Gαi-coupled receptors (19). This suggests unique functions for Gβγ regulation of the AC5/6 isoforms. Gβγ can activate AC 2, 5 and 6 in the presence of either Gαs or forskolin, but has no effect on basal AC activity (93). Prenylation of the γ subunit was also necessary, which suggests Gβγ stimulation of AC isoforms requires an interaction at the plasma membrane. Gαs and forskolin are also capable of simultaneous stimulation of AC; whether this stimulation is synergistic or additive is isoform specific (46).

The “similar yet different” aspects of AC5/6 are reflected in their physiological distribution and roles. Both isoforms are expressed in the heart, brain, kidney, liver, lung, testis, and adrenal gland, but not always at equal levels. For example, while AC5/6 are the predominant AC isoforms in the heart (120), neonates express AC6 as the predominant isoform

while adult hearts have an increase in the relative levels of AC5 expression (121). Specific locations of expression in the same organ also can vary. AC5 expression has been shown mainly in the left ventricle while AC6 is located in both the left ventricle and atria (122). In the striatum region of the brain, which is involved in locomotor function, AC5 is highly expressed compared to other AC isoforms (123). AC5 in the striatum is also involved in D2 dopamine receptor mediated responses, learning, anxiety, and morphine action (123-126).

Potential therapeutic benefit from AC5/6 modulation has shown unique differences between the isoforms in knockout models. AC5 knockout models indicate roles for AC5 behaviors resulting from chronic opioid use (126), and similar AC5-dependent signaling pathways are involved in mechanical and inflammatory pain sensation (127). AC5 knockout models also show a protective phenotype against chronic heart failure (128-130). Deletion of AC5 also has anti-aging cardiac benefits (131). Due to these AC5 KO phenotypes, selective inhibition of AC5 is a proposed treatment target against chronic heart failure (132-134). However, cardiac homogenates or myocardial isolations from failing human hearts have decreased basal cAMP levels, as well as impaired cAMP production when faced with responding to adrenergic stimulation (135, 136). Further complicating the picture, it appears that increased AC6 expression is beneficial as a chronic heart failure treatment (137-140). In fact, heart failure mortality is raised by decreases in activity and/or expression of AC6 (141-143). Thus, AC5 and AC6 selectivity is important when considering cAMP signaling changes in the development of heart failure risk.

1.7 Pharmacological Control of AC/cAMP

Many drugs control cAMP signaling for therapeutic benefit. Development of congestive

heart failure involves changes in the expression of multiple proteins associated with the AC/cAMP signaling pathway (144-146). Consistent with this, beta-blockers target β-adrenergic receptors, decrease cAMP levels, and are used as treatments for various heart conditions, such as metaprolol for hypertension and heart failure. Similarly, the μ-opioid receptor ligand morphine decreases cAMP, resulting in decreased pain sensation. In addition to these specific examples, there are various dopamine receptor ligands utilized as therapeutics for schizophrenia, Parkinson's disease, and nausea. However, direct control of cAMP production by targeting AC activity has been more difficult. For example, an AC inhibitor must be cell permeable. This has limited the kind of molecules that progress past cell-based systems and preclinical animal models, particularly since many AC inhibitors used in research have phosphate groups. Some AC inhibitor prodrug packages have been attempted as a solution for delivering phosphate-containing AC inhibitors rather than developing new ones, but currently no such attempts have clinical testing (147, 148). This includes prodrug modifications with cleavable bonds for improved cellular delivery (149).

A classic group of small molecule AC inhibitors is the P-site inhibitors, which bind in the AC catalytic cleft at the ATP binding site (the "P-site"). P-site inhibitors typically decrease AC activity through uncompetitive inhibition (150, 151). Uncompetitive inhibition by P-site inhibitors is due to the stabilization of a product-like transition state, where the inhibitor binds with pyrophosphate in the ATP binding site after cAMP product has been released. As a result, P-site inhibitors more strongly inhibit stimulated AC (152). Some classic P-site inhibitors occur within cells, especially ADP and ATP metabolites (153). More potent ATP-like P-site inhibitors can bind without a pyrophosphate molecule, and such AC inhibitors can have a greater than 10-fold stronger affinity for AC than single-phosphate analogs; for example, 2'5'-

dideoxy-3'-ATP has an IC_{50} of 40 nM while 2'5'-dideoxy-3'-AMP has an IC_{50} of 460 nM (154, 155). Another example is the noncompetitive ATP analog 2',3'-dideoxy-5'-ATP, the most potent AC P-site inhibitor, which can bind to rat brain AC with a K_i of 16 nM (156). However, classic P-site inhibitors are not typically AC isoform selective. For example, 2'-deoxy-3'-AMP is very mildly selective for AC1 over AC2 and AC6, and only under Mn^{2+}-stimulated but not Mg^{2+}-stimulated conditions (157). These are minor shifts in affinity that do not provide many clues for development of AC inhibitors that are both potent and isoform selective.

The other predominant class of direct small molecule AC inhibitors is the MANT-nucleotide inhibitors. The original MANT-nucleotide studied for AC inhibition is MANT-GTP, which has a K_i of 53nM for AC in S49 lymphoma cell membranes (158). MANT-GTP binds in the ATP-binding site like the noncompetitive P-site inhibitors, but with the nucleotide group bound in a reversed orientation (159, 160). MANT-nucleotide based inhibitors, including MANT-GTP, show potential for designing AC isoform selective compounds that target the AC catalytic site (159, 161). MANT-GTPγS blocked AC5-coupled L-type calcium channel currents when introduced inside cardiomyocytes via patch pipette, although a nonspecific AC-independent change in current was also observed (162).

Beyond these two main classes of AC inhibitors, there has also been further development targeting alternative sites of AC inhibition. One example is the non-competitive inhibitors that target the forskolin binding pocket, which typically are analogs of forskolin. The current examples of identified AC inhibitors using this approach are BODIPY-Fsk, and 6A7DA-FS (also known as iso-forskolin), which showed AC stimulations at micromolar concentrations *in vitro* but AC2 specific inhibition at nanomolar concentrations that was Mg^{2+}

dependent (163, 164). Calmidazolium, classically a calmodulin inhibitor, has also been characterized as an allosteric, nonselective AC inhibitor with unknown site(s) of action (165).

Based on these four groups, there have been attempts to develop similarly potent small molecule AC modulators, but with isoform selectivity and greater therapeutic potential. One such example, NB001, is a patented AC1 selective inhibitor (166, 167). Catalytic site AC inhibitors have been able to differentiate between targeting sAC instead of the 9 membrane bound AC isoforms (168). Some small molecules derived from P-site inhibitors, such as PMC-6 appear capable of integrating metal chelating properties at catalytic site binding to produce isoform selective inhibition (169). NKH477, a forskolin analog, can function as an AC5 activator (170). Effects of NKH477 treatment include coronary vasodilation and rescued function in the event of acute heart failure (139, 171).

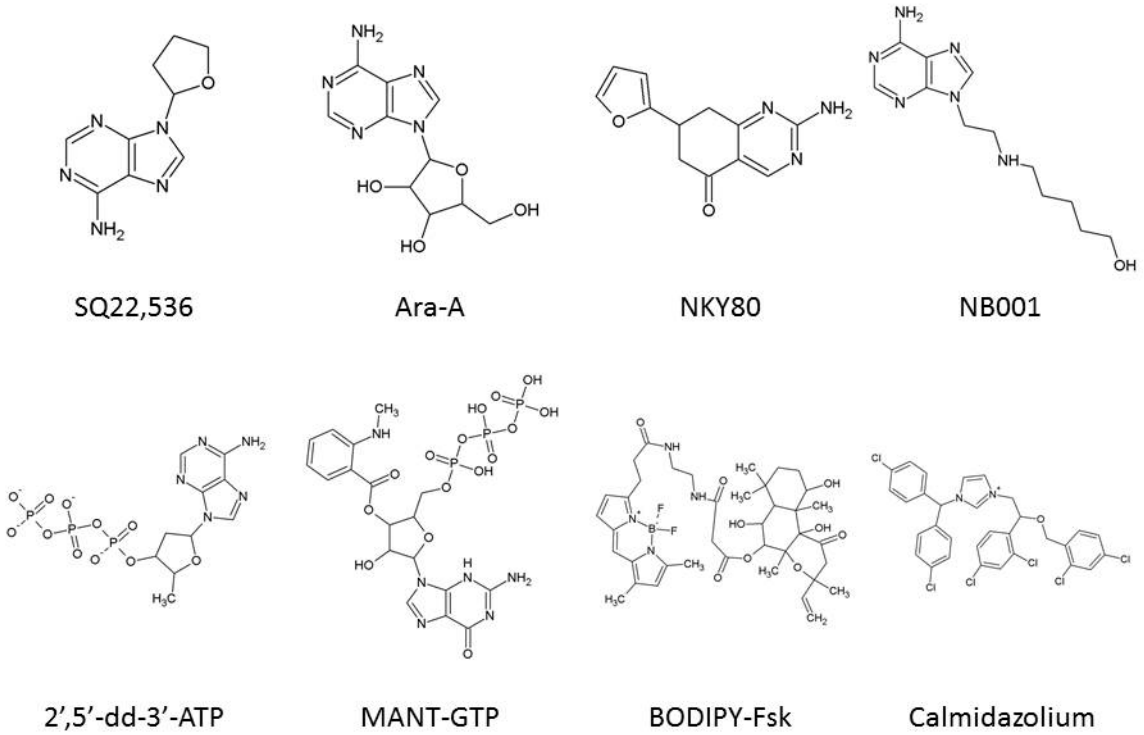

Figure 3: Chemical Structures of Small Molecule AC Inhibitors. Top row is P-site derived AC inhibitors used in the following experiments and/or previous work. Bottom row is representatives from the four main classes of small molecule AC inhibitors currently in use (P-site inhibitors, MANT-nucleotides, forskolin analogs, and calmidazolium).

1.8: Previous Work with Known and Novel AC Inhibitors

NKY80 is a P-site type inhibitor with a modified adenine structure, eliminating DNA synthesis inhibition as a potential off-target effect (148). An adenine-like motif at the C2 and C6 carbons was preserved and accurately predicted to be essential for AC5 inhibition. Consistent with this motif, NKY80 was capable of inhibiting AC5 with an IC50 in AC membrane assays of 8.3 μM, a 15-fold and 200-fold difference in potency over AC3 and AC2 respectively (172). Non-competitive mechanism of AC inhibition was also a conserved property between classic P-site inhibitors and NKY80. To further characterize the selectivity of

NKY80 against all nine isoforms of AC, we utilized a membrane assay, as NKY80 cannot function in cell-based assays of AC activity (173). We observed that NKY80 inhibition of the closely related AC5 and AC6 isoforms was more potent than inhibition of AC 1/2/3/4/7/8/9, and that the most-inhibited AC5/6 isoforms were the only clear isoform group that could be identified from these inhibition curves (174). Furthermore, virtual docking with AutoDock was used to predict how NKY80 bound within the AC catalytic pocket. Based on virtual docking results, NKY80 binds in the AC catalytic pocket as a P-site inhibitor in the ATP binding site (174). Thus, the presumed AC5 inhibitor NKY80 was an AC5/6 selective P-site inhibitor.

Due to the selectivity issues for P-site inhibitor derived compounds such as NKY80, we also wanted to assess the viability of non-adenine like small molecules inhibiting AC activity at the ATP binding site. This was considered as an alternative to identifying new sites for isoform selective AC inhibition, such as the forskolin binding pocket, or repurposing inhibitors such as calmidazolium with unknown AC interaction sites. By screening a library of 35,000 drug-like small molecules from ChemBridge using a ligand-flexible virtual docking approach at the ATP-binding site, we identified a trio of small molecules that were novel AC inhibitors. In AC membrane assays, these non-optimized molecules could inhibit AC5 at 1mM, and showed limited 2-3 fold differences in selectivity at 100μM between AC1/2/5/6 (1, 174). Although these identified inhibitor candidates did not display the desired nanomolar levels of potency or the therapeutically required AC isoform selectivity, they established proof of concept that this combination of high throughput structure-based virtual screening with medium throughput *in vitro* characterization could identify novel AC inhibitors. Such candidates can be further optimized to create more potent and selective AC inhibitors.

1.9 Significance of Proposed Research

AC1-9 all produce cAMP, are expressed in multiple tissues, and have many associated functions. However, all isoforms share a similar topology. Thus, AC isoform differences in physiological function are likely due to different regulatory profiles. A physiological example of AC isoform selective regulation is by Gβγ, since it can conditionally enhance stimulation of ACs 2, 4, 5, 6, and 7, but inhibit ACs 1, 3, and 8. Pharmacological work to identify isoform specific AC inhibitors with therapeutic potential has also provided examples of selectivity in regulation despite the shared overall AC structure, including in the catalytic C1/C2 pocket.

Isoform specific AC functions could be explained by subtle differences in isoform specific regulation, despite such regulatory details being incompletely characterized. *I hypothesize that necessity and sufficiency of AC isoforms in physiological functions is due to regulatory differences, and can be characterized through either direct regulatory modulation or via isoform specific pharmacological modulation.* Thus, I explored isoform specificity of AC5/6 from two approaches. One was to more completely characterize the small molecule AC inhibitors, primarily those allegedly able to select between AC5 and AC6. The other approach was to look at an isoform specific regulator of AC activity, the heterotrimeric Gβγ protein, to more closely examine Gβγ regulation of AC5 regarding an NT binding site that AC6 did not share, even though both isoforms are conditionally stimulated by Gβγ.

Chapter 2

Materials and Methods

2.1 Plasmids and Viruses

Rat AC1-4, human AC5, human AC6, and rat AC7 baculoviruses were described previously (14, 18, 23, 34, 175). Eukaryotic expression vectors for rat AC2, rat AC8 and human AC9 in pcDNA3.1 were described previously (102, 176). Hexa-histidine tagged Gαs was purified from *E. coli* and activated with GTPγS (23). Baculoviruses for biotinylated Gβ1 (both wildtype and mutants), Gγ2, and Gαi were used to express b-Gβ1γ2 variants as described (177, 178).

Flag-tagged AC5 pcDNA was a generous gift from Dr. Michael Kapiloff (University of Miami, FL) and has been used previously (95). YN-Gβ1 was a generous gift from Catherine Berlot (Weis Center for Research, Danville, PA) and consists of Gβ1 fused to the first 157 aa of YFP (YN). The YN-Gβ1-W99A pcDNA plasmid was generated by PCR mutagenesis. NT23-27 was generated by mutagenesis of the Gβ1 aa 23-27 sequence KACAD to ANCAA and cloned into the YN-Gβ1 or YN-Gβ1-W99A pcDNA template to produce either YN-Gβ1-NT23-27 or YN-Gβ1-NT23-27/W99A double mutant pcDNA plasmid.

2.2 Antibodies

Antibodies used were rabbit anti-Gβ (Santa Cruz), mouse anti-GST (Santa Cruz), rabbit anti-H6 (Bethyl), rabbit anti-GFP (Cell Signaling), and anti-Flag (Sigma).

2.3 Small Molecules

Forskolin and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich. The AC inhibitors SQ22,536 (Enzo Biosciences), Ara-A under the trade name Vidarabine (Tokyo Chemical Industry), and NB001 (Sigma-Aldrich) were purchased from the indicated vendor. All small molecules were purchased in solid form and suspended in DMSO. In membrane AC assays (see below), the final concentration of DMSO in adenylyl cyclase assays was always less than 5%.

2.4 Virtual Ligand Docking

Global and site-directed docking of selected AC inhibitor ligands was performed using AutoDock 4.2 (Scripps, La Jolla, CA). Here, known AC5 inhibitors SQ22,536 and Ara-A were docked to either the entire surface of AC structures or just to the ATP binding site. The structures of AC used were PDB ID's 1CJT and 1TL7, which are crystallized chimeras of the AC5 C1 domain and AC2 C2 domain. In the former "blind docking" procedure (179), we used a cubic grid of size 60 Å (with spacing of 0.375 Å) centered on the C1/C2 domain. For site directed docking to the ATP-bound site, docking was restricted to a cubic grid with sides 20 Å in length, in order to encompass the ATP binding site as the docking region. This box was centered on the crystallized P-site inhibitor (1CJT) or MANT-GTP inhibitor (1TL7). Prior to their use in AutoDock docking runs, ligands were preprocessed with AutoDock Tools including assignment of Gasteiger atomic charges and torsions (180, 181).

AutoDock 4.2 was then used to dock the ligands onto a grid large enough to encompass the entire surface of the AC catalytic site from crystal structure 1CJT. An LGA hybrid with 256

runs was used, with maximum generation of 10,000, and population size of 150. A cutoff of 2 Å RMSD was used for clustering. Predicted poses were analyzed in terms of their energetic binding potential. Hydrogen bonds were defined by a donor-acceptor distance cutoff of 3.5 Å and a donor-hydrogen-acceptor angle between 150° and 180°. Van der Waals contacts were defined by a carbon-carbon distance cutoff of 5.0 Å.

A combination of the ICM-browser (Molsoft LLC) and VMD 1.9 (Theoretical and Computational Biophysics Group at the University of Illinois at Urbana-Champaign) (182), both freely available, were used for visual analysis ofl docking results and image rendering.

2.5 AC Sequence Alignment

Alignment of two or more AC sequences using BLAST (*http://*blast.ncbi.nlm.nih.gov/) was utilized to compare primary sequence for the domains of various AC isoforms. The primary sequences of the C1/C2 domains used in a crystallized AC structure (PDB ID 1CJT) were aligned with the corresponding C1/C2 sequences of human AC1-9. Various regions of AC5 C1 and C2 were also aligned with AC2 C1 and C2 sequences containing identified Gβγ interaction and/or activation sites.

2.6 C1/C2 Domain and G Protein Purifications

Proteins 5C1(670)H_6, $H_6$5C2, and GαsH_6, were expressed in *Escherichia coli* and purified as described previously (32, 183, 184). GST-tagged proteins were expressed in *Escherichia coli* and purified using glutathione agarose resin as described previously (99). Non-tagged or biotin-tagged $G\beta_1\gamma_2$ was coexpressed with $G\alpha_iH_6$ in clonal isolates of

Spodoptera frugiperda (*Sf9*) insect cells and purified on nickel-NTA columns, followed by either overnight dialysis or ion exchange as described previously (33).

2.7 Sf9 AC Expression

Clonal isolates of *Sf9* cells were maintained in cell suspension flasks with SF-900 II SFM media at 27°C. Baculoviruses encoding AC isoforms or G protein subunits were amplified for 5-7 days using 500µl of storage virus stock with 50ml of Sf9 cells in log phase in SF-900 II SFM media with 50 µg/ml gentamicin at a concentration of 1.5-2 × 10^6 cells/ml to produce a baculovirus working stock with a minimum titer of 1 × 10^7 pfu/ml. 25ml of the appropriate AC isoform baculovirus was used to infect 1 liter of Sf9 cells at a concentration of 1.5-2 X 10^6 cells/ml. After 48 hours of infection, Sf9 cells were harvested by centrifugation at 1000 g for 10 minutes at 4°C.

2.8 Sf9 Membrane Preparation

Harvested Sf9 cells were suspended in ice-cold lysis buffer consisting of 20 mM HEPES (pH 8.0), 150 mM NaCl, 5 mM EDTA, 1 mM EGTA, 2 mM DTT, and protease inhibitors phenylmethylsulfonyl fluoride, tosyl phenylalanyl chloromethyl ketone, leupeptin, lima bean trypsin inhibitor, and aprotinin. Cells were lysed by nitrogen cavitation at 500 psi for 30 minutes at 4°C. Cell lysates were centrifuged at 750g for 10 minutes to remove intact cells and nuclei. The supernatants were centrifuged at 100,000g for 30 minutes, and the resulting pellets were resuspended and washed in buffer consisting of 20 mM HEPES (pH 8.0), 2 mM DTT, 200 mM sucrose, and protease inhibitors as previously described. Membranes were homogenized with a Dounce homogenizer and centrifuged again at 100,000g for 30 minutes.

Resuspended Sf9 membrane pellets had concentration determined by Bradford assay, with membranes then immediately frozen in liquid nitrogen and stored at -80°C in one-use aliquots for future adenylyl cyclase activity assays (see below).

2.9 HEK293 and COS-7 Cell Transfections and Membrane Preparations

HEK293 or COS-7 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C with 5% CO_2. All volumes and amounts are for transfection in 10cm plates for membrane preparations. HEK293 (3×10^6 cells) and COS-7 cells (2×10^6 cells) were seeded 24 hours prior to transfection in 10cm dish. Medium was replaced the next day with fresh DMEM (no penicillin/streptomycin) and cells were transfected with the appropriate plasmids (10 µg DNA total per 10cm plate) using Lipofectamine 2000 in a 1:3 µg DNA: µg lipofectamine ratio. Cells were incubated at 37°C for 4-6 hours, the media was replaced, and membranes prepared approximately 42 hours (COS-7 cells) or 42-48 hours (HEK293 cells) after transfection.

Following transfections, cells were rinsed and harvested in cold PBS then pelleted by centrifugation at 3000g, 4°C for 5 min. Cell pellets were aspirated and resuspended in 20 mM HEPES, 1 mM EDTA, 2 mM MgCl2, 1 mM DTT, 250 mM sucrose, and protease inhibitors. Cells were incubated on ice for 10-30 min, subjected to dounce homogenization, and centrifuged at 1800g for 5 min at 4°C to pellet nuclei. The supernatants were centrifuged at 60,000g, 4°C for 20 min. The resulting membrane pellet was resuspended in 20 mM HEPES, 1 mM EDTA, 2 mM MgCl2, 1 mM DTT, 250 mM sucrose, and protease inhibitors. HEK293/COS-7 membrane concentrations were determined by Bradford assay. Membranes

were immediately used in adenylyl cyclase activity assays or frozen in liquid nitrogen and stored at -80°C in one-use aliquots for future assays.

2.10 Adenylyl Cyclase Membrane Activity Assays

As performed and described previously (185, 186), Sf9, COS-7, or HEK membrane preparations (see above) were incubated for 10 minutes at 30°C with an AC mix containing [α-^{32}P]ATP, 10 mM $MgCl_2$, and appropriate activators, including forskolin or GTPγS-Gαs with or without the indicated concentrations of Gβγ. When applicable, inhibitors in DMSO (1µl) were added on ice before the addition of activators. The activators used were 50µM forskolin for Sf9 membranes of AC1-7, 100µM calcium and 300µM calmodulin for HEK membranes of AC8, 50nM Gαs for membranes expressing AC1-8 or 300nM Gαs for HEK membranes of AC9. Final volume of these reactions was 50 µl. Reactions were stopped with 850 µl of a solution of 2.5% SDS, 50 mM ATP, and 1.75 mM cAMP. Nucleotides in each reaction sample were then separated by sequential column chromatography on Dowex and Alumina resins to isolate [^{32}P]cAMP product, using [^{3}H]cAMP to monitor column recovery rates. Separated samples were collected in scintillation vials with scintillation fluid, and production of cAMP was measured by scintillation counting using a dual dpm program to count [^{3}H]cAMP and [^{32}P]cAMP isolated from each sample.

2.11 Intact cAMP Accumulation Assays

HEK293 cells were transfected with either AC1 or a pcDNA vector control in poly-lysine coated 6-well plates. 48 hours post transfection, cells were labeled with [^{3}H]adenine for 3-4 hours, washed, and incubated with 1 mM 1-methyl-3-isobutylxanthine (IBMX) and either

NB001 or a DMSO vehicle control at 37°C for 10 min. Forskolin (10 μM) and ionomycin (10 μM) were incubated for an additional 30 min. Reactions were stopped upon addition of ice-cold 5% trichloroacetic acid (TCA), 1 mM ATP, and 100 μM cAMP. Nucleotides were then separated by column chromatography to isolate [^{3}H]cAMP product, using [^{32}P]cAMP to monitor column recovery rates. [^{3}H]cAMP and [^{32}P]cAMP separation and counting were performed as described above for AC membrane activity assays.

2.12 Pull-Down Binding Assays

GST or GST-tagged AC NT (full-length or truncations) were incubated with purified Gβγ subunits in 50 μl of binding buffer (20 mM HEPES, pH 8.0, 1 mM EDTA, 5 mM $MgCl_2$, 2 mM DTT, 100 mM NaCl, 0.1% $C_{12}E_9$). The proteins were incubated for 30 min at 4°C followed by addition of 100 μl of 20% glutathione-agarose beads. After rotating for 2 hours at 4°C, the resin was washed three times with wash buffer (20 mM HEPES, pH 8.0, 1 mM EDTA, 5 mM $MgCl_2$, 2 mM DTT, 100 mM NaCl, 0.05% $C_{12}E_{9)}$. Bound proteins were eluted with 15 mM glutathione and analyzed by SDS-PAGE and Western blotting.

For biotin-tagged protein pulldowns, purified biotin-tagged Gβγ was incubated with His-tagged 5C1(670)H_6 in 50μl of binding buffer for 30 min at 4°C. After incubation, 100 μl of 20% streptavidin-agarose beads was added to the samples, rotated for 2 hours at 4°C, and the resin was subsequently washed three times with wash buffer. Bound proteins were eluted from the streptavidin-agarose by direct addition of 1X Laemmli buffer and analyzed by SDS-PAGE and Western blotting.

2.13 Flag-AC5 Immunoprecipitation

Human Flag-tagged AC5 was transfected in HEK293 cells (10 cm dish/IP). After 40 hours, HEK293 cells were rinsed with phosphate-buffered saline, resuspended in lysis buffer (50 mM HEPES, pH 8.0, 1 mM EDTA, 1 mM $MgCl_2$, 1 mM DTT, 150 mM NaCl, 0.5% $C_{12}E_9$, and protease inhibitors), and homogenized using a 23-gauge syringe. Cellular debris was removed by centrifugation, and 30 µl of anti-Flag agarose was added. Samples were rotated at 4°C for 2 h, and then washed three times with lysis buffer that contained only 0.05% $C_{12}E_9$. Proteins were eluted from anti-Flag resin with SDS-PAGE sample buffer, and analyzed by SDS-PAGE and Western blotting.

2.14 Gβγ Binding Overlays

BSA controls, His-tagged AC5/6 C1 or C2 domains were run on SDS-PAGE gels and transferred to polyvinyldifluoride membranes. After blocking with 5% milk for 1 hour, PVDF membranes were incubated overnight with 10 µg purified Gβγ in 3 ml of overlay wash buffer (Tris-buffered saline pH 7.4, 0.1% Tween-20, and 1mM DTT) overnight at 4°C. Bound Gβγ was detected by Western blotting.

Chapter 3

Characterized Isoform Selectivity of Adenylyl Cyclase Inhibitors

Results figures in this chapter have been reprinted with permission from the following publications (text describing results partially reprinted with permission from Brand *et al.* only):

Brand CS, Hocker HJ, Gorfe AA, Cavasotto CN, Dessauer CW. Isoform Selectivity of Adenylyl Cyclase Inhibitors: Characterization of Known and Novel Compounds. J Pharmacol Exp Ther. November 2013, 347:265-275.

Conley JM, **Brand CS**, Bogard AS, Pratt EP, Xu R, Hockerman GH, Ostrom RS, Dessauer CW, Watts VJ. Development of a high-throughput screening paradigm for the discovery of small molecule modulators of adenylyl cyclase: Identification of an adenylyl cyclase 2 inhibitor. J Pharmacol Exp Ther. November 2013, 347:276-287.

3.1 Rationale

AC inhibitors, through decreasing cAMP production, have therapeutic potential. This is supported by the use of Gαs-coupled GPCR antagonists, such as beta blockers for treating heart diseases, or Gαi-coupled GPCR agonists, such as opioids for treating pain. However, in order for AC inhibitors to have much therapeutic potential, they should be selective for an AC isoform or subset of isoforms. Otherwise, due to the many cAMP-associated physiological functions, off-target effects would be probable. Similar issues are seen for the above GPCR

ligands that target upstream of AC. Previously developed AC inhibitors have been shown to be selective while only comparing a subset of AC isoforms; the incomplete characterization of such an approach has already been observed for NKY80.

There are other small molecule inhibitors allegedly specific for AC5 that are related to NKY80; namely, SQ22,536 and Ara-A. These small molecules have been proposed to be AC5 selective compared to AC2 and AC3, three relatively dissimilar isoforms with regards to overall regulatory patterns (157, 172, 173). Additionally, SQ22,536 and Ara-A have worked for intact cell AC inhibition, an issue for many selective AC inhibitors (187). However, neither compound was tested against all 9 transmembrane AC isoforms. Since these inhibitors are also derived from P-site inhibitors, they also have similar adenine structures which bring up the issues of potential off-target effects. These potential issues are not restricted to alleged AC5 inhibitors; the allegedly AC1-specific inhibitor NB001 has a chemical structure extremely similar to ATP. Thus, we proposed to more vigorously characterize the isoform selectivity of small molecule AC inhibitors such as SQ22,536, Ara-A, and NB001.

3.2 "AC5" Inhibitors Are Actually AC5/6 Selective Inhibitors

SQ22,536 and Ara-A are three adenine-like inhibitors that are reportedly selective for AC5 (157, 172, 173) (the chemical structure of these ligands is shown in Figures 4A and 5A while the 3D structure of the ligand-AC complexes obtained from docking is shown in Figures 4B and 5B). However, these inhibitors have never been tested against all nine membrane-bound AC isoforms. Using an *in vitro* AC activity assay with Sf9 or HEK293 membranes expressing a given AC isoform, we tested these inhibitors for their potency against each transmembrane AC.

SQ22,536 was the first P-site inhibitor analog without phosphates developed, and is essentially a dideoxyadenosine analog (Figure 4A). It has been previously shown to be selective for AC5 over AC 2 or 3 (157, 172). In addition to confirming these results, we show that SQ22,536 has high potency for AC 5 and 6 with 10-15 fold selectivity over the next closest isoform, AC1. Potency and efficacy for AC 2, 3, 4, 7, 8, and 9 are further reduced, with less than 50% inhibition of AC8 and 9 at 1 mM. Although inhibition of AC 8 and 9 appears to level off slightly, this is likely only due to our inability to further increase inhibitor concentrations and reach complete saturation. Importantly, inhibition by SQ22,536 is not significantly different for AC5 and AC6 (Figure 4C and Table 1).

Ara-A, also known as vidarabine, is an adenosine analog (Figure 5A) that is used clinically as an antiviral, with a mechanism that is unrelated to inhibition of AC/cAMP signaling (188). Ara-A was shown previously to be selective for AC5 over AC 2 and 3 when expressed in Sf9 membranes, and has been suggested to inhibit AC5 more potently than AC6 in cardiomyocytes (189). We show that, similar to SQ22,536, Ara-A most potently inhibits AC 5 and 6 (Figure 5C and Table 1). Additionally, for any given concentration of Ara-A, we observe less than a 2-3 fold difference in the inhibition of AC5 versus AC6. Therefore SQ22,536 and Ara-A, in addition to NKY80, are actually AC5/6 selective inhibitors.

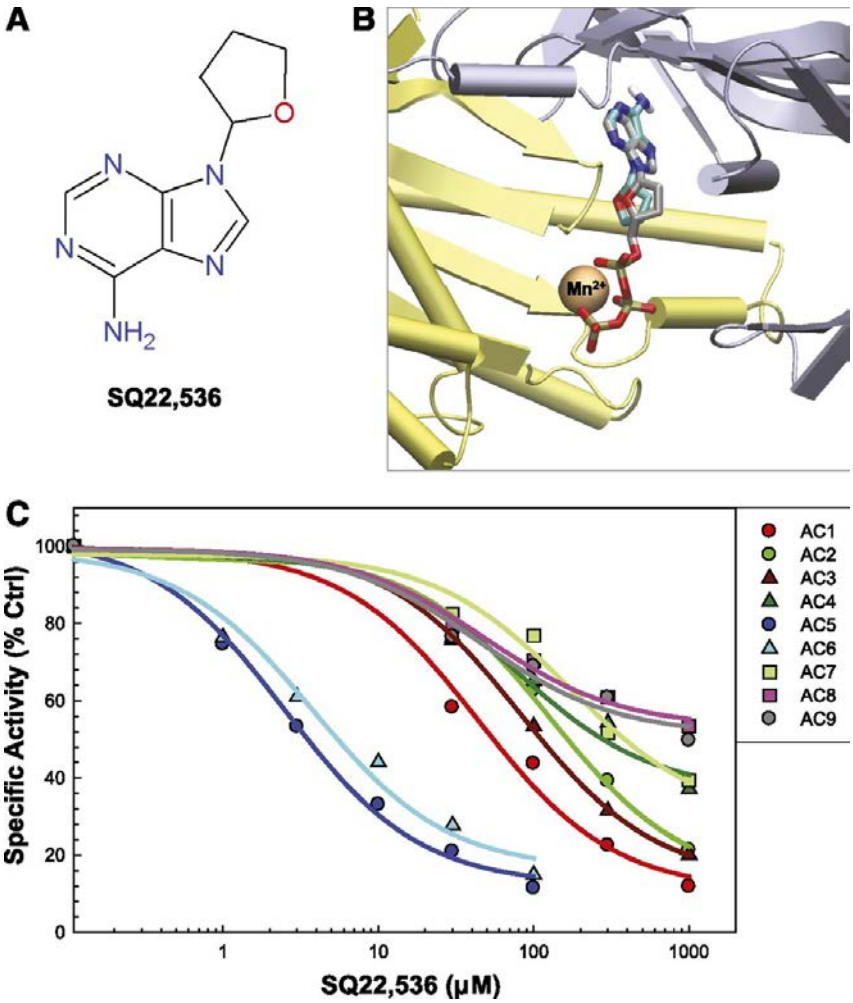

Figure 3: AC Inhibition Profile of SQ22,536. A, Chemical structure of SQ22,536. **B,** Virtual docking of SQ22,536 to a P-site inhibitor-bound conformation of AC (PDB 1CJT). SQ 22,536 is shown with cyan carbons; the crystallized position of 2',3'-dd-ATP ligand shown with grey carbons for reference. C1 domain is yellow; C2 domain is silver. **C,** Inhibition profile of SQ22,536 for all 9 AC isoforms. Inhibition curves for each AC is shown as a calculated fit to the means of each concentration (indicated by symbols and grouped with colors by AC family; n=3, performed in duplicate; error bars removed for clarity). Membranes from Sf9 cells expressing AC 1-7 were stimulated by 50 µM forskolin. Membranes from HEK293 cells expressing AC 8 and 9 were stimulated by 300 µM calmodulin, and 300 nM Gαs, respectively. Reproduced from **Brand CS**, Hocker HJ, Gorfe AA, Cavasotto CN, Dessauer CW. Isoform Selectivity of Adenylyl Cyclase Inhibitors: Characterization of Known and Novel Compounds. J Pharmacol Exp Ther. November 2013, 347:265-275 (1).

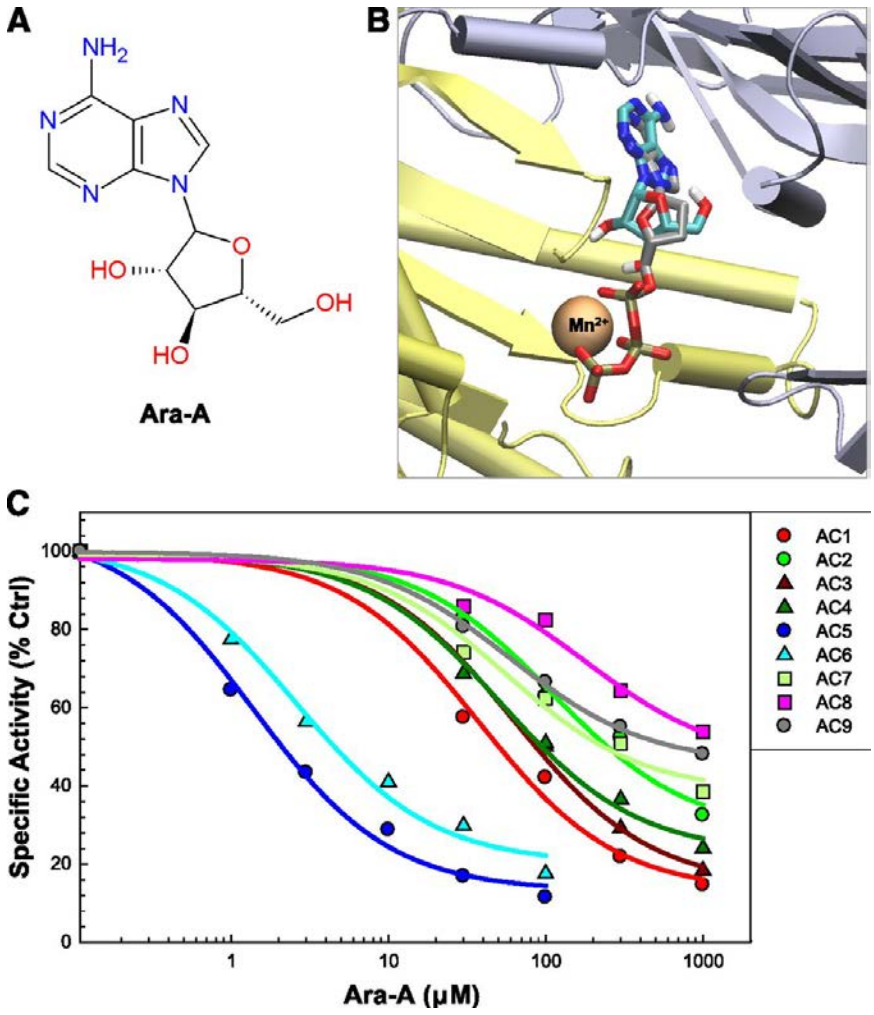

Figure 4: Ara-A Does Not Discriminate Between AC5 and AC6. A, Chemical structure of Ara-A. **B,** Virtual docking of Ara-A to the 2'3'-dd-ATP bound conformation of AC as described for Figure 3B. **C,** Complete AC isoform inhibition profile of Ara-A. Inhibition curves for each AC isoform shown as fit to means of AC activity assays (n=3). Membranes and stimulation conditions were as described in Figure 3C (n=3, performed in duplicate). Reproduced from **Brand CS**, Hocker HJ, Gorfe AA, Cavasotto CN, Dessauer CW. Isoform Selectivity of Adenylyl Cyclase Inhibitors: Characterization of Known and Novel Compounds. J Pharmacol Exp Ther. November 2013, 347:265-275 (1).

Table 2: Pharmacological Profile for Inhibition of Adenylyl Cyclase Isoforms

Inhibitor	pIC_{50} +/- S. E. (IC_{50} in μM)								
	AC1	AC2	AC3	AC4	AC5	AC6	AC7	AC8	AC9
SQ22,536	4.27 +/- 0.09** (54)	3.71 +/- 0.23** (210)	3.96 +/- 0.09** (110)	3.55 +/- 0.04** (280)	5.46 +/- 0.08 (3.5)	5.25 +/- 0.14 (5.8)	3.38 +/- 0.16** (440)	#, (>1000)	#, (>1000)
Ara-A	4.31 +/- 0.09** (50)	3.61 +/- 0.05** (250)	4.07 +/- 0.01** (85)	4.03 +/- 0.22** (100)	5.67 +/- 0.05 (2.2)	5.34 +/- 0.13* (4.7)	3.64 +/- 0.07** (230)	#, (>1000)	#, (>1000)

Experiments performed as described in Fig 1C-3C. Values are reported as pIC_{50} +/- S.E., where pIC_{50} is calculated as -log(IC_{50}). n=3, each performed in duplicate. Statistics (t-test) were performed on pIC_{50} of the indicated isoform versus that of AC5. *$p < 0.05$, **$p < 0.001$, $^{\#}$ Did not reach 50% activity at 1 mM inhibitor; ^ Did not reach 50% inhibition in one experiment. Table adapted from **Brand CS**, Hocker HJ, Gorfe AA, Cavasotto CN, Dessauer CW. Isoform Selectivity of Adenylyl Cyclase Inhibitors: Characterization of Known and Novel Compounds. J Pharmacol Exp Ther. November 2013, 347:265-275 (1).

3.3 Mutation of Serine 942 in AC2 Forskolin Pocket Does Not Alter Inhibition

Global docking of SQ22,536 and Ara-A predicted that these inhibitors frequently (~70% and ~50%, respectively, see Table 2) target the ATP binding site of the AC C1/C2 domains (1CJT). Similar docking to the MANT-GTP bound conformation of AC (1TL7) was less predictive, suggesting that these molecules bind to a similar AC conformation as their classical P-site predecessors.

The catalytic and forskolin sites are pseudosymmetrically related. Although forskolin is an AC activator, inhibitors that target the forskolin binding site have been identified (163, 164). Since our virtual screening focused only on the ATP site, we wanted to rule out that SQ22,536 and the other molecules that inhibited AC activity do not exert their actions through interactions with the forskolin pocket. To this end, we mutated residues within the forskolin-binding pocket. From the crystal structure of AC, Ser942 in the C2 domain of AC2 interacts with forskolin but does not contribute to C1-C2 interaction (12). Since substitution of this residue by proline was predicted to prevent hydrogen bonding to a nearby water molecule and distort the forskolin binding pocket, we mutated Ser942 to proline in AC2 and prepared membranes from COS-7 cells expressing the wild-type and mutant proteins. Ser942P activity had decreased sensitivity to stimulation by forskolin compared to wild-type AC2, while basal activity and stimulation by Gαs were not significantly altered (Figure 6A, 5B). Synergistic AC2 activation by both forskolin and Gαs was also impaired in the Ser942P mutant. However, the mutation had no effect on inhibition of Gαs-stimulated AC2 by SQ22,536 (Figure 6C), supporting an interaction of these inhibitors with the ATP binding site. Forskolin pocket mutation also had no effect on inhibition of Gαs-stimulated AC2 by CB-7833407, a novel AC

inhibitor without adenosine-like structure identified previously in a structure-based virtual screen of the ATP binding site of AC.

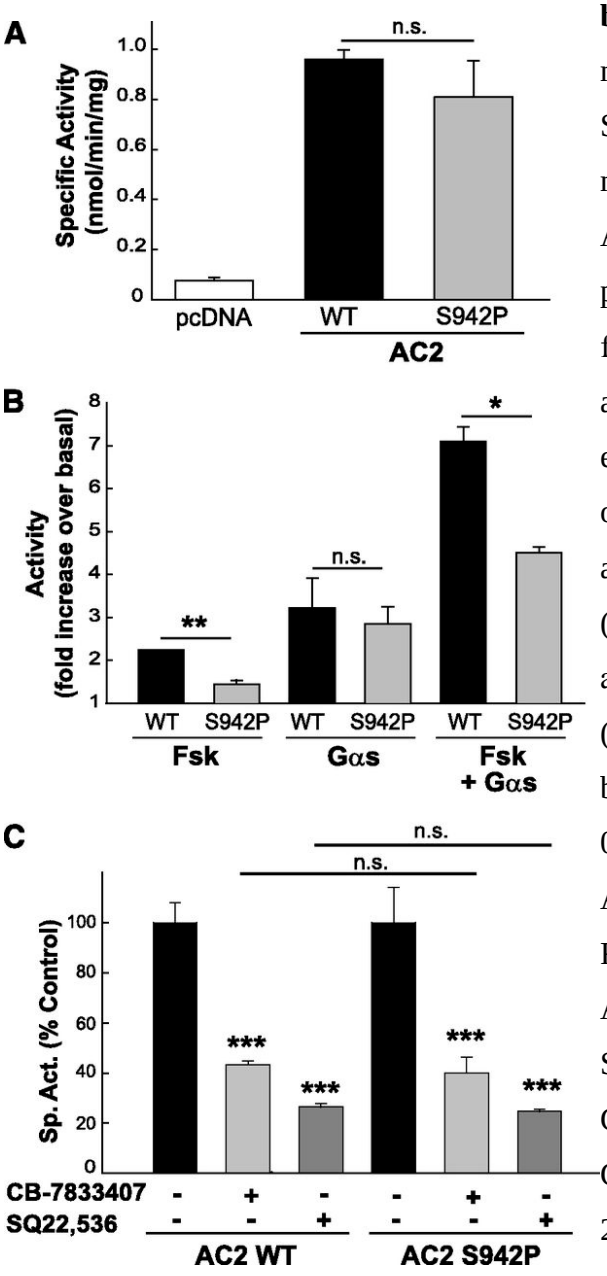

Figure 5: Mutation of AC2 Forskolin Binding Pocket Does Not Impair Inhibition by SQ22,536. A, Basal activity of COS-7 membranes expressing AC2 wildtype, AC2 S942P mutant, or a pcDNA control was measured. **B,** COS-7 membranes expressing AC2 wildtype, AC2 S942P mutant, or a pcDNA control were incubated with 10 µM forskolin and/or 50 nM activated Gαs and AC2 activity was measured. **C,** COS-7 membranes expressing AC2 wild-type, AC2 S942P mutant, or a pcDNA control were preincubated in the absence or presence of the indicated inhibitor (100 µM SQ22,536 or 500 µM CB-7833407) and then stimulated with 50 nM Gαs. Statistics (paired t-test) for inhibition of AC2 WT/S942P by the indicated AC inhibitor, *$p < 0.05$, **$p < 0.01$, and for differences between inhibition of AC2 WT vs. S942P, n.s. - not significant. Reproduced from **Brand CS**, Hocker HJ, Gorfe AA, Cavasotto CN, Dessauer CW. Isoform Selectivity of Adenylyl Cyclase Inhibitors: Characterization of Known and Novel Compounds. J Pharmacol Exp Ther. November 2013, 347:265-275 (1).

Table 3: Global Docking of Small Molecule Inhibitors to AC Catalytic Domains

Inhibitor	% of Docking Hits at Indicated Site (% of Total Docks)			
	AC Without Bound Forskolin		AC With Bound Forskolin	
	ATP Binding Site	Fsk Binding Site	ATP Binding Site	Fsk Binding Site
SQ22,536	69.9%	0.8%	68.7%	0.8%
Ara-A	47.7%	10.9%	42.6%	0.8%

Global docking with AutoDock performed as described in Materials and Methods using the 2'3'ddATP-bound conformation of AC (1CJT). Percentages are based upon 256 independent docking runs. Table adapted from **Brand CS**, Hocker HJ, Gorfe AA, Cavasotto CN, Dessauer CW. Isoform Selectivity of Adenylyl Cyclase Inhibitors: Characterization of Known and Novel Compounds. J Pharmacol Exp Ther. November 2013, 347:265-275 (1).

3.4 "AC1 Inhibitor" NB001 Does Not Directly Inhibit AC1 Activity

To gain further insight into the selectivity of different AC inhibitors, we also examined NB001, which is reported to be an AC1 selective ligand with therapeutic potential as an analgesic (US patent #8,124,599). In HEK cells stably expressing AC1, NB001 inhibited AC activity when stimulated with forskolin and the calcium ionophore calimycin (166). We also observed NB001 inhibition of cAMP accumulation in HEK cells transiently expressing AC1 when stimulated with ionomycin and forskolin (Figure 7B). However, this experiment performed both by us and Wang *et al.*. detects cAMP accumulation within the cell and does not necessarily reflect solely AC1 activity. We were unable to inhibit AC1 activity *in vitro* by NB001 when assayed in membranes prepared from HEK293 cells expressing AC1, as used in the cAMP accumulation assays (Figure 7C). This lack of direct action of NB001 on AC1 was irrespective of activation conditions, including stimulation by forskolin, calmodulin, or the combination of forskolin and calmodulin to mimic the effects of forskolin and ionomycin in intact cells. This suggests that although NB001 can reduce cAMP accumulation in cells in an AC1-dependent manner, the ligand is not acting through direct inhibition of AC1 activity.

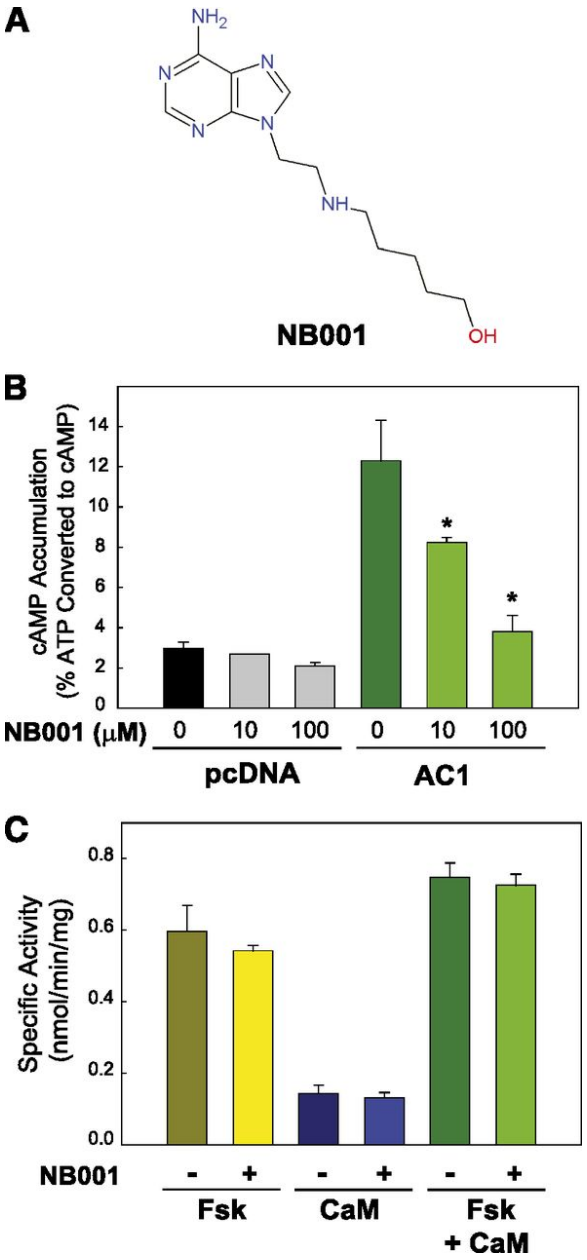

Figure 6: NB001 Decreases AC1-dependent cAMP Accumulation but Does Not Directly Inhibit AC1. A, Chemical structure of NB001. **B,** HEK293 cells expressing AC1 or pcDNA control were incubated with NB001 (10 or 100 μM) or vehicle prior to stimulation with 10 μM forskolin and 10 μM ionomycin. Statistics (t-test) for NB001 inhibition, *$p < 0.05$. **C,** NB001 (100 μM) or vehicle control were incubated with membranes from HEK293 cells expressing AC1 and stimulated as indicated. Reproduced from **Brand CS**, Hocker HJ, Gorfe AA, Cavasotto CN, Dessauer CW. Isoform Selectivity of Adenylyl Cyclase Inhibitors: Characterization of Known and Novel Compounds. J Pharmacol Exp Ther. November 2013, 347:265-275 (1).

3.5 Small Molecules from Cell-Based Screen Show Direct AC2 Inhibition

Given the nonspecific effects observed in this work and cited literature, it is not surprising that various groups are looking for alternative screening methods to identify isoform specific small molecule AC inhibitors. One such target is AC2, due to its inhibition producing effects on skeletal muscle development (190-192), both neuroendocrine and colorectal cancer types (193-195), and IL-6 responses in airway smooth muscle (196-198). As such, Conley *et al.* identified novel AC2 inhibitors through a cell-based screen for small molecules from NIH clinical libraries that blocked AC2-specific stimulation of cAMP accumulation by treatment with PMA. This method produced two promising AC2 inhibitor candidates, tranilast and SKF-83566 (2). SKF-83566 is also a D1 dopamine receptor antagonist, but at thousand-fold higher concentrations than those that directly inhibit AC2 activity (199).

However, we had also shown via NB001 that AC inhibitors which test well in cell-based screens may not directly inhibit AC activity. As such, in addition to their work identifying these compounds through in-cell screens, we tested their AC2 inhibitor candidates *in vitro* in assays using Sf9 membranes. Both tranilast and SKF-83566 directly inhibit AC2 activity, and continue to do so in an isoform-specific manner (Figure 8A). In comparison to the AC5/6 inhibitor NKY80, the range of difference in AC2 vs. AC5 inhibition at a single concentration was more pronounced for both SKF-83566 and tranilast. This differentiates these novel candidates from BODIPY-forskolin, a potent AC2 inhibitor yet one that lacks isoform selectivity (163, 164, 200). Thus, our results validated that their cell-based PMA stimulation assay identified novel isoform selective AC inhibitors that act directly on AC activity.

In addition, there was some question as to the mechanism of SKF-83566 inhibition of AC2. The reason for this was that SKF-83566 was the most promising candidate pulled out of

their PMA-stimulation based inhibitor screen. We tested the inhibition of AC2 by SKF-83566 at set concentrations of 75 or 200 μM or control, and increasing concentrations of ATP. When plotted, our results showed a stable Km value but variable Vmax values, indicating that SKF-83566 candidate was a noncompetitive AC2 inhibitor (Figure 8C). Notably, classic P-site inhibitors as well as SQ22,536, Ara-A, and NKY80 are also noncompetitive inhibitors. Therefore, like our previously mentioned structure-based virtual screen hits, it is possible that SKF-83566 is also a small molecule catalytic P-site inhibitor lacking adenine or ATP-like chemical structure.

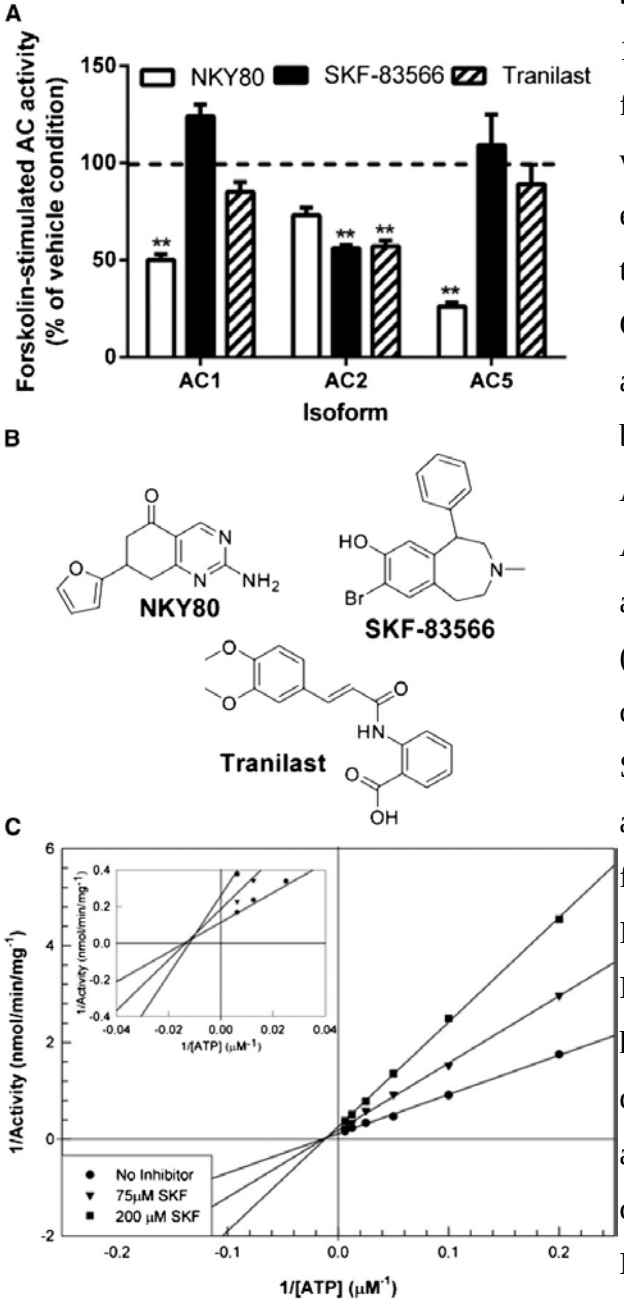

Figure 7: Inhibition of AC Activity *in vitro* by Novel AC2 Inhibitors. (A) The effects of 100 μM NKY80, SKF-83566, and tranilast on forskolin-stimulated (30 μM) AC activity were measured in membranes of Sf9 cells expressing AC1, AC2, or AC5. $^{**}P < 0.01$ (*t* test compared with vehicle condition). (B) Chemical structures of NKY80, SKF-83566, and tranilast. Chemical structures were drawn by JM Conley. (C) Double-reciprocal plot of AC activity from Sf9 membranes expressing AC2 (5 μg) in the presence of 100 nM $G\alpha_s$ and in the absence or presence of SKF-83566 (75 μM or 200 μM) and the indicated concentrations of ATP. The data suggest that SKF-83566 noncompetitively inhibited AC2 activity with respect to ATP. Reproduced from Conley JM, **Brand CS**, Bogard AS, Pratt EP, Xu R, Hockerman GH, Ostrom RS, Dessauer CW, Watts VJ. Development of a high-throughput screening paradigm for the discovery of small molecule modulators of adenylyl cyclase: Identification of an adenylyl cyclase 2 inhibitor. J Pharmacol Exp Ther. November 2013, 347:276-287 (2).

3.6 Summary

We have shown that allegedly AC5-selective inhibitors SQ22,536 and Ara-A can inhibit both AC5 and AC6. This has implications for the therapeutic repurposing of Ara-A; the inhibitor blocks both AC5/6 activity, yet was proposed to decrease AC5 activity in cardiomyocyte models of heart failure where AC6 inhibition has been suggested to not be beneficial, or even detrimental. We identified the site of action as the ATP-binding site in the catalytic C1/C2 domains of cyclase, suggesting a P-site inhibitor method of inhibition. We also showed that the small molecule inhibitor NB001 does not inhibit AC1 directly, despite decreasing AC1-dependent cAMP accumulation within cells. Lastly, we have characterized novel AC2 inhibitors that were identified by chemical screening of clinical compound libraries and validated their direct effects on AC2 inhibition. Overall, the potential flaws from incomplete characterization of selectivity in AC inhibitors have been highlighted, and a cell-based AC inhibitor screening method has been validated in part through such characterization.

Chapter 4

Adenylyl Cyclase Isoform Specificity of NT Interactions and Regulation by Gβγ

4.1 Rationale

The heterotrimeric Gβγ protein subunit has isoform specific regulatory effects on AC. Gβγ can enhance the activation of Gαs- or forskolin-stimulated AC 2, 4, 5, 6, and 7 (18-21, 35). In addition, Gβγ can also inhibit the activity of AC 1, 3, and 8 (18, 36, 37). Both Gαs and Gβγ subunits are needed to observe a full stimulatory AC6 response to the Gs-coupled receptor agonist isoproterenol (93). This suggests that Gβγ generated from activation of Gαs plays a regulatory role for enhancement of AC5/6 activity. This differs from the conditional Gβγ stimulation of AC2/4/7, where the Gβγ subunit is thought to be provided via activation of Gαi-coupled GPCRs (19). Differences in Gβγ regulation of AC depending on the GPCR source have also been observed via live cell imaging (201). Thus, Gβγ was an avenue for looking at differences in AC isoform activity control by the same physiological regulator.

A binding site for inactive G protein heterotrimer on the NT of AC5 (AC5NT) was previously identified, but not required for Gβγ stimulation of AC5 (95). Gβγ binding to residues 66-137 of AC5NT is synergistically enhanced in the presence of GDP-Gαs and the formation of a heterotrimer (95). Similar scaffolding interactions between AC and heterotrimeric G proteins have previously been proposed (202, 203). Other Gβγ effectors, such as PLCβ, GIRK channels, and RACK1, are also capable of scaffolding G protein heterotrimers

(67, 204, 205). The binding of Gαs-βγ at the AC5NT is independent from Gβγ stimulation of AC5, supporting a model where inactive heterotrimer occupies the NT and a separate event results in Gβγ stimulation of AC5. Although both AC5 and the closely related AC6 are stimulated by Gβγ, AC6 stimulation by Gβγ requires residues 77-151 of AC6NT (93).

In the inactive Gα-βγ conformation, regions on the Gα and Gβγ subunits required for effector regulation are concealed in a bound heterotrimer. After activation, such regions are exposed and can interact with downstream effector proteins such as AC (26). The Gβγ structure includes β-sheet WD40 motif repeats, or "blades", and a "hotspot" area where the turns between blades intersect. The "hotspot" on Gβγ is required for interactions with either the alpha subunit when inactive, or numerous effectors when active (88, 177, 206).

We hypothesized that Gβγ requires the "hotspot" to stimulate AC5, but utilizes a different surface to interact with AC5NT. A similar scaffold/stimulation mechanism occurs for Gβγ and Gαi regulation of GIRK channels (66, 67). We also examined Gβγ hotspot roles in stimulation of AC6 and if these properties differed from AC5 regulation by Gβγ, since AC5/6 are closely related isoforms.

4.2 Gβγ Binds to Various AC Isoform N-Termini

Gβγ binding to the NT of AC has been observed previously for AC5 and AC6, either alone or as a Gs heterotrimer (93, 95). The NT of AC isoforms are varied in their size and sequence, and the resepective AC isoforms also vary in the regulatory function of Gβγ (Figure 9A). To determine if Gβγ binding is a conserved property of all AC NT, GST pulldowns were performed using GST-tagged AC-NT and purified Gβγ. As shown in Figure 9B, Gβγ binds to

the NT of all AC isoforms tested, including AC1, 2, 3, 5, 6, and 9. Previous studies have also identified an interaction between AC8 and Gβ2 (94).

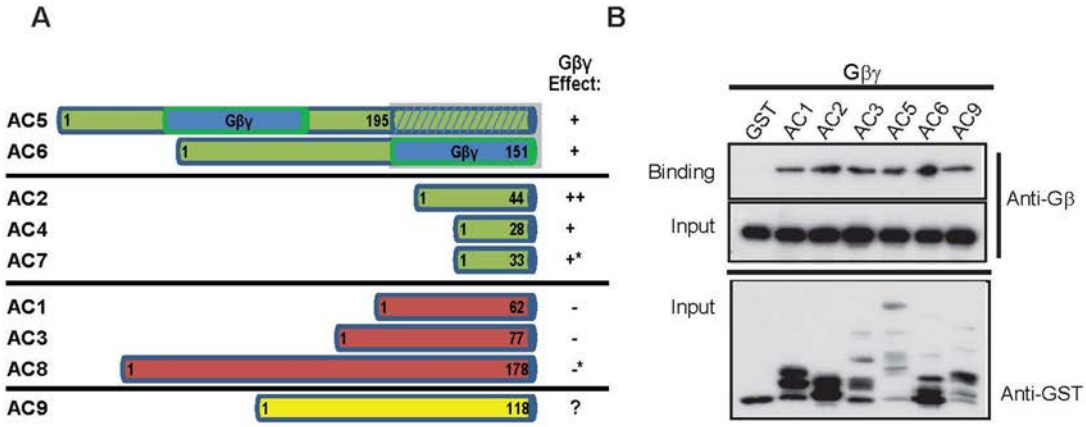

Figure 9: Gβγ Binds to Various AC N-Termini. A, Schematic of AC N-Termini grouped by similar isoforms. Regulation by Gβγ is indicated, with regulation previously in the literature shown indirectly indicated by *, and shown directly otherwise. **B,** Pulldown of GST-tagged AC NT's with bound Gβγ. The final concentration of GST or GST-tagged NT's was 2 µM. Inputs and elutes from GST pulldowns were analyzed by Western blotting. Figure 8B experiment performed by Rachna Sadana.

4.3 Gβγ Scaffolding versus Stimulation of AC5, but Not AC6, Activity are Separable Events

With the AC5NT binding site for Gβγ being a proposed heterotrimeric scaffolding site, (95), we predicted that scaffolding at the AC5NT and activation of AC5 were separable events. In order to separate these events, we utilized alanine-substituted mutants of the Gβγ "hotspot" region required for effector interactions (Figure 10A) (90, 204). Mutations within the Gβγ hotspot did not alter binding to AC5NT in GST pulldown assays (Figure 10B). However, when

assayed for their ability to increase Gαs-stimulated AC5 activity in vitro, Gβγ hotspot mutants were unable to stimulate AC5 (Figure 10C). Histidine 311 serves as a control as it is located largely outside the traditional hotspot of Gβγ, located in the junction between blades 6 and 7 (204). The inability of hotspot mutants to conditionally stimulate AC5 was independent of the Gβγ scaffolding site within the AC5NT (aa 66-137) (Figure 10D). This suggests that AC5NT binding and stimulation of AC5 by Gβγ are indeed separable events.

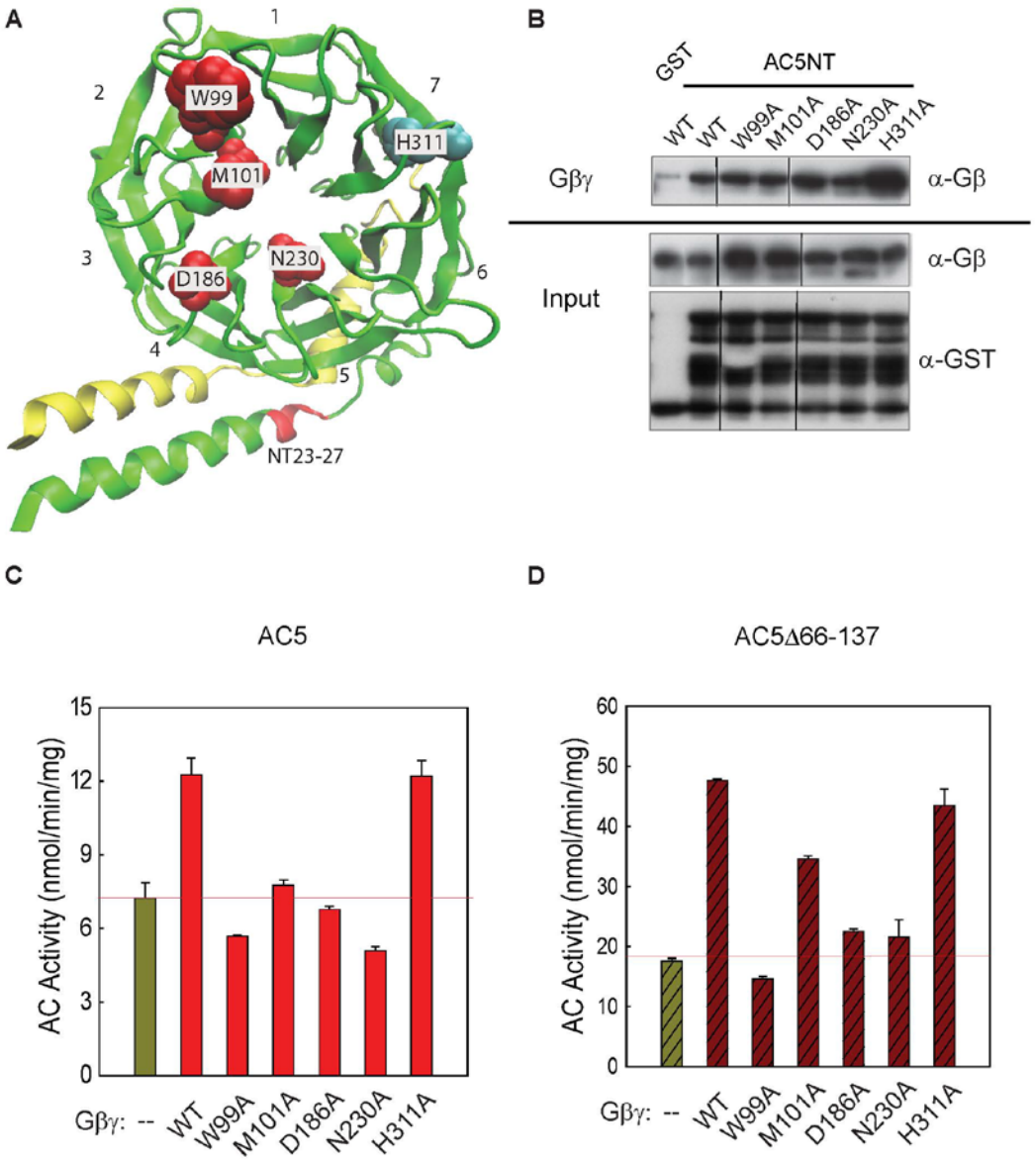

Figure 10: Gβγ Hotspot Mutants Bind to AC5NT But Are Unable to Stimulate AC5. A, Schematic of Gβγ structure, indicating sites of the alanine-substitution hotspot and NT23-27 mutants used in this paper. Image rendered using Visual Molecular Dynamics (VMD 1.9). **B,** Pulldown of GST or GST-tagged AC5NT (2 μM) incubated with 300 nM wild-type Gβγ (WT) or the indicated Gβγ hotspot mutants. Elutes from GST pulldowns were analyzed by Western blotting. Experiment performed by Rachna Sadana. **C,** AC activity assay of Sf9 membranes expressing AC5 were stimulated with 50 nM Gαs +/- 300 nM Gβγ. **D,** AC activity assay with Sf9 membranes expressing AC5 with NT residues 66-137 deleted (Δ66-137) were stimulated with 50 nM Gαs +/- 300 nM Gβγ.

As AC5 and AC6 are closely related AC isoforms, each containing a NT binding site for Gβγ, we examined NT binding versus stimulation of AC6 by Gβγ hotspot mutants. Unlike AC5, hotspot mutants of Gβγ displayed both reduced binding to the AC6NT (Figure 11A) and prevented conditional stimulation of AC6 (Figure 11B). Thus, Gβγ interactions differ between AC5 and AC6.

Previous work had shown that Gβγ could conditionally stimulate AC5/6 in the presence of either Gαs or forskolin (93). We show that the Gβγ hotspot is also required for AC5 and AC6 conditional stimulation by forskolin (Figure 11C and 11D), suggesting that the requirement for the hotspot is due to direct interactions of Gβγ with AC, independent of Gαs.

Since Gβγ bound to the NT of many AC and the hotspot was necessary for binding to the AC6NT but not AC5NT, we screened the other AC NT with wildtype vs. W99A hotspot Gβγ to determine the isoform specificity of hotspot necessity for Gβγ/ACNT binding. The hotspot mutation of Gβγ caused decreased binding of all non-AC5 NT, although the relative effect of W99A varied depending on the AC NT tested (Figure 11E). Thus, the relative hotspot necessity for AC NT binding by Gβγ is isoform-specific.

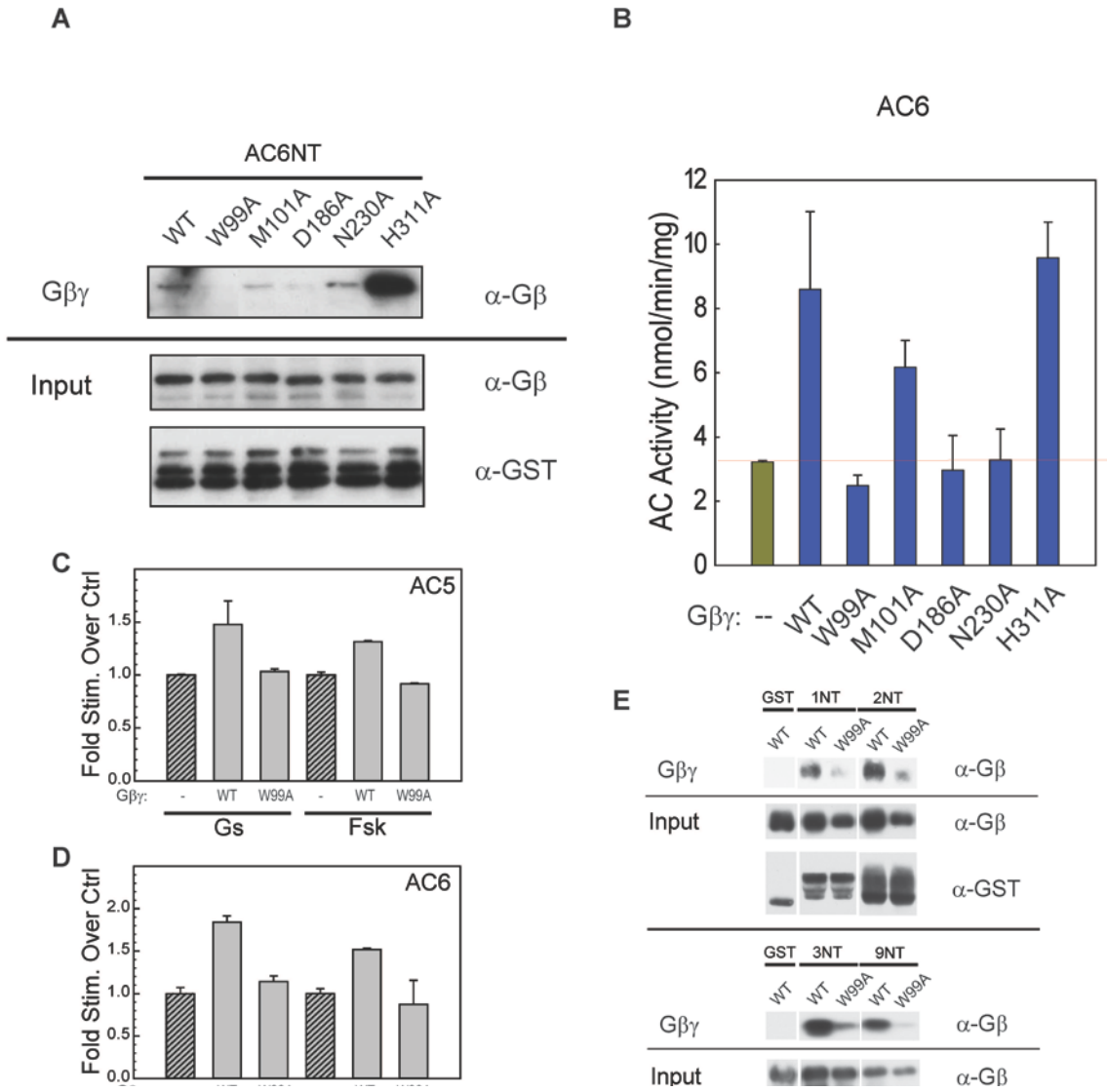

Figure 11: Gβγ Hotspot Mutants Interfere with Binding to AC NT, Stimulation of AC6. **A,** Pulldown of GST or GST-tagged AC6NT (2 μM) incubated with 300 nM wild-type Gβγ (WT) or the indicated Gβγ hotspot mutants. Elutes from GST pulldowns were analyzed by Western blotting. **B,** AC activity assay with Sf9 membranes expressing AC6 were stimulated with 50 nM Gαs +/- 300 nM Gβγ. **C**, **D**, AC activity assay with Sf9 membranes expressing AC5 (C) or AC6 (D) were stimulated with either 50nM Gαs or 50μM forskolin +/- 100 nM Gβγ. **E**, Pulldown of GST-tagged AC NT of the isoform indicated (4 μM for 1NT/2NT pulldowns, 2 μM for 3NT/9NT pulldowns), incubated with 300nM wildtype (WT) or hotspot mutant (W99A) Gβγ. Elutes from GST pulldowns were analyzed by Western blotting.

4.4 Pharmacological Targeting of Gβγ Supports a Unique AC5 Regulatory Mechanism Compared to Other AC Isoforms.

In addition to alanine-scanning mutants of Gβγ, we also used a different method of targeting Gβγ-AC interactions. The small SIGK peptide has been used previously to disrupt select hotspot-dependent Gβγ interactions with effector proteins (177, 206). Addition of SIGK prevented Gβγ regulation of several AC isoforms, including Gβγ inhibition of AC1 and stimulation of AC2 and AC6 (Figure 12B and 12D). However, SIGK was surprisingly unable to block AC5 stimulation by Gβγ, even at higher concentrations of SIGK peptide (Figure 12B and 12C). The AC isoform differences from pharmacological targeting of the Gβγ hotspot support a unique AC5-Gβγ regulatory mechanism.

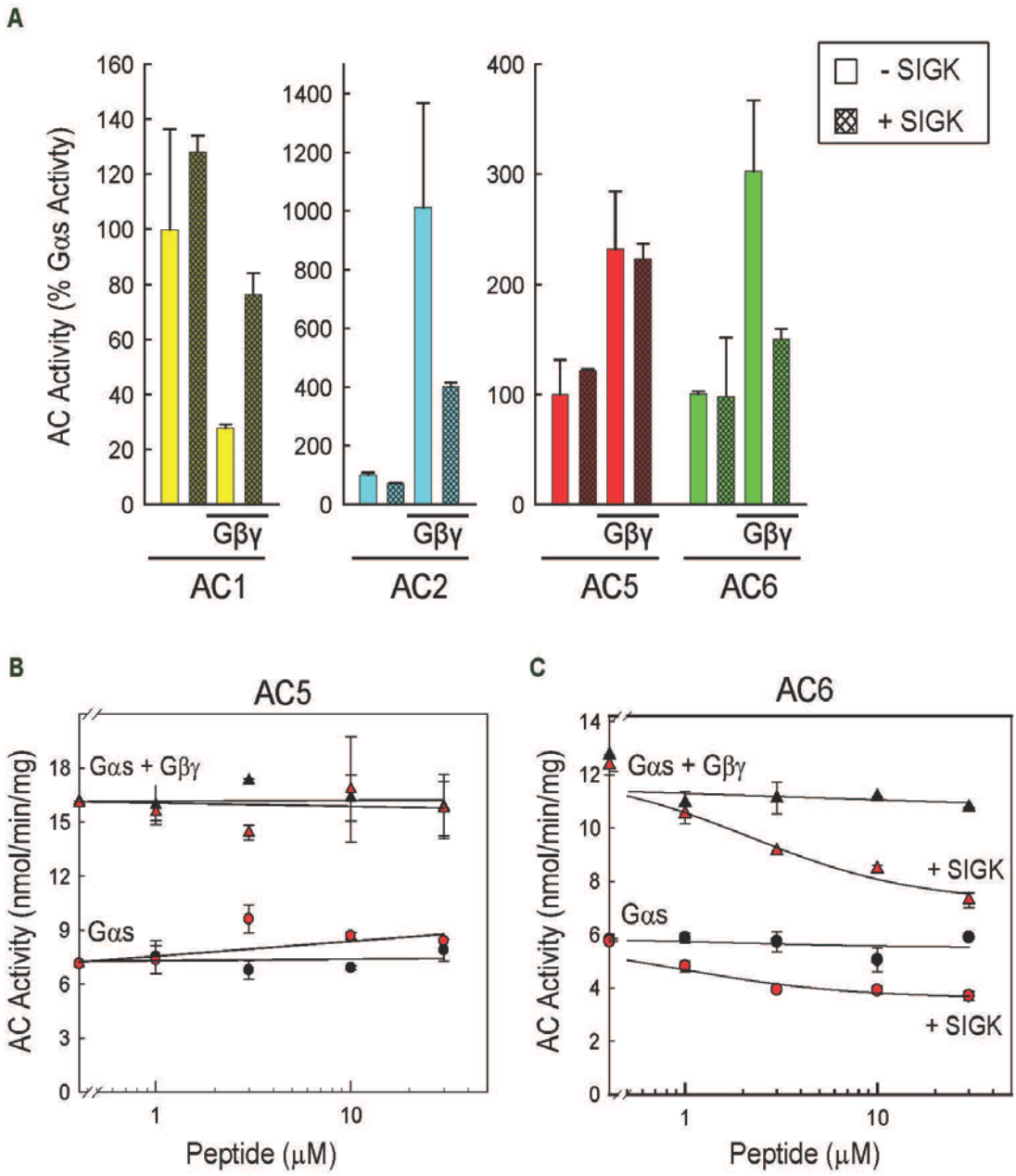

Figure 12: SIGK Peptide Blocks Gβγ Regulation of AC in an Isoform-Specific Manner. A, Sf9 membranes expressing the indicated AC isoform were stimulated with 50 nM Gαs +/- Gβγ (50 nM Gβγ for AC1 and AC2; 300 nM for AC5 and AC6). AC assays were in the presence or absence of 10 μM SIGK peptide. **C, D,** SIGK inhibition curves with Sf9 membranes expressing AC5 (**C**) or AC6 (**D**). AC containing Sf9 membranes were stimulated with 50 nM Gαs +/- 300 nM Gβγ and the indicated concentrations of SIGK peptide (red) or a control peptide (SIGK L9A, black).

4.5 Small Molecule Gβγ Inhibitor Gallein Does Not Block AC Activity

In addition to hotspot Gβγ inhibition via use of the SIGK peptide, novel small molecule inhibitors of Gβγ has been developed with varying selectivities. A screen for small molecule inhibitors of Gβγ that could compete with SIGK for the Gβγ hotspot resulted in a diverse set of nine candidates, with high nanomolar to micromolar IC50's for blocking Gβγ regulatory actions (207). One of these candidates was gallein. Gallein has been shown to inhibit PLCβ2/3, PI3Kγ, GRK2, and the GEF pREX1, affecting phenotypes including prevention of heart failure, roles in prostate cancer metastasis, and neutrophil movement (207-212). Although that is still a fairly nonselective range of effects, gallein does have selectivity in that it is unable to affect Gβγ-mediated regulation of ERK1/2, N-type calcium channels, and GIRK channels, according to a combination of published work (207) and unpublished observations that have been noted in published reviews (213). This inhibition of Gβγ by gallein appears to occur via binding in a reversible, noncovalent competitive mechanism at the Gβγ hotspot (214).

We attempted to test the specificity of gallein for AC isoforms tested against SIGK. Based on our results, gallein had no effect on stimulation of AC2, AC5, or AC6 by Gβγ (Figure 13). The selectivity between AC isoforms shown by the SIGK peptide was not replicated by the more specific Gβγ inhibitor gallein; thus, differences in the role of the Gβγ hotspot in regulation of AC isoforms are more subtle than differences in Gβγ hotspot mediated regulation between different effectors.

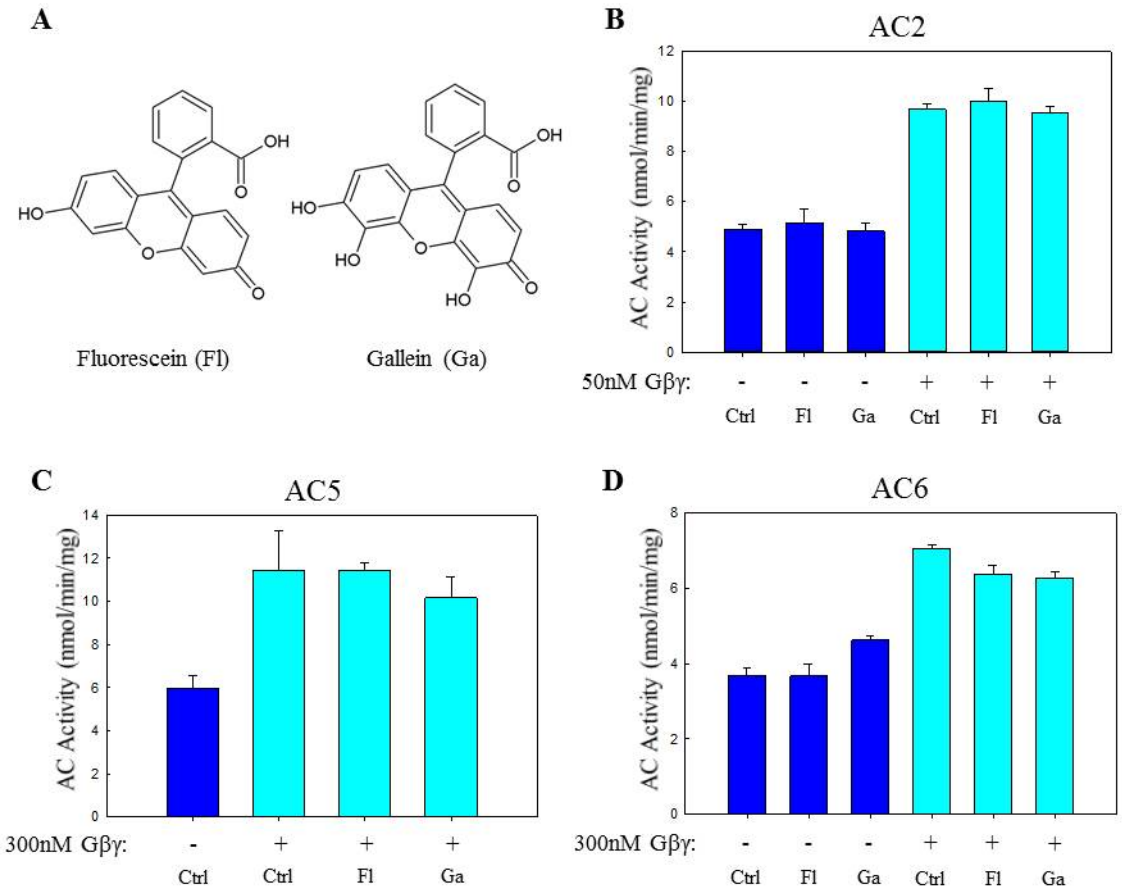

Figure 13: Gβγ Hotspot Inhibitor Gallein Does Not Block Gβγ Stimulation of AC. A, Chemical structures of the small molecule Gβγ inhibitor gallein (Ga), and the small molecule control fluorescein (Fl). **B,** Sf9 membranes expressing AC2 were stimulated with 50 nM Gαs +/- 50 nM Gβγ in the presence of gallein (Ga), the hotspot non-interacting small molecule analog fluorescin (Fl), or a DMSO control (Ctrl). **C,** Sf9 membranes expressing AC5 were stimulated with 50 nM Gαs +/- 300 nM Gβγ. AC5 stimulated with Gβγ was performed in the presence of gallein (Ga), the hotspot non-interacting small molecule analog fluorescin (Fl), or a DMSO control (Ctrl). **D,** Sf9 membranes expressing AC6 were stimulated with 50 nM Gαs +/- 300 nM Gβγ in the presence of gallein (Ga), the hotspot non-interacting small molecule analog fluorescin (Fl), or a DMSO control (Ctrl).

4.6 Gβγ Binds to AC5/6 Catalytic Domains

Although multiple Gβγ binding sites on AC5 were expected, only the AC5NT heterotrimer scaffolding site had previously been identified. We assumed based upon homology with AC6NT that a second site necessary for activation was likely present in AC5NT. However, due to expression issues in *E. coli*, we have never been able to test this region directly (aa residues 195-238). Gβγ interacts with multiple sites in AC2 to promote conditional stimulation, including the C1 and C2 catalytic domains (36, 54, 55). Therefore, we used several strategies to determine if Gβγ interacted with the C1/C2 domains of AC5 and AC6. Streptavidin pulldowns of biotin-tagged purified Gβγ showed strong interactions with the H_6-tagged C1 domain of AC5 that was independent of the W99A mutation (Figure 14A). However, due to nonspecific binding issues, this method could not be used to determine if AC5-C2 bound to Gβγ. To overcome this limitation, we performed a Gβγ binding overlay assay where the C1 and C2 domains were separated by SDS-PAGE, renatured, and incubated with WT or W99A purified Gβγ (Figure 14B, 14C). The detection of AC5-C1 interactions was weak but did not reproducibly depend on mutation of W99, similar to what was observed by streptavidin pulldowns. A much stronger interaction was observed with AC5-C2 using this assay, likely due to its greater capacity for renaturation (data not shown). Thus, Gβγ is capable of interacting with all three cytoplasmic domains of AC5. Compared to WT Gβγ, the W99A hotspot mutant showed a 2-3 fold decrease in binding affinity for the 5C2 domain (Figure 14C). Similarly, Gβγ also bound to the AC6 C1 and C2 domains (Figure 14D). The W99A-Gβγ mutant displayed impaired interactions with both AC6-C1 and AC6-C2, with an approximately 5-6 fold decrease in binding affinity for AC6-C2 compared to WT (Figure 14E). Thus, Gβγ

binds to the C1/C2 catalytic domains of both AC5 and AC6, with a high dependency on the hotspot for interactions with the C2 domain.

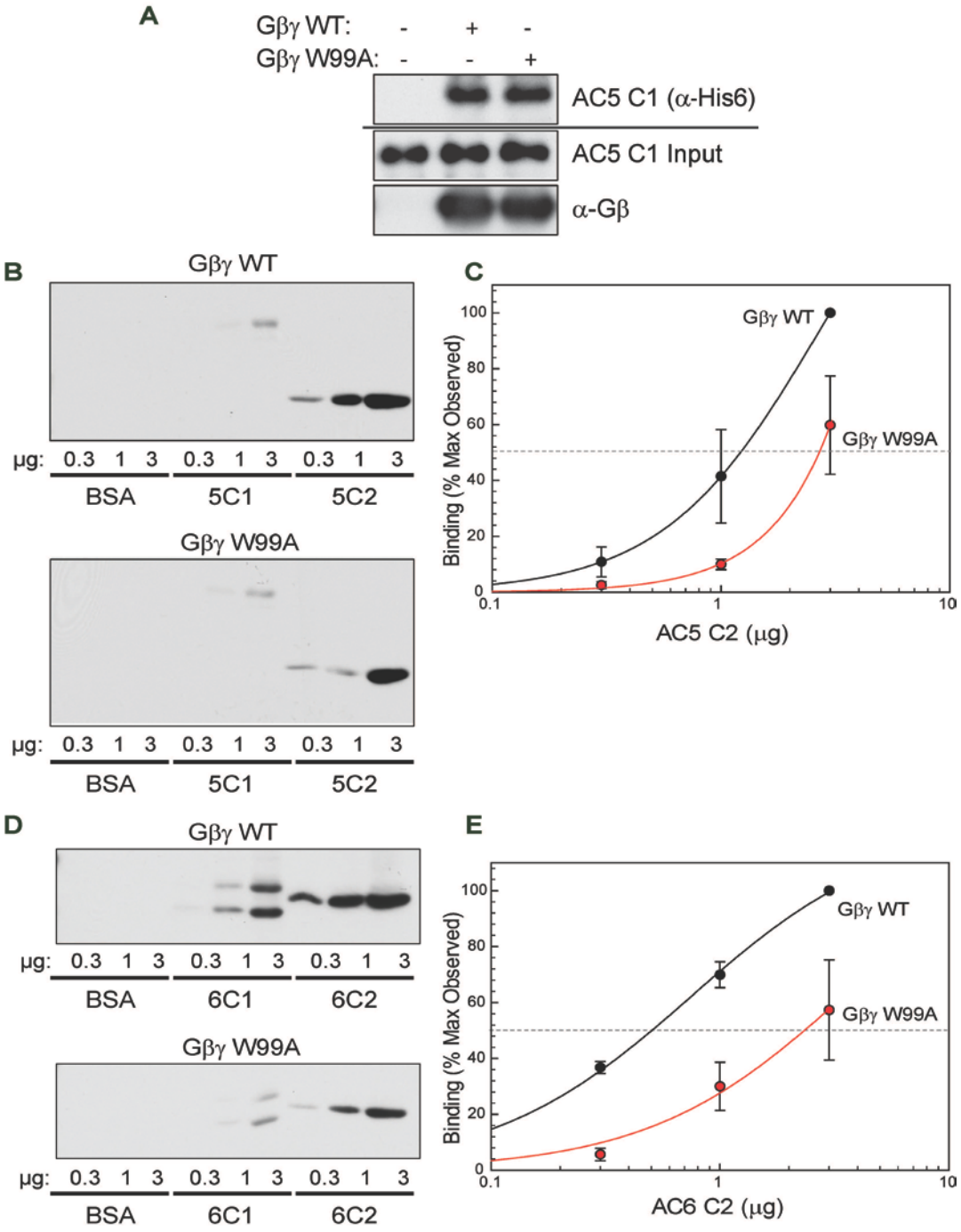

Figure 14: Gβγ Interacts with AC5/6 Catalytic Domains. A, Streptavidin pulldown of 300 nM biotin-tagged Gβγ wildtype or mutant incubated with 500 nM His-tagged AC5-C1. Pulldowns analyzed by SDS-PAGE and Western blot. **B and D,** Far-Western Gβγ assay using AC5-C1 and AC6-C2 **(B)** or AC6-C1 and AC6-C2 **(D)** immobilized on PVDF membrane and probed with Gβγ WT or W99A. **C and E,** Quantification of relative WT or W99A Gβγ binding to AC5-C2 **(C)** or AC6-C2 **(E)** domains.

4.7 AC5-Gβγ Interactions Depend on Multiple Sites within Gβγ.

Previously, Yuan *et al*. have shown that Gβγ can contact PLCβ using the NT of Gβγ (residues 23-27) when the hotspot is otherwise occupied, allowing Gβγ to stimulate PLCβ when simultaneously bound by a Gα subunit as well as a receptor-independent activator of G protein signaling (AGS8) (204). Therefore we wanted to determine if Gβγ uses multiple interaction surfaces to regulate AC5 and/or AC6. Mutation of residues 23-27 of the Gβ NT greatly impaired the ability of Gβγ to stimulate AC activity, with only marginal effects on binding to the AC5/6 NT (Figure 15). Thus, multiple regions of Gβγ are required for regulation of AC5/6 activity.

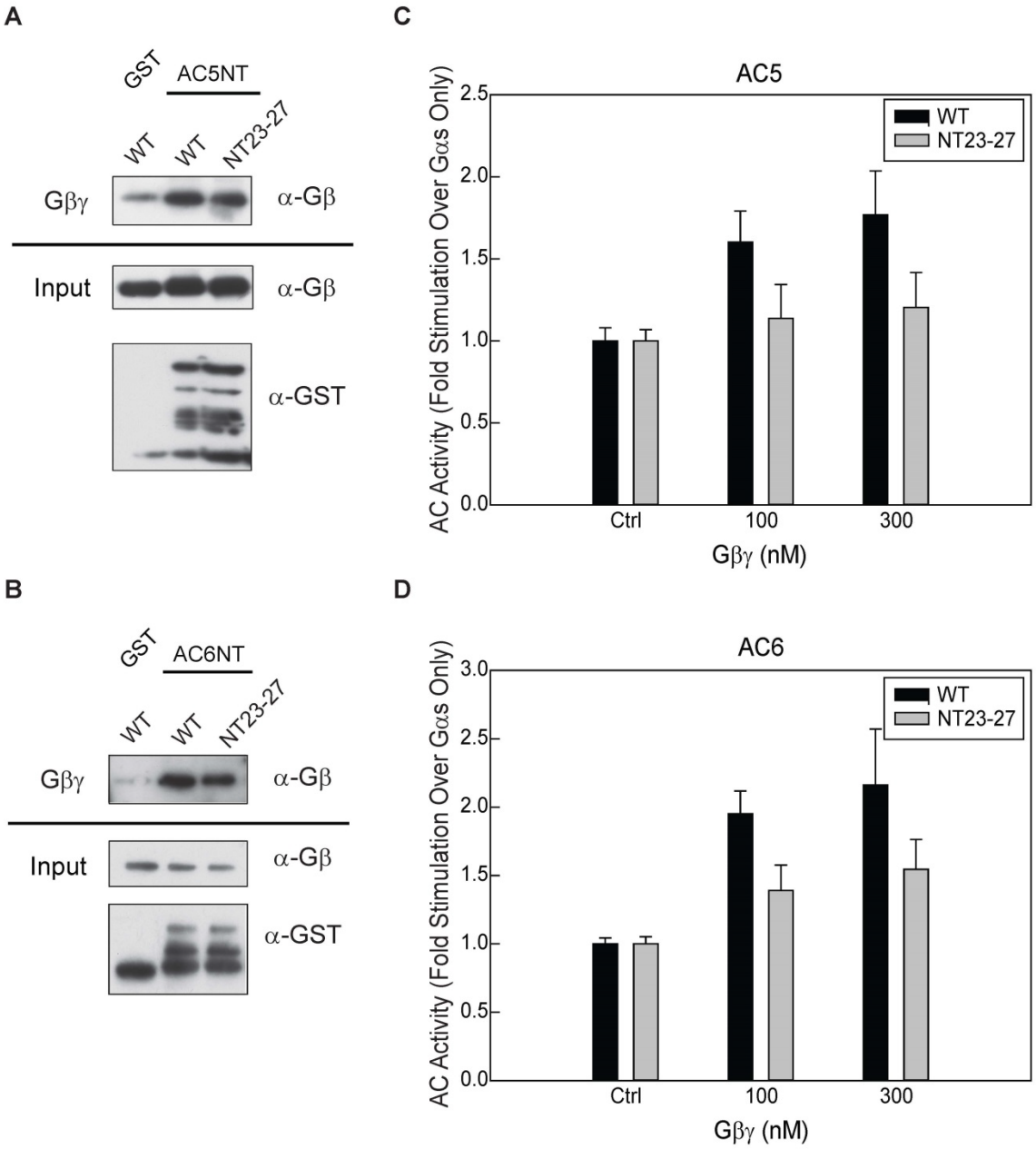

Figure 15: Mutation of Gβ NT Inhibits Stimulation of AC5/6, but Not Binding to AC5/6 NT. A and B, Pulldown of GST-tagged AC5NT **(A)** or AC6NT **(B)** with purified Gβγ wildtype or Gβ(NT23-27)γ mutant. Final concentrations were 2 μM GST-ACNT and 300 nM Gβγ. Elutes from GST pulldowns were analyzed by Western blotting. **C,D,** AC activity assay with Sf9 membranes expressing AC5 (C) or AC6 (D) were stimulated with 50 nM Gαs and indicated concentration of Gβγ.

Endogenous Gαs and Gβγ are readily detectable in immunoprecipitations of Flag-tagged AC5 from HEK293 cells (95). To determine which surface(s) of Gβγ are required, we analyzed the interactions of Flag-tagged AC5 with YN-tagged Gβ (YN-Gβγ) to differentiate mutants from endogenous wild-type Gβγ. YN-Gβγ was expressed significantly less than endogenous levels, but could clearly be pulled down upon immunoprecipitation of AC5. This interaction was not significantly reduced with Gβγ-W99A or Gβγ-NT23-27, but both mutations were required to observe a significant reduction in AC5 association (Figure 16). Similar to *in vitro* AC activity assays, both the hotspot and NT surfaces of Gβγ were required to interact with AC5 in cells. Therefore, Gβγ regulation of AC5 is not a simple two-site mechanism, but rather utilizes multiple surfaces of Gβγ and AC5 for scaffolding and enzyme stimulation.

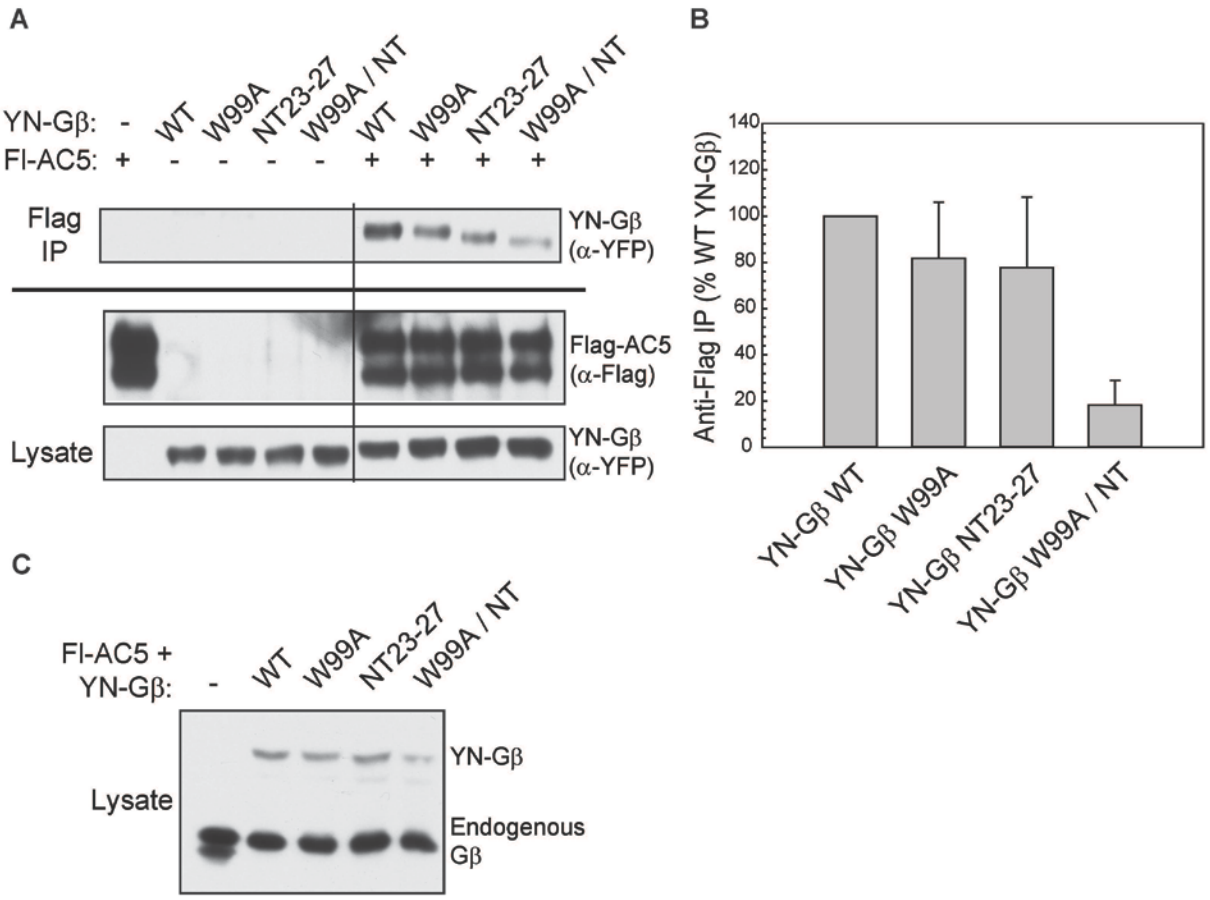

Figure 16: Multiple Sites on Gβγ Are Involved in Binding to AC5 in HEK293 Cells. A, Immunoprecipitation of Flag-tagged AC5 and YN-Gβγ wildtype or mutants. HEK293 cells were transfected with the indicated plasmids, lysed, and subjected to an immunoprecipitation using anti-Flag agarose resin. Associated proteins were analyzed by Western blotting. **B,** Quantification of Flag-AC5/YN-Gβγ IP-Westerns. **C,** Lysate samples from immunoprecipitation of Flag-tagged AC5 and YN-Gβγ wildtype or mutants, with analysis by Western blotting to compare expression of YN-Gβγ to endogenous Gβγ.

4.8 Summary

We have explored Gβγ interactions and regulation of AC isoforms, particularly the differences in scaffolding versus stimulation of AC5. The Gβγ "hotspot" is necessary for AC5 stimulation but not solely required for interaction at the AC5NT. This contrasts with AC6, where the Gβγ hotspot is required for all known AC6 interaction sites. Furthermore, Gβγ bound to a wide variety of AC NTs; hotspot dependent interactions with AC NT varied with the isoform of AC. Gβγ hotspot competition by the peptide SIGK blocked Gβγ regulation of various differentially regulated AC isoforms, but surprisingly did not affect Gβγ regulation of AC5. We also determined that the very selective small molecule Gβγ inhibitor gallein was unable to affect Gβγ stimulation of AC2/5/6. Thus, we were restricted to the lessons learned from SIGK competition for Gβγ's hotspot; namely, that AC5 appears to have a unique characteristic in how it is regulated by Gβγ.

For both AC5 and AC6, Gβγ interacts with all three intracellular domains of AC; NT, C1 and C2. The large numbers of interaction sites on AC5/6 mirrors the multiple points of contact on Gβγ, as Gβγ NT mutants much like their hotspot counterparts were unable to fully stimulate AC5/6. This emphasizes the multiple domains on both AC and Gβγ involved in interactions for the stimulatory effect observed, a phenomenon supported by observing AC5/Gβγ binding in cells via immunoprecipitation when single surfaces of Gβγ were mutated, but not when both the hotspot and NT of Gβγ were disrupted. The AC5 and AC6 interactions with Gβγ are summarized in Table 4. Overall, the mechanistic difference of AC5 is that it has an NT binding site for Gβγ not directly involved in stimulation, a property not shared by other AC isoforms.

Table 4: Results Summary of AC5/6 Interactions with Gβγ

	AC5				AC6			
Gβγ	**5NT Binding**	**5C1 Binding**	**5C2 Binding**	**AC5 Stim.**	**6NT Binding**	**6C1 Binding**	**6C2 Binding**	**AC6 Stim.**
WT	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**
NT 23-27	**Yes**			**No**	**Yes**			**No**
W99A	**Yes**	**Yes**	**Dec.**	**No**	**No**	**Dec.**	**Dec.**	**No**
M101A	**Yes**			**Dec.**	**No**			**Dec.**
D186A	**Yes**			**No**	**No**			**No**
N230A	**Yes**			**No**	**No**			**No**
H311A	**Inc.**			**Yes**	**Inc.**			**Yes**

Yes = binding or stimulation (Stim.) observed. No = binding or stimulation not observed. Inc. = binding or stimulation observed, higher than wildtype (WT). Dec. = binding or stimulation observed was significantly lower than that of wildtype.

Chapter 5

Discussion

5.1 Summary of Conclusions

Based on inhibition curves against all 9 AC isoforms, alleged AC5 inhibitors SQ22,536 and Ara-A are actually AC5/6 inhibitors. Thus, their use in studying AC5 specific physiological roles is questionable, particularly in the terms of congestive heart failure where AC6 inhibition in cardiomyocytes may have effects opposite to that of AC5 inhibition. Also, allegedly isoform selective AC inhibitors such as NB001 may inhibit cAMP signaling in an isoform specific manner in cells without directly targeting the AC itself. These results showed the need to complement cell based novel drug screens with precise *in vitro* characterizations of inhibitor candidates. Building upon this concept, a novel AC2 inhibitor from a cell-based inhibitor screen was characterized *in vitro*, showing direct effects with greater inhibition of AC2 compared to AC1/5, and a noncompetitive inhibitory mechanism.

In tandem with study of pharmacological modulators of AC and their isoform specificity, I further explored isoform specific aspects of AC regulation by the physiological regulator Gβγ. Gβγ binds to a wide variety of AC isoforms, displaying isoform-specific variation in the requirement of the Gβγ "hotspot" region in mediating these interactions. In addition, whereas hotspot mutants of Gβγ demonstrated the necessity of the hotspot for AC5 stimulation but not for interactions with the 1-195 aa of AC5 NT, the hotspot was required for both AC6 NT binding and stimulation of AC6. Thus, G protein heterotrimeric binding to the

AC5 NT does not appear to require the Gβγ hotspot directly, consistent with its coverage in interactions with Gαs-GDP. Furthermore, this lack of hotspot necessity is specific to the AC5 NT at this time. The ability of the SIGK peptide to block Gβγ regulation of AC1/2/6 but not AC5 supports a unique AC5 regulatory property. Gβγ NT mutants are unable to stimulate AC5/6 *in vitro*. AC5 and Gβγ were immunoprecipitated from cells even if the Gβγ hotspot or NT were mutated, but not if both Gβγ surfaces were mutated. These suggest multiple interaction sites on both AC isoforms and the Gβγ regulator. In addition to the AC5/6 NT, Gβγ binds to the C1/C2 domains of both AC5 and AC6 in what appears to be a hotspot dependent manner, consistent with Gβγ stimulating both of these isoforms via catalytic domain interactions. As such, Gβγ stimulates AC5 and AC6 similarly in hotspot-dependent regulatory interactions, and the AC5 NT appears to be isoform specific in its separate, hotspot-independent binding of inactive Gβγ.

5.2 Pharmacological Selectivity of AC5/6

Results indicate that although SQ22,536 and Ara-A inhibit AC by binding at the catalytic site as has been proposed, their inhibition is AC5/6 selective. The lack of selectivity for SQ22,536 and Ara-A between AC5 and AC6 is consistent with recent developments on the effects of AC inhibitors on cardiac knockout models. Studies have proposed AC5-specific roles in chronic heart failure development and in control of heart rate during parabolic flight using Ara-A (189, 215). However, in other recent studies, inhibition of cardiac AC activity by SQ22,536 and Ara-A is the same in wild-type and AC5 knockout animals (216). Additionally, AC5KO animals had lower basal AC activity levels compared to wildtypes, but no change in stimulated AC activity levels. This was despite no changes in mRNA expression of the cardiac

AC isoforms. As AC5/6 are the predominant AC isoforms in cardiomyocytes, it appears that upregulated AC6 activity and wildtype AC5/6 activity were similarly inhibited by Ara-A.

Similar issues have occurred previously using these inhibitors to identify AC5-specific function. Based on work using pharmacological inhibition by SQ22,536, the authors proposed AC5 was primarily responsible for cAMP signaling involved in renin secretion in kidney cells (217). Later knockout models clarified both AC5 and AC6 were involved in renin secretion (218), and additional cAMP signaling roles in the kidney have been specific to AC3 and AC6 rather than AC5 (219, 220). Our work sheds additional light on the weaknesses of poorly characterized inhibitors to ascribe physiological roles to specific AC isoforms.

Additional off-target effects are also of concern. Ara-A is clinically used under the name vidarabine as an antiviral that inhibits DNA synthesis and AMP activated protein kinase α-2 (188, 221). While small molecule drugs can be clinically repurposed, there remains the concern of off-target effects. Ara-A is an adenosine-like molecule, and the ATP binding sites of DNA polymerases and AC are not highly divergent. It is notable that clinical data has resulted in vidarabine's current use being restricted to a limited dose range, and only for treatment of viral eye infections. Similarly, SQ22,536 displays AC-independent effects on neurogenesis and superoxide production (222, 223). As many ligand-based AC inhibitor screens have been at the AC catalytic site, small molecule inhibitors have both been derived from and themselves resemble ATP-like compounds. Information on whether known AC inhibitors have any reactivity with kinase inhibitors competitive for ATP is also lacking. The many ATP binding sites in the human biochemical milieu make for unpredictable off-target effects.

5.3 Characterizing Isoform Specific AC/cAMP Modulation

The NB001 inhibitor decreases cAMP in an AC1-dependent manner in cells. However, direct inhibition of AC1 *in vitro* was not observed. Such work shows the drawbacks when attempting to characterize drug candidates, particularly AC inhibitors, in cell-based assays. Such assays can identify compounds with therapeutic value; irrespective of its actual target, NB001 does decrease behavioral symptoms of neuropathic pain. AC1 is involved in such pain sensation (224, 225), but also in long-term potentiation aspects of memory (226). NB001 also does not have effects on motor function or spatial memory (166), and a similar separation for NB001's inhibition of pain signaling without impairment of anxiety behaviors was observed in models of pain induced by irritable bowel syndrome (166, 227). Such behavioral studies are consistent with an indirect effect beyond inhibiting AC1 activity. Recent use of NB001 has explored allegedly AC1-specific function in long term potentiation in the development of chronic pain (228). Thus, it is important to suggest a mechanism or alternative target for NB001's AC1-dependent effects in cells that cannot be replicated *in vitro*.

AC1's role in neuropathic pain is thought to be through a cAMP increase caused by calmodulin stimulation of AC1. NB001 inhibited calmodulin-stimulated AC1 activity in cells but not that of AC8, which is also stimulated by calmodulin (166). Since we could not replicate this AC1 inhibition in cell-free assays, it is worth noting other potential NB001 targets within cells. Another kinase activated by calmodulin, CaMKIV, is capable of selectively inhibiting AC1 but not AC8 activity in neurons (229). Thus, NB001 stimulating CaMKIV, and subsequent AC1 inhibition by CaMKIV is a possible link between an AC1-dependent decrease in cAMP and decreased neuropathic pain upon treatment with NB001. Alternatively, the ATP-like structure of NB001 may be metabolized into an AC1-specific inhibitor product. Such a

modification to “activate” NB001 in cells would explain NB001 inhibition of AC1 *in vivo* but not the *in vitro* membrane assay setting, where such a modifying enzyme was not present.

Examples such as NB001 highlight the due diligence needed to characterize hits from high throughput screens, particularly when such screens are ligand-based. I characterized SKF-83566 and, to a lesser extent, tranilast after another group identified them as cellular AC2 inhibitors using a cell-based stimulatory screen, showing that unlike NB001 these hits were able to directly inhibit AC2 activity. The characterizations of SKF-83566 demonstrate how to use *in vitro* characterization of AC inhibitor candidates as a supplemental selectivity screen in combination with a high throughput virtual or cell-based screen.

The differences between *in vitro* and *in vivo* AC signaling relate to physiological regulator effects and functions as well. For AC5/6 and Gβγ regulation itself, initial cell-based characterizations were used to determine if the effect was stimulatory or inhibitory, with misleading results. Cellular transfection of Gβγ subunits with either AC5 or AC6 suggested inhibition of these isoforms (230). However, in hindsight this work’s overexpression of Gβγ could regulate any number of targets eventually resulting in inhibition of AC5/6; opening up the cell cytoplasm to free calcium ions or activating inhibitory PKC isoforms via PLCβ are two potential ways this could happen. Later characterizations of AC5/6 regulation by Gβγ either *in vitro* or in cells with endogenous Gβγ clarified the direct conditional stimulation of Gβγ regulation on AC5/6 (93).

5.4 AC5/Gβγ Specificity of Mechanism

AC5 has a unique interaction site with Gβγ at the AC5 NT. This site, not required for regulatory actions, appears to be a scaffolding site for Gαs/Gβγ heterotrimer, while Gβγ is also

capable of interacting with the AC catalytic domains for stimulatory effects. Gβγ differential effects on AC isoforms already included stimulation of AC 2/4/5/6/7 and inhibition of AC 1/3/8. Compared to the other AC isoforms, AC5/6 are very closely related, but do have differences in their regulation by heterotrimeric G protein subunits. Specifically from this work, the observed difference is that the AC6 NT both requires the Gβγ hotspot for binding interactions and is necessary for AC6 stimulation by Gβγ, whereas the AC5 NT neither requires the Gβγ hotspot for binding nor is necessary for AC5 to be stimulated by Gβγ. AC5 and AC6 also have differences in their Gα regulatory properties. Gαi inhibits Gas-stimulated AC5 and AC6, while only the basal activity of AC5 is Gαi-inhibited. In addition, Gαs stimulation curves of human AC5 and AC6 suggested cooperative stimulation of human AC5 with a Hill coefficient of 1.4, but negligible cooperation in Gαs stimulation of human AC6 (23). It appears that AC5 has unique regulatory properties via both Gα and Gβγ subunits.

It is worth mentioning that the AC5 NT used in our *in vitro* GST-pulldown binding assays may be missing a binding site analogous to that on the AC6 NT; the purified GST-5NT domain consists of aa 1-195 of AC5 (Figure 9A). Some structure prediction programs propose that AC5 does not begin its first transmembrane spanning domain until after aa residue 238. A region of AC6 NT 77-151, which has been mapped as required for Gβγ stimulation of AC6, shares homology with aa 200-238 of AC5. That region of AC5 may be a distal site on the AC5 NT where Gβγ both binds in a hotspot-dependent manner and is utilized for Gβγ stimulation of AC5. This could also potentially explain why purified C1/C2 domains of AC5, even with AC5 NT with aa 1-195 present, is unable to be stimulated by Gβγ (95).

At first glance, lack of SIGK competition indicates unique AC5 regulation by Gβγ. AC5 is not the only effector target of Gβγ with hotspot interactions that SIGK competition cannot

disrupt; voltage-gated calcium channel regulation by Gβγ is also unaffected by SIGK (206). However, it is unclear precisely why SIGK is unable to block Gβγ stimulation of AC5, but able to disrupt regulation of other AC isoforms. According to the model of heterotrimeric G protein "breathing", heterotrimeric interactions exist between Gβγ and both the Gα NT as well as the Gα Switch 2 helix. This allows a certain degree of heterotrimer flexibility, and has been supported by significantly stronger binding between the G protein subunits when both interactions are combined relative to when either Gα region is mutated (231, 232). It is possible that AC5/Gβγ interactions are less "breathable", decreasing the ability for SIGK to compete for the hotspot. The SIGK peptide interacts with residues on 6 out of 7 blades of the WD40 barrel-like structure at the hotspot (207), but AC5 interactions with other areas such as Gβ NT residues 23-27 may help to overcome competition by SIGK. Gβγ residues 23-27 are utilized for regulation of PLCβ (178), and for interaction with AGS8 under an alternative signaling mechanism when the hotspot is otherwise occupied (204). Such an alternative signaling mechanism could also explain AC5-specific utilization of Gβ NT 23-27 when mutation of this area affected Gβγ stimulation of both AC5 and AC6. PI3K appears to interact with the Gβ NT at residues 31-45, another unexplored region for AC/Gβγ interactions (233). AC5 may also interact more strongly with hotspot residues on blade 6 of Gβγ, a region that does not contact SIGK. None of the alanine-substitution hotspot mutants of Gβγ used in the results shown here are located on blade 6 as well.

Since only the NT of AC5/6 differ in their dependency on the Gβγ hotspot for interactions, it is possible that loss of SIGK efficacy is a 5NT mediated effect. However, we show that AC5 and AC6 have multiple interaction sites with Gβγ. This is analogous to AC2, the AC isoform most thoroughly mapped for Gβγ binding. In addition to our observed binding

of Gβγ to AC2NT, binding sites have been mapped to the C1 domain and C2 domains of AC2 (36, 52, 54, 55). These identified sites are summarized in Figure 17. AC5/6 show 65% homology to the site located within the C1a domain of AC2 (aa 339-360), while sites located within the C1b and C2 domains of AC2 are poorly conserved or not present in AC5/6 (55). The AC2 aa 493-514 sequence is significant in that it is a so-called PFAHL motif that was required for stimulation of AC2 by Gβγ; it shows only 26% homology with AC5, but is part of the very flexible C1b region of the C1 domain. Although the AC2 NT requirement for stimulation of AC2 by Gβγ is not ruled out, this supports stimulation by Gβγ of AC requiring C1/C2 catalytic domain modulations. Similar to AC5, AC2 has a so-called QEHA motif which is a binding site for Gβγ that was not required for stimulation of AC2 by Gβγ. However, unlike the AC5 NT heterotrimer binding site, the AC2 QEHA motif is located on the C2 domain. Therefore, even if other AC isoforms can scaffold inactive G proteins like AC5 can at its NT, the location of that anchoring may be isoform specific.

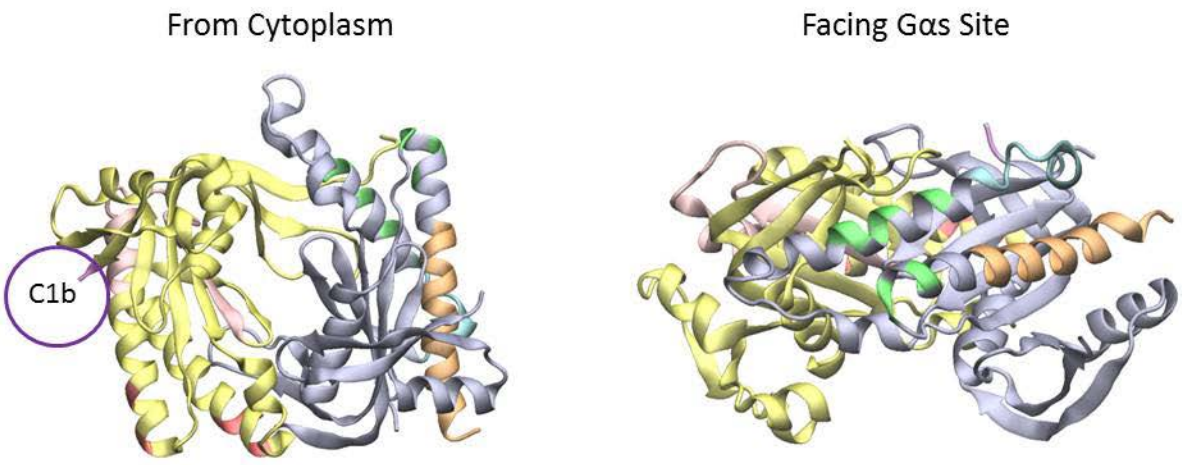

Figure 17: Summary of Identified Gβγ Binding Sites on AC2. C1 domain is yellow, C2 domain is silver. C1a interacting sequence is pink. C2a KF loop is cyan. QEHA domain is orange. AC5 Gαi binding site is red, Gαs binding site is green. C1b, where PFAHL motif is located, is disorded and its general location is indicated by the purple circle. Note that the areas conserved between AC5 and AC2, namely C1b and C2a regions such as the KF loop (QEHA domain is not conserved), are either too dynamic for crystallization or near where the plasma membrane would be.

Examining the C1/C2 crystal structure, it appears that there are Gβγ binding sites for AC5 on opposite ends of the catalytic shell. AC5 has the longest NT by amino acid sequence of all the isoforms, but even ~200 amino acids is not sufficiently long to realistically wrap around the entire C1/C2 catalytic core. It has been shown previously in cells that AC5 and AC2 are able to form functional AC heterodimers (234). This AC5/AC2 heterodimer was also more sensitive to stimulation by Gαs or forskolin, possibly suggesting enhanced Gβγ mediated potentiation of AC5/2 signaling. Furthermore, previously observed cooperative stimulation of AC5 by Gαs can be explained by the presence of AC5 dimers (23). In such a situation, it is unclear physiologically whether AC5 would homodimerize or produce heterodimers such as

AC5/AC2, since both are capable of stimulation by both Gαs and Gβγ. In addition, AC6 fragments have been able to immunoprecipitate AC1, AC2, or AC5 from cells, suggesting the possibility of AC5/6 heterodimers (235). In such cases, since AC2/5/6 are all stimulated by Gβγ to some degree, AC5 NT scaffolding inactive Gαs/Gβγ heterotrimer could facilitate Gβγ stimulation of a AC heterodimer through spatial proximity of Gβγ to the C1/C2 catalytic core, with active Gβγ stimulating AC through a combination of interactions with the C1 domain of one AC isoform and the C2 domain of a second isoform. In summary, the proposed mechanism of Gβγ regulation of AC5 is inactive Gαs/βγ heterotrimer is scaffolded to AC5 NT aa 66-137, and following activation the G protein subunits interact with the AC5 catalytic domains to enhance catalytic activity (Figure 18). For both AC5 and AC6, stimulation by Gβγ may involve interaction with distal NT sites, and/or close vicinity to AC catalytic domain regions such as C1b and C2a that have sections near the plasma membrane.

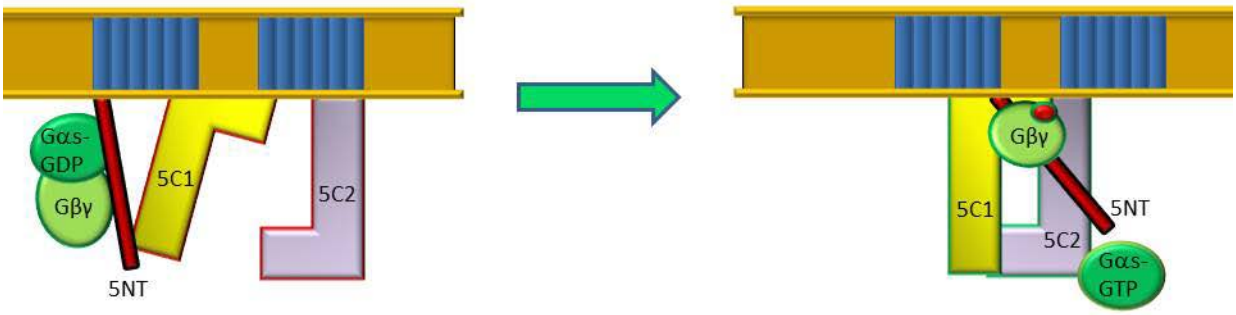

Figure 18: Proposed Mechanism for AC5 NT Scaffolding and AC5 Stimulation by Gβγ. Inactive G protein heterotrimer is scaffolded to the AC5 NT (left). After G protein activation, Gαs stimulates AC5 at the C2 domain, while Gβγ stimulates AC5 at the plasma membrane by interacting with the 5C1/5C2 domains in a hotspot-dependent manner, and possibly interaction with a distal 5NT site (right).

Similarly, the distal C-terminus of GIRK1 also anchors Gβγ, but is not required for Gβγ regulation of GIRK1; it was proposed that such anchoring is for localized recruitment of Gβγ (69). As another example, the scaffolding protein WDR26 binds both Gβγ and PLCβ2, mediating Gβγ regulation by recruitment of PLCβ2 from the cytosol to the plasma membrane (236). Although currently such an anchoring function in AC appears to be specific to the AC5 isoform, other AC isoforms may anchor Gβγ independent of any regulatory role. Gβγ binds to AC9NT in a hotspot-dependent manner in our pulldown binding assays, even though Gβγ may lack a direct regulatory effect on human AC9 (25, 237).

5.5 Proposed Function of AC5 NT Scaffolding

The function of an AC NT on cAMP producing activity could be to 1) localize regulators such as Gβγ nearby the C1/C2 domains, or 2) through direct interaction of the NT with the C1/C2 domains. Further AC isoform specificity is brought by indirect AC regulator function in a way besides stimulation or inhibition of cAMP production. For example, heterotrimeric G proteins bound to the AC5 NT may undergo effects similar to those observed for PKC anchored to AKAP79. When bound to AKAP79, PKC's regulatory profile is modified (238). Not surprisingly, an AKAP anchoring PKA and a downstream target significantly enhanced the likelihood of that target's phosphorylation by PKA (239). In terms of AC5/6, this implies a potential AKAP-scaffolded effect on feedback inhibition by PKA. Localized ion channels such as Na+/H+ exchanger 1 can shield AC from changes in pH; thus, another interpretation is that the AC5 NT protects G protein heterotrimers from undesired stimuli (240). Gβγ has a wide variety of physiological targets beyond AC; anchoring to the AC5 NT could also restrict heterotrimeric G protein regulation to AC and local pool of targets. That cardiac

overexpression of Gαs results in AC-independent activation of L-type calcium channels supports this idea (241). Similarly, AC8 NT aids in enhanced actin based signaling at the plasma membrane that is restricted to AC8 movement in cholesterol-enriched domains (242).

In cells, both heterotrimeric G protein subunits were needed to see full AC6 stimulation by the Gαs-coupled GPCR agonist isoproterenol (93). Since AC5 and AC6 are both stimulated by Gβγ, but only AC5 has a heterotrimer scaffolding site, our proposed AC5 scaffold-primed signaling adds another dimension to the composition of isoform-specific signaling complexes. It is of interest to consider whether a localized cAMP signaling pathway involves AC5, which could self-scaffold a Gαs heterotrimer, or AC6, which may rely on a scaffolding protein to provide heterotrimeric G protein. Supporting this idea, through a Gβγ dependent process, EP1 receptors are able to induce D1 receptor signaling stimulating AC7; it was shown that disruption of such complexes produced separate signals that stimulated AC6 activity (243).

Complexes with β2-AR have been identified that include heterotrimeric G protein, AC, PKA and an opposing PP2A phosphatase, and Cav1.2 L-type calcium channel to be targeted by PKA (244). AKAP79/150 interacts with upstream β-AR receptors, as well as AC5/6 at their NT (103, 104). Fully functional AC2/AKAP79 signaling complexes are involved in signaling produced by the hormone relaxin (245). Similarly, a larger complex facilitated by AKAP79/150 may contain all necessary components for signaling from a Gαs-coupled GPCR to PKA and downstream effectors via AC5 generation of cAMP (Figure 19). AC5 would provide Gαs heterotrimer to AKAP79/150 complexes, which are capable of β-AR/AKAP79/AC5 mediated blockade of TRPV1 channel desensitization in HEK293 cells and PGE2/AKAP150/AC5 mediated blockade of TRPV1 channel desensitization in mouse dorsal root ganglia (108).

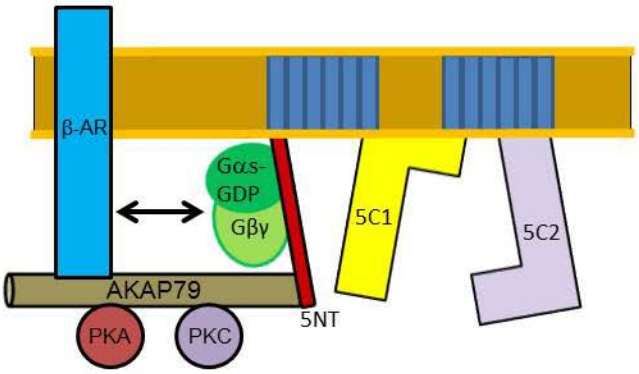

Figure 19: Potential Role for AC5NT/Gβγ Scaffolding in Signaling from AC5 Complexes. Inactive G protein heterotrimer may scaffold to the AC5 NT as part of larger GPCR/G Protein/AC5/cAMP signaling complexes, such as those formed by AKAP79.

5.6 Broader Potential Roles for AC Scaffolded Complexes

It is possible that G protein anchoring sites such as the AC5 NT are important for transmission of isoform specific trafficking information for signaling complexes including AC5 and heterotrimeric G proteins. The idea of localized signaling complexes involving AC for cAMP production has been proposed for some time (202). A combination of immunoprecipitations and BRET studies showed a complex between β2-AR receptors, AC2, and heterotrimeric G protein subunits that was stable in the absence or presence or receptor stimulation, and that was maintained before transportation to the plasma membrane, indicating that AC/G protein complexes are preformed (246-248). Specifically, AC2 and GPCRs initially formed into a complex at the ER, followed by the associated G protein heterotrimer. Furthermore, isoprenylation of the γ subunit of Gβγ is necessary for its plasma membrane

targeting, but heterotrimer formation with a Gα subunit is also required (249). Thus, the appropriate G protein heterotrimer may be needed for correct localization of associated GPCRs and ACs.

AC/G protein scaffolded complexes may integrate the formation of AC dimers. As mentioned above, functional AC5/2 heterodimers in cells were more sensitive to stimulation by Gαs or forskolin (234). Complexes consisting of β2-AR, D4DR, heterotrimeric G proteins, and AC2 have been functionally identified in cells via immunoprecipitation and BRET (250). This complex showed increased BRET signal between Gβγ and AC2 from isoproterenol stimulation of β2-AR, but not from dopamine stimulation of D4DR; this is in contrast to dogma that Gβγ mediated stimulation of AC2 comes from Gαi coupled GPCRs like the D4DR (19). Since these are proteins expressed via cell transfection, AC5/AC2 dimers for 5NT scaffolding of Gαs/Gβγ heterotrimer may be required for stimulation of AC2 by Gαs-coupled Gβγ.

5.7 AC5 vs. AC6 Complex Signaling

It is worth noting that while AC5 and AC6 are both highly expressed in cardiomyocytes, they have differing physiological relevance with regards to cardiac stress-induced hypertrophy, handling of induced calcium release, and cardiac repolarization/store reuptake (251). AC5 and AC6 expression in cardiomyocytes also have visible differences in localization (252). Differential PDE4 expression can compartmentalize cAMP signaling, with different cardiovascular cell types having varying PDE4 level changes to produce specific phenotypic effects (253). Such specificity suggests localized AC/PDE pairs to dynamically control cAMP accumulation.

Adrenergic receptors, heterotrimeric G proteins, and AC2/5/6 have all been found in cardiomyocytes at the nuclear membrane with intact cAMP signaling capability; although complex formation was not directly shown, signaling was β-AR isoform specific (254). AC5 but not AC6 associates with mAKAPβ in the heart at the nuclear membrane, providing AC5 specific hypertrophic signaling near T-tubules including AC5-specific feedback inhibition by PKA (113). AC5 self-scaffolding heterotrimeric G protein may be needed in such a complex for proper signaling from adrenergic GPCRs at the T-tubule, as RNAi disruption of mAKAPβ prevented adrenergic-stimulated cardiac hypertrophy (112). Similarly, AC5 but not AC6 knockouts resulted in loss of sympathetic adrenergic signaling, and prevention of chronic heart failure state induced via aortic stress band (128, 129, 141). Pathological hypertrophic signaling in the heart muscle is needed for such a heart failure state. AC5 knockout also results in decreased stress-induced aging of the cardiomyocyte, a phenotype that may be due to an increased Akt (130, 131). AC5 knockouts include disruption of parasympathetic Gαi based signaling as well (128), although Gαi ability to scaffold to the AC5 NT is significantly weak compared to Gαs (95). Notably, Gαi regulation of cardiac AC5 can be receptor independent (255). Although an AC5 specific effect, it is unclear if 5NT scaffolding is involved in Gαi effects on AC5.

Initial development of chronic heart failure is associated with increased GRK activity (another potential Gβγ target), uncoupling of β-adrenergic receptor signal, and notably in the context of this manuscript, decreased AC6 mRNA (256). In contrast to AC5, AC6 knockout increases heart failure mortality in animal models (142, 143), and AC6 expression is actually beneficial in preventing stress-induced development of heart failure (257, 258). Many of these benefits from AC6 expression are replicated using a catalytically inactive mutant (259). Both

AC5 and AC6 have NT binding to AKAP79, in a complex strongly associated with both adrenergic signaling and cardiac calcium handling (103). In the context of our results, AC5 NT specific assembly of adrenergic/AKAP79 signaling complexes is an interesting possibility.

Caveolin versus noncaveolin distribution of AC isoforms also involves signaling complexes with different functions. Isoproterenol or forskolin, but not other Gαs-coupled receptor agonists, stimulate cAMP production in an AC6-dependent manner in cardiomyocytes (260). This selectivity for AC6 may result from AC6 localization in caveolae; transportation of β2-AR out of such caveolae caused decoupling of that receptor (and associated agonists) from AC6 stimulation (260, 261). Nitric oxide inhibition of AC6 in cardiomyocytes has similar dependency on caveolin-based AC6 complexes (262). AC6 or AC8 regulation by the effects of capacitive calcium entry also requires isoform specific complexes in cholesterol rich domains (263, 264). Caveolae are also responsible via maintenance of signaling complexes for cardiomyocyte protection from ischemic damage; such protection was lost with disruption of caveolae and caveolin-1 protein (265). Isoform specificity in Gβγ regulation of AC5/6 via caveolin versus noncaveolin localization was not studied, but cannot be ruled out since Gβγ is unable to directly stimulate soluble C1/C2 domains of AC5 (95).

5.8 AC5/6 Physiology and Potential AC/Gβγ Complex Roles

Gβγ is limited in the cell, and may be competed for by various Gα subunits in cardiomyocytes (266). The Gαs to Gαi ratio in a heart failure risk state, for example, can be between 1:10 and 1:40 (267). Scaffolding on AC of Gαs and Gβγ could maintain local pools of Gαs for adrenergic signaling. One of the AC5 heart knockout models had approximately 60% loss of Gαs concentration (141). Loss of AC5 could also mean loss of G protein that is

otherwise scaffolded and used for signaling in cardiac stress-induced hypertrophy. Treatments for preventing heart failure that target Gβγ typically focus not on Gβγ regulation of AC but rather effects on β-AR desensitization via Gβγ recruitment of GRK2 and PI3K (268). Cardiac and adrenal gland blockade of adrenergic signaling through gallein inhibition of Gβγ, which we have shown does not affect AC2/5/6 activity, attenuated β-adrenergic dependent development of heart failure in cardiomyocytes (269). Thus, AC activity independent functions such as scaffolding may be relevant to Gβγ signaling in progression of heart failure.

Beyond AC5/6 and G protein roles in heart failure development, there is also a Gβγ-dependent AC sensitization phenotype seen in neuronal tissues (37, 270). This sensitization, or enhanced AC stimulation by Gαs-coupled receptors following prolonged activation of Gαi-coupled receptors, required the presence of Gβγ subunits (271). AC5 sensitization is also closely related to AC5-specific opioid dependency/withdrawal behaviors that have been disrupted in AC5 knockout models (126). This Gβγ effect appears to be more organizational in nature rather than direct interactions with Gαs; Gαs was needed for sensitization of AC5, and blocking Gβγ prevented AC5 sensitization, but Gαs/Gβγ interactions were not required (272). This supports an indirect function of Gβγ on AC5 sensitization; Gβγ mediated construction and trafficking of AC5-specific signaling complexes as suggested above is one such explanation.

AC/G protein heterotrimer signaling can also be hijacked in cases of pathological invasion, considering cAMP signaling involvement in so many cellular functions. One example is the bacteria *Bartonella henselae* and its BepA protein that is secreted as a result of endothelial cell invasion. Release of BepA in endothelial cells results in pathological anti-apoptotic effects that are associated with increased cAMP (273, 274). BepA's effects were shown to occur via conditional stimulation of AC2/7 in the presence of Gαs, in a mechanism

very similar to that employed for conditional stimulation of AC isoforms by Gβγ, including that forskolin also was sufficient for BepA to increase cAMP via AC stimulation (275).

5.9 Future Directions—AC Inhibitor Development

There are limited explanations for what determines isoform selectivity of the AC5/6 inhibitors studied to date, considering the conservation of the AC catalytic site. The C1 α1 structural helix is a rare region of lack of isoform conservation; as noted previously, this subdomain not only is closely homologous only between AC5/6, but is less homologous for isoforms the inhibitors are least potent on, such as AC8/9 (1). This helix is involved in metal chelation during cAMP synthesis. As such, designing inhibitors that can either interact with this helix or that have metal chelating properties are one avenue for ligand-based AC5/6 inhibitor optimization of potency. One published example is the inhibitor PMC-6 (169), but such a strategy should also work with optimizing novel non-adenine based small molecules.

This work further studied AC inhibitors that, for the most part, inhibit AC by interactions within the catalytic pocket. This may be one reason for the difficulties in finding highly potent non-ATP like small molecule inhibitors. For further AC5/6 inhibitor discovery, the Gαi binding site on the C1 domain is a potential alternative therapeutic target site. This may provide novel AC1/5/6 inhibitors, as those are the isoforms inhibited by Gαi binding. Further selectivity may occur depending on whether inhibitors identified can discriminately involve AC NT in the bounding inhibitory conformation.

In addition to strategies for finding novel isoform selective AC inhibitors, current inhibitors can be used in research to further characterize isoform specific physiological roles by pharmacological inhibition of AC5/6. For example, although AC5 and AC6 are the most highly

expressed isoforms in cardiomyocytes, AC4 and AC9 are also expressed in lesser amounts. Despite lower expression, AC9 does have some function in cardiac repolarization. Specifically, AC9 is involved in a complex with the ion channel KCNQ1 and AKAP Yotiao in a complex that mediates PKA phosphorylation of KCNQ1 repolarization (109-111). Disruption of such a complex results in phenotypes resembling long QT syndrome, an abnormal heartbeat. In cardiomyocytes, use of the AC5/6 inhibitors and a combination of wildtype vs. AC4/9 knockout animals could be used to further characterize the necessity and/or sufficiency of AC isoforms in cardiac calcium handling, stress responses, and contractile control.

5.10 Future Directions—AC/Heterotrimeric G Protein Regulation

It is interesting that 5NT was identified as the least hotspot-dependent binding site of the AC NT tested. AC9 has no clear effects on its activity by Gβγ, so it is surprising that the G protein subunit's binding to 9NT was so affected by hotspot mutation. Further study is needed to understand the significance of hotspot-dependent Gβγ interactions with AC9, and whether the 9NT serves as a scaffold to anchor Gβγ for other effectors similar to the function of WDR26 (236).

In the AC5/Gβγ regulatory mechanism proposed, a lot of assumption is placed upon the likely 5NT distal binding site that may be required for stimulatory effects. Ways to confirm this site has a similar function to the 6NT binding site for Gβγ will be required to clarify that the aa 66-137 scaffolding site on 5NT is not the only AC5 NT region involved in Gβγ function.

Most importantly, via immunoprecipitations, multiple interactions between AC5 and Gβγ have been shown. However, despite much discussion above about AC and Gβγ involvement in larger signaling complexes, these have not been shown directly yet. Further

work should examine the dynamics of AC5/Gβγ interaction in cells. In addition, showing AC5/Gβγ dependent formation of complexes in physiologically relevant cellular models can clarify the exact effects of AC5 specific heterotrimeric G protein scaffolding, and the selectivity of such scaffolding in AC isoform selective cAMP signaling.

Bibliography

1. Brand CS, Hocker HJ, Gorfe AA, Cavasotto CN, Dessauer CW. Isoform selectivity of adenylyl cyclase inhibitors: characterization of known and novel compounds. J Pharmacol Exp Ther. 2013;347(2):265-75.

2. Conley JM, Brand CS, Bogard AS, Pratt EP, Xu R, Hockerman GH, Ostrom RS, Dessauer CW, Watts VJ. Development of a high-throughput screening paradigm for the discovery of small-molecule modulators of adenylyl cyclase: identification of an adenylyl cyclase 2 inhibitor. J Pharmacol Exp Ther. 2013;347(2):276-87.

3. Sutherland EW, Rall TW. Fractionation and characterization of a cyclic adenine ribonucleotide formed by tissue particles. J Biol Chem. 1958;232(2):1077-91.

4. Walsh DA, Perkins JP, Krebs EG. An adenosine 3',5'-monophosphate-dependant protein kinase from rabbit skeletal muscle. J Biol Chem. 1968;243(13):3763-5.

5. de Rooij J, Zwartkruis FJ, Verheijen MH, Cool RH, Nijman SM, Wittinghofer A, Bos JL. Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. Nature. 1998;396(6710):474-7.

6. Sadana R, Dessauer CW. Physiological Roles for G Protein-Regulated Adenylyl Cyclase Isoforms: Insights from Knockout and Overexpression Studies. NeuroSignals. 2009;17:5-22.

7. Oki N, Takahashi SI, Hidaka H, Conti M. Short term feedback regulation of cAMP in FRTL-5 thyroid cells. Role of PDE4D3 phosphodiesterase activation. J Biol Chem. 2000;275(15):10831-7.

8. Conti M, Richter W, Mehats C, Livera G, Park JY, Jin C. Cyclic AMP-specific PDE4 phosphodiesterases as critical components of cyclic AMP signaling. J Biol Chem. 2003;278(8):5493-6.

9. Post SR, Hilal-Dandan R, Urasawa K, Brunton LL, Insel PA. Quantification of signalling components and amplification in the beta-adrenergic-receptor-adenylate cyclase pathway in isolated adult rat ventricular myocytes. Biochem J. 1995;311 (Pt 1):75-80.

10. Kamenetsky M, Middelhaufe S, Bank EM, Levin LR, Buck J, Steegborn C. Molecular Details of cAMP Generation in Mammalian Cells: A Tale of Two Systems. J Mol Biol. 2006;362(4):623-39.

11. Krupinski J, Coussen F, Bakalyar HA, Tang WJ, Feinstein PG, Orth K, Slaughter C, Reed RR, Gilman AG. Adenylyl cyclase amino acid sequence: possible channel- or transporter-like structure. Science. 1989;244(4912):1558-64.

12. Tesmer JJ, Sunahara RK, Gilman AG, Sprang SR. Crystal structure of the catalytic domains of adenylyl cyclase in a complex with Gsalpha.GTPgammaS. Science. 1997;278(5345):1907-16.

13. Sunahara RK, Dessauer CW, Gilman AG. Complexity and diversity of mammalian adenylyl cyclases. Annual Review of Pharmacology and Toxicology. 1996;36:461-80.

14. Tang WJ, Krupinski J, Gilman AG. Expression and characterization of calmodulin-activated (type I) adenylylcyclase. Journal of Biological Chemistry. 1991;266(13):8595-603.

15. Choi EJ, Wong ST, Hinds TR, Storm DR. Calcium and muscarinic agonist stimulation of type-I adenylyl cyclase in whole cells. Journal of Biological Chemistry. 1992;267:12440.

16. Cali JJ, Zwaagstra JC, Mons N, Cooper DM, Krupinski J. Type VIII adenylyl cyclase. A Ca2+/calmodulin-stimulated enzyme expressed in discrete regions of rat brain. J Biol Chem. 1994;269(16):12190-5.

17. Choi EJ, Xia Z, Storm DR. Stimulation of the type III olfactory adenylyl cyclase by calcium and calmodulin. Biochemistry. 1992;31(28):6492-8.

18. Tang WJ, Gilman AG. Type-specific regulation of adenylyl cyclase by G protein beta gamma subunits. Science. 1991;254(5037):1500-3.

19. Federman AD, Conklin BR, Schrader KA, Reed RR, Bourne HR. Hormonal stimulation of adenylyl cyclase through Gi-protein beta-gamma subunits. Nature. 1992;356(6365):159-61.

20. Taussig R, Quarmby LM, Gilman AG. Regulation of purified type I and type II adenylylcyclases by G protein beta gamma subunits. J Biol Chem. 1993;268(1):9-12.

21. Bayewitch ML, Avidor-Reiss T, Levy R, Pfeuffer T, Nevo I, Simonds WF, Vogel Z. Differential Modulation of Adenylyl Cyclases I and II by Various Gbeta Subunits. J Biol Chem. 1998;273(4):2273-6.

22. Guillou JL, Nakata H, Cooper DM. Inhibition by calcium of mammalian adenylyl cyclases. J Biol Chem. 1999;274(50):35539-45.

23. Chen-Goodspeed M, Lukan AN, Dessauer CW. Modeling of G alpha(s) and G alpha(i) regulation of human type V and VI adenylyl cyclase. J Biol Chem. 2005;280(3):1808-16.

24. Dessauer CW, Chen-Goodspeed M, Chen J. Mechanism of G alpha(i)-mediated inhibition of type V adenylyl cyclase. J Biol Chem. 2002;277(32):28823-9.

25. Hacker BM, Tomlinson JE, Wayman GA, Sultana R, Chan G, Villacres E, Disteche C, Storm DR. Cloning, Chromosomal Mapping, and Regulatory Properties of the Human Type 9 Adenylyl Cyclase (ADCY9). Genomics. 1998;50(1):97-104.

26. Sprang SR, Chen Z, Du X, Stephen RS. Structural Basis of Effector Regulation and Signal Termination in Heterotrimeric G[alpha] Proteins. Advances in Protein Chemistry. Volume 74: Academic Press; 2007. p. 1-65.

27. Levitzki A, Klein S. G-protein subunit dissociation is not an integral part of G-protein action. Chembiochem. 2002;3(9):815-8.

28. Bunemann M, Frank M, Lohse MJ. Gi protein activation in intact cells involves subunit rearrangement rather than dissociation. Proc Natl Acad Sci U S A. 2003;100(26):16077-82.

29. Frank M, Thumer L, Lohse MJ, Bunemann M. G Protein activation without subunit dissociation depends on a G{alpha}(i)-specific region. J Biol Chem. 2005;280(26):24584-90.

30. Harry A, Chen Y, Magnusson R, Iyengar R, Weng G. Differential regulation of adenylyl cyclases by G alpha s. J Biol Chem. 1997;272(30):19017-21.

31. Taussig R, Iniguez-Lluhi JA, Gilman AG. Inhibition of adenylyl cyclase by Gi alpha. Science. 1993;261:218-21.

32. Dessauer CW, Tesmer JJ, Sprang SR, Gilman AG. Identification of a Gialpha binding site on type V adenylyl cyclase. J Biol Chem. 1998;273(40):25831-9.

33. Kozasa T, Gilman AG. Purification of recombinant G proteins from Sf9 cells by hexahistidine tagging of associated subunits. Characterization of alpha 12 and inhibition of adenylyl cyclase by alpha z. J Biol Chem. 1995;270(4):1734-41.

34. Taussig R, Tang WJ, Hepler JR, Gilman AG. Distinct patterns of bidirectional regulation of mammalian adenylyl cyclases. J Biol Chem. 1994;269(8):6093-100.

35. Iniguez-Lluhi JA, Simon MI, Robishaw JD, Gilman AG. G protein beta gamma subunits synthesized in Sf9 cells. Functional characterization and the significance of prenylation of gamma. J Biol Chem. 1992;267(32):23409-17.

36. Diel S, Klass K, Wittig B, Kleuss C. Gbetagamma activation site in adenylyl cyclase type II. Adenylyl cyclase type III is inhibited by Gbetagamma. J Biol Chem. 2006;281(1):288-94.

37. Steiner D, Avidor-Reiss T, Schallmach E, Saya D, Vogel Z. Inhibition and superactivation of the calcium-stimulated isoforms of adenylyl cyclase: role of Gbetagamma dimers. J Mol Neurosci. 2005;27(2):195-203.

38. Iwami G, Kawabe J, Ebina T, Cannon PJ, Homcy CJ, Ishikawa Y. Regulation of adenylyl cyclase by protein kinase A. J Biol Chem. 1995;270(21):12481-4.

39. Chen Y, Harry A, Li J, Smit MJ, Bai X, Magnusson R, Pieroni JP, Weng G, Iyengar R. Adenylyl cyclase 6 is selectively regulated by protein kinase A phosphorylation in a region involved in Galphas stimulation. Proc Natl Acad Sci U S A. 1997;94(25):14100.

40. Kawabe J, Iwami G, Ebina T, Ohno S, Katada T, Ueda Y, Homcy CJ, Ishikawa Y. Differential Activation of Adenylyl-Cyclase by Protein-Kinase-C Isoenzymes. Journal of Biological Chemistry. 1994;269(24):16554-8.

41. Mou TC, Masada N, Cooper DM, Sprang SR. Structural basis for inhibition of mammalian adenylyl cyclase by calcium. Biochemistry. 2009;48(15):3387-97.

42. Hu B, Nakata H, Gu C, de Beer T, Cooper DMF. A Critical Interplay between Ca2+ Inhibition and Activation by Mg2+ of AC5 Revealed by Mutants and Chimeric Constructs. J Biol Chem. 2002;277(36):33139-47.

43. Mitterauer T, Hohenegger M, Tang WJ, Nanoff C, Freissmuth M. The C2 Catalytic Domain of Adenylyl Cyclase Contains the Second Metal Ion (Mn2+) Binding Site. Biochemistry. 1998;37(46):16183-91.

44. Tesmer JJ, nbsp, G, Sunahara RK, Johnson RA, Gosselin G, Gilman AG, Sprang SR. Two-Metal-Ion Catalysis in Adenylyl Cyclase. Science. 1999;285(5428):756-60.

45. Seamon KB, Daly JW. Forskolin: its biological and chemical properties. Adv Cyclic Nucleotide Protein Phosphorylation Res. 1986;20:1-150.

46. Sutkowski EM, Tang WJ, Broome CW, Robbins JD, Seamon KB. Regulation of forskolin interactions with type I, II, V, and VI adenylyl cyclases by Gs alpha. Biochemistry. 1994;33(43):12852-9.

47. Dessauer CW, Scully TT, Gilman AG. Interactions of forskolin and ATP with the cytosolic domains of mammalian adenylyl cyclase. J Biol Chem. 1997;272(35):22272-7.

48. Yan S-Z, Huang Z-H, Andrews RK, Tang W-J. Conversion of Forskolin-Insensitive to Forskolin-Sensitive (Mouse-Type IX) Adenylyl Cyclase. Mol Pharmacol. 1998;53(2):182-7.

49. Ueda N, Iniguez-Lluhi JA, Lee E, Smrcka AV, Robishaw JD, Gilman AG. G protein beta gamma subunits. Simplified purification and properties of novel isoforms. J Biol Chem. 1994;269(6):4388-95.

50. Steiner D, Saya D, Schallmach E, Simonds WF, Vogel Z. Adenylyl cyclase type-VIII activity is regulated by G[beta][gamma] subunits. Cellular Signalling. 2006;18(1):62-8.

51. Chen JQ, Devivo M, Dingus J, Harry A, Li JR, Sui JL, Carty DJ, Blank JL, Exton JH, Stoffel RH, Inglese J, Lefkowitz RJ, Logothetis DE, Hildebrandt JD, Iyengar R. A region of adenylyl cyclase 2 critical for regulation by G protein beta-gamma subunits. Science. 1995;268:1166.

52. Weng G, Li J, Dingus J, Hildebrandt JD, Weinstein H, Iyengar R. Gbeta Subunit Interacts with a Peptide Encoding Region 956-982 of Adenylyl Cyclase 2. Cross-Linking Of

The Peptide To Free Gbeta Gamma But Not The Heterotrimer. J Biol Chem. 1996;271(43):26445-8.

53. Chen YB, Weng GZ, Li JR, Pieroni HA, Dingus J, Hildebrandt JD, Guarnieri F, Weinstein H, Iyengar R. A surface on the G protein alpha subunit involved in interactions with adenylyl cyclases. Proc Natl Acad Sci U S A. 1997;94:2711.

54. Diel S, Beyermann M, Navarro Llorens JM, Wittig B, Kleuss C. Two Interaction Sites on Mammalian Adenylyl Cyclase Type I and II: modulation by calmodulin and Gbetagamma. Biochemical Journal. 2008.

55. Boran AD, Chen Y, Iyengar R. Identification of new Gbetagamma interaction sites in adenylyl cyclase 2. Cell Signal. 2011;23(9):1489-95.

56. Krapivinsky G, Kennedy ME, Nemec J, Medina I, Krapivinsky L, Clapham DE. Gbeta binding to GIRK4 subunit is critical for G protein-gated K+ channel activation. J Biol Chem. 1998;273(27):16946-52.

57. Logothetis DE, Kurachi Y, Galper J, Neer EJ, Clapham DE. The beta gamma subunits of GTP-binding proteins activate the muscarinic K+ channel in heart. Nature. 1987;325(6102):321-6.

58. Wickman KD, Iniguez-Lluhl JA, Davenport PA, Taussig R, Krapivinsky GB, Linder ME, Gilman AG, Clapham DE. Recombinant G-protein beta gamma-subunits activate the muscarinic-gated atrial potassium channel. Nature. 1994;368(6468):255-7.

59. Schreibmayer W, Dessauer CW, Vorobiov D, Gilman AG, Lester HA, Davidson N, Dascal N. Inhibition of an inwardly rectifying K+ channel by G-protein alpha-subunits. Nature. 1996;380(6575):624-7.

60. Peleg S, Ivanina T, Dessauer CW, Dascal N. G(ai GDP) regulates the basal activity of G protein-activated K+ channels (GIRK; Kir3). Biophysical Journal. 2002;82(1):592A-A.

61. Peleg S, Varon D, Ivanina T, Dessauer CW, Dascal N. G alpha(i) controls the gating of the G protein-activated K+ channel, GIRK. Neuron. 2002;33(1):87-99.

62. Ivanina T, Rishal I, Varon D, Mullner C, Frohnwieser-Steinecke B, Schreibmayer W, Dessauer CW, Dascal N. Mapping the G beta gamma-binding sites in GIRK1 and GIRK2 subunits of the G protein-activated K+ channel. J Biol Chem. 2003;278(31):29174-83.

63. Rishal I, Keren-Raifman T, Yakubovich D, Ivanina T, Dessauer CW, Slepak VZ, Dascal N. Na+ promotes the dissociation between G alpha(GDP) and G beta gamma, activating G protein-gated K+ channels. Journal of Biological Chemistry. 2003;278(6):3840-5.

64. Ivanina T, Varon D, Peleg S, Rishal I, Porozov Y, Dessauer CW, Keren-Raifman T, Dascal N. G alpha(i1) and G alpha(i3) differentially interact with, and regulate, the G protein-activated K+ channel. J Biol Chem. 2004;279(17):17260-8.

65. Rishal I, Porozov Y, Yakubovich D, Varon D, Dascal N. Gbetagamma-dependent and Gbetagamma-independent basal activity of G protein-activated K+ channels. J Biol Chem. 2005;280(17):16685-94.

66. Rubinstein M, Peleg S, Berlin S, Brass D, Keren-Raifman T, Dessauer CW, Ivanina T, Dascal N. Divergent regulation of GIRK1 and GIRK2 subunits of the neuronal G protein gated K+ channel by GalphaiGDP and Gbetagamma. J Physiol. 2009;587(Pt 14):3473-91.

67. Berlin S, Keren-Raifman T, Castel R, Rubinstein M, Dessauer CW, Ivanina T, Dascal N. G alpha(i) and G betagamma jointly regulate the conformations of a G betagamma effector, the neuronal G protein-activated K+ channel (GIRK). J Biol Chem. 2010;285(9):6179-85.

68. Berlin S, Tsemakhovich VA, Castel R, Ivanina T, Dessauer CW, Keren-Raifman T, Dascal N. Two distinct aspects of coupling between Galpha(i) protein and G protein-activated K+ channel (GIRK) revealed by fluorescently labeled Galpha(i3) protein subunits. J Biol Chem. 2011;286(38):33223-35.

69. Kahanovitch U, Tsemakhovich V, Berlin S, Rubinstein M, Styr B, Castel R, Peleg S, Tabak G, Dessauer CW, Ivanina T, Dascal N. Recruitment of Gbetagamma controls the basal activity of G-protein coupled inwardly rectifying potassium (GIRK) channels: crucial role of distal C terminus of GIRK1. J Physiol. 2014;592(Pt 24):5373-90.

70. Pitcher JA, Inglese J, Higgins JB, Arriza JL, Casey PJ, Kim C, Benovic JL, Kwatra MM, Caron MG, Lefkowitz RJ. Role of beta gamma subunits of G proteins in targeting the beta-adrenergic receptor kinase to membrane-bound receptors. Science. 1992;257(5074):1264-7.

71. Goodman OB, Jr., Krupnick JG, Santini F, Gurevich VV, Penn RB, Gagnon AW, Keen JH, Benovic JL. Beta-arrestin acts as a clathrin adaptor in endocytosis of the beta2-adrenergic receptor. Nature. 1996;383(6599):447-50.

72. Boyer JL, Waldo GL, Harden TK. Beta gamma-subunit activation of G-protein-regulated phospholipase C. J Biol Chem. 1992;267(35):25451-6.

73. Camps M, Carozzi A, Schnabel P, Scheer A, Parker PJ, Gierschik P. Isozyme-selective stimulation of phospholipase C-beta 2 by G protein beta gamma-subunits. Nature. 1992;360(6405):684-6.

74. Camps M, Hou C, Sidiropoulos D, Stock JB, Jakobs KH, Gierschik P. Stimulation of phospholipase C by guanine-nucleotide-binding protein beta gamma subunits. Eur J Biochem. 1992;206(3):821-31.

75. Schnabel P, Camps M, Carozzi A, Parker PJ, Gierschik P. Mutational analysis of phospholipase C-beta 2. Identification of regions required for membrane association and stimulation by guanine-nucleotide-binding protein beta gamma subunits. Eur J Biochem. 1993;217(3):1109-15.

76. Smrcka AV, Sternweis PC. Regulation of purified subtypes of phosphatidylinositol-specific phospholipase C beta by G protein alpha and beta gamma subunits. J Biol Chem. 1993;268(13):9667-74.

77. Ikeda SR. Voltage-dependent modulation of N-type calcium channels by G-protein beta gamma subunits. Nature. 1996;380(6571):255-8.

78. Furukawa T, Miura R, Mori Y, Strobeck M, Suzuki K, Ogihara Y, Asano T, Morishita R, Hashii M, Higashida H, Yoshii M, Nukada T. Differential interactions of the C terminus and the cytoplasmic I-II loop of neuronal Ca2+ channels with G-protein alpha and beta gamma subunits. II. Evidence for direct binding. J Biol Chem. 1998;273(28):17595-603.

79. Herlitze S, Garcia DE, Mackie K, Hille B, Scheuer T, Catterall WA. Modulation of Ca2+ channels by G-protein beta gamma subunits. Nature. 1996;380(6571):258-62.

80. Jeong SW, Ikeda SR. Sequestration of G-protein beta gamma subunits by different G-protein alpha subunits blocks voltage-dependent modulation of Ca2+ channels in rat sympathetic neurons. J Neurosci. 1999;19(12):4755-61.

81. Stephens L, Smrcka A, Cooke FT, Jackson TR, Sternweis PC, Hawkins PT. A novel phosphoinositide 3 kinase activity in myeloid-derived cells is activated by G protein beta gamma subunits. Cell. 1994;77(1):83-93.

82. Stephens LR, Eguinoa A, Erdjument-Bromage H, Lui M, Cooke F, Coadwell J, Smrcka AS, Thelen M, Cadwallader K, Tempst P, Hawkins PT. The G beta gamma sensitivity of a PI3K is dependent upon a tightly associated adaptor, p101. Cell. 1997;89(1):105-14.

83. Blackmer T, Larsen EC, Bartleson C, Kowalchyk JA, Yoon EJ, Preininger AM, Alford S, Hamm HE, Martin TF. G protein betagamma directly regulates SNARE protein fusion machinery for secretory granule exocytosis. Nat Neurosci. 2005;8(4):421-5.

84. Gerachshenko T, Blackmer T, Yoon EJ, Bartleson C, Hamm HE, Alford S. Gbetagamma acts at the C terminus of SNAP-25 to mediate presynaptic inhibition. Nat Neurosci. 2005;8(5):597-605.

85. Welch HC, Coadwell WJ, Ellson CD, Ferguson GJ, Andrews SR, Erdjument-Bromage H, Tempst P, Hawkins PT, Stephens LR. P-Rex1, a PtdIns(3,4,5)P3- and Gbetagamma-regulated guanine-nucleotide exchange factor for Rac. Cell. 2002;108(6):809-21.

86. Sternweis PC. The purified alpha subunits of Go and Gi from bovine brain require beta gamma for association with phospholipid vesicles. J Biol Chem. 1986;261:631-7.

87. Wu G, Bogatkevich GS, Mukhin YV, Benovic JL, Hildebrandt JD, Lanier SM. Identification of Gbetagamma binding sites in the third intracellular loop of the M(3)-muscarinic receptor and their role in receptor regulation. J Biol Chem. 2000;275(12):9026-34.

88. Wall MA, Coleman DE, Lee E, Iniguez-Lluhi JA, Posner BA, Gilman AG, Sprang SR. The structure of the G protein heterotrimer Gi alpha 1 beta 1 gamma 2. Cell. 1995;83(6):1047-58.

89. Sondek J, Bohm A, Lambright DG, Hamm HE, Sigler PB. Crystal structure of a G A protein beta/gamma dimer at 2.1 A resolution. Nature. 1996;379:369.

90. Ford CE, Skiba NP, Bae H, Daaka Y, Reuveny E, Shekter LR, Rosal R, Weng G, Yang C-S, Iyengar R, Miller RJ, Jan LY, Lefkowitz RJ, Hamm HE. Molecular Basis for Interactions of G Protein {beta} Subunits with Effectors. Science. 1998;280(5367):1271-4.

91. Li Y, Sternweis PM, Charnecki S, Smith TF, Gilman AG, Neer EJ, Kozasa T. Sites for Galpha binding on the G protein beta subunit overlap with sites for regulation of phospholipase Cbeta and adenylyl cyclase. J Biol Chem. 1998;273(26):16265-72.

92. Panchenko MP, Saxena K, Li Y, Charnecki S, Sternweis PM, Smith TF, Gilman AG, Kozasa T, Neer EJ. Sites important for PLCbeta2 activation by the G protein betagamma subunit map to the sides of the beta propeller structure. J Biol Chem. 1998;273(43):28298-304.

93. Gao X, Sadana R, Dessauer CW, Patel TB. Conditional stimulation of type V and VI adenylyl cyclases by G protein beta gamma subunits. J Biol Chem. 2007;282(1):294-302.

94. Crossthwaite AJ, Ciruela A, Rayner TF, Cooper DMF. A direct interaction between the N terminus of adenylyl cyclase AC8 and the catalytic subunit of protein phosphatase 2A. Mol Pharmacol. 2006;69(2):608-17.

95. Sadana R, Dascal N, Dessauer CW. N terminus of type 5 adenylyl cyclase scaffolds Gs heterotrimer. Mol Pharmacol. 2009;76(6):1256-64.

96. Lai HL, Lin TH, Kao YY, Lin WJ, Hwang MJ, Chern Y. The N terminus domain of type VI adenylyl cyclase mediates its inhibition by protein kinase C. Mol Pharmacol. 1999;56(3):644-50.

97. Chou JL, Huang CL, Lai HL, Hung AC, Chien CL, Kao YY, Chern Y. Regulation of type VI adenylyl cyclase by Snapin, a SNAP25-binding protein. J Biol Chem. 2004;279(44):46271-9.

98. Wang S-C, Lai H-L, Chiu Y-T, Ou R, Huang C-L, Chern Y. Regulation of type V adenylate cyclase by Ric8a, a guanine nucleotide exchange factor. Biochem J. 2007;406(3):383-8.

99. Salim S, Sinnarajah S, Kehrl JH, Dessauer CW. Identification of RGS2 and type V adenylyl cyclase interaction sites. J Biol Chem. 2003;278(18):15842-9.

100. Kao YY, Lai HL, Hwang MJ, Chern Y. An Important functional role of the N terminus domain of type VI adenylyl cyclase (ACVI) in Gia-mediated inhibition. Journal of Biological Chemistry. 2004.

101. Simpson RE, Ciruela A, Cooper DMF. The Role of Calmodulin Recruitment in Ca2+ Stimulation of Adenylyl Cyclase Type 8. J Biol Chem. 2006;281(25):17379-89.

102. Piggott LA, Bauman AL, Scott JD, Dessauer CW. The A-kinase anchoring protein Yotiao binds and regulates adenylyl cyclase in brain. Proc Natl Acad Sci U S A. 2008;105(37):13835-40.

103. Bauman AL, Soughayer J, Nguyen BT, Willoughby D, Carnegie GK, Wong W, Hoshi N, Langeberg LK, Cooper DM, Dessauer CW, Scott JD. Dynamic regulation of cAMP synthesis through anchored PKA-adenylyl cyclase V/VI complexes. Mol Cell. 2006;23(6):925-31.

104. Efendiev R, Samelson BK, Nguyen BT, Phatarpekar PV, Baameur F, Scott JD, Dessauer CW. AKAP79 interacts with multiple adenylyl cyclase (AC) isoforms and scaffolds AC5 and -6 to alpha-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptors. J Biol Chem. 2010;285(19):14450-8.

105. Jeske NA, Diogenes A, Ruparel NB, Fehrenbacher JC, Henry M, Akopian AN, Hargreaves KM. A-kinase anchoring protein mediates TRPV1 thermal hyperalgesia through PKA phosphorylation of TRPV1. Pain. 2008;138(3):604-16.

106. Schnizler K, Shutov LP, Van Kanegan MJ, Merrill MA, Nichols B, McKnight GS, Strack S, Hell JW, Usachev YM. Protein kinase A anchoring via AKAP150 is essential for TRPV1 modulation by forskolin and prostaglandin E2 in mouse sensory neurons. J Neurosci. 2008;28(19):4904-17.

107. Zhang X, Li L, McNaughton PA. Proinflammatory mediators modulate the heat-activated ion channel TRPV1 via the scaffolding protein AKAP79/150. Neuron. 2008;59(3):450-61.

108. Efendiev R, Bavencoffe A, Hu H, Zhu MX, Dessauer CW. Scaffolding by A-kinase anchoring protein enhances functional coupling between adenylyl cyclase and TRPV1 channel. J Biol Chem. 2013;288(6):3929-37.

109. Marx SO, Kurokawa J, Reiken S, Motoike H, D'Armiento J, Marks AR, Kass RS. Requirement of a macromolecular signaling complex for beta adrenergic receptor modulation of the KCNQ1-KCNE1 potassium channel. Science. 2002;295(5554):496-9.

110. Chen L, Marquardt ML, Tester DJ, Sampson KJ, Ackerman MJ, Kass RS. Mutation of an A-kinase-anchoring protein causes long-QT syndrome. Proc Natl Acad Sci U S A. 2007;104(52):20990-5.

111. Li Y, Chen L, Kass RS, Dessauer CW. The A-kinase anchoring protein Yotiao facilitates complex formation between adenylyl cyclase type 9 and the IKs potassium channel in heart. J Biol Chem. 2012;287(35):29815-24.

112. Pare GC, Bauman AL, McHenry M, Michel JJ, Dodge-Kafka KL, Kapiloff MS. The mAKAP complex participates in the induction of cardiac myocyte hypertrophy by adrenergic receptor signaling. J Cell Sci. 2005;118(Pt 23):5637-46.

113. Kapiloff MS, Piggott LA, Sadana R, Li J, Heredia LA, Henson E, Efendiev R, Dessauer CW. An adenylyl cyclase-mAKAPbeta signaling complex regulates cAMP levels in cardiac myocytes. J Biol Chem. 2009;284(35):23540-6.

114. Dodge-Kafka KL, Soughayer J, Pare GC, Carlisle Michel JJ, Langeberg LK, Kapiloff MS, Scott JD. The protein kinase A anchoring protein mAKAP coordinates two integrated cAMP effector pathways. Nature. 2005;437(7058):574-8.

115. Carter BD, Medzihradsky F. Go mediates the coupling of the mu opioid receptor to adenylyl cyclase in cloned neural cells and brain. Proc Natl Acad Sci U S A. 1993;90(9):4062-6.

116. Kawabe J, Ebina T, Toya Y, Oka N, Schwencke C, Duzic E, Ishikawa Y. Regulation of type V adenylyl cyclase by PMA-sensitive and -insensitive protein kinase C isoenzymes in intact cells. FEBS Letters. 1996;384(3):273-6.

117. Lai HL, Yang TH, Messing RO, Ching YH, Lin SC, Chern Y. Protein kinase C inhibits adenylyl cyclase type VI activity during desensitization of the A2a-adenosine receptor-mediated cAMP response. J Biol Chem. 1997;272(8):4970-7.

118. Zimmermann G, Taussig R. Protein kinase C alters the responsiveness of adenylyl cyclases to G protein alpha and betagamma subunits. J Biol Chem. 1996;271(43):27161-6.

119. Beazely MA, Watts VJ. Galphaq-coupled receptor signaling enhances adenylate cyclase type 6 activation. Biochem Pharmacol. 2005;70(1):113-20.

120. Defer N, Best-Belpomme M, Hanoune J. Tissue specificity and physiological relevance of various isoforms of adenylyl cyclase. Am J Physiol Renal Physiol. 2000;279(3):F400-16.

121. Espinasse I, Iourgenko V, Defer N, Samson F, Hanoune J, Mercadier JJ. Type V, but not type VI, adenylyl cyclase mRNA accumulates in the rat heart during ontogenic development. Correlation with increased global adenylyl cyclase activity. J Mol Cell Cardiol. 1995;27(9):1789-95.

122. Wang T, Brown MJ. Differential expression of adenylyl cyclase subtypes in human cardiovascular system. Mol Cell Endocrinol. 2004;223(1-2):55-62.

123. Lee KW, Hong JH, Choi IY, Che Y, Lee JK, Yang SD, Song CW, Kang HS, Lee JH, Noh JS, Shin HS, Han PL. Impaired D2 dopamine receptor function in mice lacking type 5 adenylyl cyclase. J Neurosci. 2002;22(18):7931-40.

124. Kheirbek MA, Britt JP, Beeler JA, Ishikawa Y, McGehee DS, Zhuang X. Adenylyl cyclase type 5 contributes to corticostriatal plasticity and striatum-dependent learning. J Neurosci. 2009;29(39):12115-24.

125. Kim KS, Lee KW, Baek IS, Lim CM, Krishnan V, Lee JK, Nestler EJ, Han PL. Adenylyl cyclase-5 activity in the nucleus accumbens regulates anxiety-related behavior. J Neurochem. 2008;107(1):105-15.

126. Kim KS, Lee KW, Im JY, Yoo JY, Kim SW, Lee JK, Nestler EJ, Han PL. Adenylyl cyclase type 5 (AC5) is an essential mediator of morphine action. Proc Natl Acad Sci U S A. 2006;103(10):3908-13.

127. Kim KS, Kim J, Back SK, Im JY, Na HS, Han PL. Markedly attenuated acute and chronic pain responses in mice lacking adenylyl cyclase-5. Genes Brain Behav. 2007;6(2):120-7.

128. Okumura S, Kawabe J, Yatani A, Takagi G, Lee MC, Hong C, Liu J, Takagi I, Sadoshima J, Vatner DE, Vatner SF, Ishikawa Y. Type 5 adenylyl cyclase disruption alters not only sympathetic but also parasympathetic and calcium-mediated cardiac regulation. Circulation Research. 2003;93(4):364-71.

129. Okumura S, Takagi G, Kawabe J, Yang G, Lee MC, Hong C, Liu J, Vatner DE, Sadoshima J, Vatner SF, Ishikawa Y. Disruption of type 5 adenylyl cyclase gene preserves cardiac function against pressure overload. Proc Natl Acad Sci U S A. 2003;100(17):9986-90.

130. Okumura S, Vatner DE, Kurotani R, Bai Y, Gao S, Yuan Z, Iwatsubo K, Ulucan C, Kawabe J, Ghosh K, Vatner SF, Ishikawa Y. Disruption of type 5 adenylyl cyclase enhances desensitization of cyclic adenosine monophosphate signal and increases Akt signal with chronic catecholamine stress. Circulation. 2007;116(16):1776-83.

131. Yan L, Vatner DE, O'Connor JP, Ivessa A, Ge H, Chen W, Hirotani S, Ishikawa Y, Sadoshima J, Vatner SF. Type 5 adenylyl cyclase disruption increases longevity and protects against stress. Cell. 2007;130(2):247-58.

132. Pavan B, Biondi C, Dalpiaz A. Adenylyl cyclases as innovative therapeutic goals. Drug Discov Today. 2009;14(19-20):982-91.

133. Pierre S, Eschenhagen T, Geisslinger G, Scholich K. Capturing adenylyl cyclases as potential drug targets. Nat Rev Drug Discov. 2009;8(4):321-35.

134. Ho D, Yan L, Iwatsubo K, Vatner DE, Vatner SF. Modulation of beta-adrenergic receptor signaling in heart failure and longevity: targeting adenylyl cyclase type 5. Heart failure reviews. 2010;15(5):495-512.

135. Bristow MR, Ginsburg R, Minobe W, Cubicciotti RS, Sageman WS, Lurie K, Billingham ME, Harrison DC, Stinson EB. Decreased catecholamine sensitivity and beta-adrenergic-receptor density in failing human hearts. N Engl J Med. 1982;307(4):205-11.

136. Feldman MD, Copelas L, Gwathmey JK, Phillips P, Warren SE, Schoen FJ, Grossman W, Morgan JP. Deficient production of cyclic AMP: pharmacologic evidence of an important cause of contractile dysfunction in patients with end-stage heart failure. Circulation. 1987;75(2):331-9.

137. Lai NC, Roth DM, Gao MH, Fine S, Head BP, Zhu J, McKirnan MD, Kwong C, Dalton N, Urasawa K, Roth DA, Hammond HK. Intracoronary delivery of adenovirus encoding adenylyl cyclase VI increases left ventricular function and cAMP-generating capacity. Circulation. 2000;102(19):2396-401.

138. Lai NC, Roth DM, Gao MH, Tang T, Dalton N, Lai YY, Spellman M, Clopton P, Hammond HK. Intracoronary adenovirus encoding adenylyl cyclase VI increases left ventricular function in heart failure. Circulation. 2004;110(3):330-6.

139. Lai NC, Tang T, Gao MH, Saito M, Takahashi T, Roth DM, Hammond HK. Activation of cardiac adenylyl cyclase expression increases function of the failing ischemic heart in mice. J Am Coll Cardiol. 2008;51(15):1490-7.

140. Phan HM, Gao MH, Lai NC, Tang T, Hammond HK. New signaling pathways associated with increased cardiac adenylyl cyclase 6 expression: implications for possible congestive heart failure therapy. Trends Cardiovasc Med. 2007;17(7):215-21.

141. Tang T, Lai NC, Roth DM, Drumm J, Guo T, Lee KW, Han PL, Dalton N, Gao MH. Adenylyl cyclase type V deletion increases basal left ventricular function and reduces left

ventricular contractile responsiveness to beta-adrenergic stimulation. Basic Res Cardiol. 2006;101(2):117-26.

142. Tang T, Gao MH, Lai NC, Firth AL, Takahashi T, Guo T, Yuan JX, Roth DM, Hammond HK. Adenylyl cyclase type 6 deletion decreases left ventricular function via impaired calcium handling. Circulation. 2008;117(1):61-9.

143. Tang T, Lai NC, Wright AT, Gao MH, Lee P, Guo T, Tang R, McCulloch AD, Hammond HK. Adenylyl cyclase 6 deletion increases mortality during sustained beta-adrenergic receptor stimulation. J Mol Cell Cardiol. 2013;60:60-7.

144. Iwase M, Uechi M, Vatner DE, Asai K, Shannon RP, Kudej RK, Wagner TE, Wight DC, Patrick TA, Ishikawa Y, Homcy CJ, Vatner SF. Cardiomyopathy induced by cardiac Gs alpha overexpression. Am J Physiol Heart Circ Physiol. 1997;272(1):H585-9.

145. Engelhardt S, Hein L, Wiesmann F, Lohse MJ. Progressive hypertrophy and heart failure in beta1-adrenergic receptor transgenic mice. Proc Natl Acad Sci U S A. 1999;96(12):7059-64.

146. Antos CL, Frey N, Marx SO, Reiken S, Gaburjakova M, Richardson JA, Marks AR, Olson EN. Dilated cardiomyopathy and sudden death resulting from constitutive activation of protein kinase a. Circ Res. 2001;89(11):997-1004.

147. Griffith TM, Chaytor AT, Edwards DH, Daverio F, McGuigan C. Enhanced inhibition of the EDHF phenomenon by a phenyl methoxyalaninyl phosphoramidate derivative of dideoxyadenosine. Br J Pharmacol. 2004;142(1):27-30.

148. Laux WH, Pande P, Shoshani I, Gao J, Boudou-Vivet V, Gosselin G, Johnson RA. Pro-nucleotide inhibitors of adenylyl cyclases in intact cells. J Biol Chem. 2004;279(14):13317-32.

149. Lelle M, Hameed A, Ackermann LM, Kaloyanova S, Wagner M, Berisha F, Nikolaev VO, Peneva K. Functional Non-Nucleoside Adenylyl Cyclase Inhibitors. Chemical biology & drug design. 2014.

150. Dessauer CW, Gilman AG. The catalytic mechanism of mammalian adenylyl cyclase. Equilibrium binding and kinetic analysis of P-site inhibition. J Biol Chem. 1997;272(44):27787-95.

151. Dessauer CW, Tesmer JJ, Sprang SR, Gilman AG. The interactions of adenylate cyclases with P-site inhibitors. Trends Pharmacol Sci. 1999;20(5):205-10.

152. Tesmer JJ, Dessauer CW, Sunahara RK, Murray LD, Johnson RA, Gilman AG, Sprang SR. Molecular basis for P-site inhibition of adenylyl cyclase. Biochemistry. 2000;39(47):14464-71.

153. Desaubry L, Shoshani I, Johnson RA. 2',5'-Dideoxyadenosine 3'-polyphosphates are potent inhibitors of adenylyl cyclase. Journal of Biological Chemistry. 1996;271:2380.

154. Desaubry L, Shoshani I, Johnson RA. Inhibition of Adenylyl Cyclase by a Family of Newly Synthesized Adenine Nucleoside 3'-Polyphosphates. J Biol Chem. 1996;271(24):14028-34.

155. Desaubry L, Johnson RA. Adenine Nucleoside 3'-Tetraphosphates Are Novel and Potent Inhibitors of Adenylyl Cyclases. J Biol Chem. 1998;273(38):24972-7.

156. Shoshani I, Boudou V, Pierra C, Gosselin G, Johnson RA. Enzymatic synthesis of unlabeled and beta-(32)P-labeled beta-L-2', 3'-dideoxyadenosine-5'-triphosphate as a potent inhibitor of adenylyl cyclases and its use as reversible binding ligand. Journal of Biological Chemistry. 1999;274(49):34735.

157. Johnson RA, Desaubry L, Bianchi G, Shoshani I, Lyons E, Jr., Taussig R, Watson PA, Cali JJ, Krupinski J, Pieroni JP, Iyengar R. Isozyme-dependent Sensitivity of Adenylyl Cyclases to P-site-mediated Inhibition by Adenine Nucleosides and Nucleoside 3'-Polyphosphates. J Biol Chem. 1997;272(14):8962-6.

158. Gille A, Seifert R. MANT-substituted guanine nucleotides: a novel class of potent adenylyl cyclase inhibitors. Life Sci. 2003;74(2-3):271-9.

159. Gille A, Lushington GH, Mou TC, Doughty MB, Johnson RA, Seifert R. Differential inhibition of adenylyl cyclase isoforms and soluble guanylyl cyclase by purine and pyrimidine nucleotides. J Biol Chem. 2004;279(19):19955-69.

160. Mou TC, Gille A, Fancy DA, Seifert R, Sprang SR. Structural basis for the inhibition of mammalian membrane adenylyl cyclase by 2 '(3')-O-(N-Methylanthraniloyl)-guanosine 5 '-triphosphate. J Biol Chem. 2005;280(8):7253-61.

161. Mou TC, Gille A, Suryanarayana S, Richter M, Seifert R, Sprang SR. Broad specificity of mammalian adenylyl cyclase for interaction with 2',3'-substituted purine- and pyrimidine nucleotide inhibitors. Mol Pharmacol. 2006;70(3):878-86.

162. Rottlaender D, Matthes J, Vatner SF, Seifert R, Herzig S. Functional adenylyl cyclase inhibition in murine cardiomyocytes by 2'(3')-O-(N-methylanthraniloyl)-guanosine 5'-[gamma-thio]triphosphate. J Pharmacol Exp Ther. 2007;321(2):608-15.

163. Pinto C, Hubner M, Gille A, Richter M, Mou TC, Sprang SR, Seifert R. Differential interactions of the catalytic subunits of adenylyl cyclase with forskolin analogs. Biochem Pharmacol. 2009;78(1):62-9.

164. Erdorf M, Mou TC, Seifert R. Impact of divalent metal ions on regulation of adenylyl cyclase isoforms by forskolin analogs. Biochem Pharmacol. 2011;82(11):1673-81.

165. Haunso A, Simpson J, Antoni FA. Small ligands modulating the activity of mammalian adenylyl cyclases: a novel mode of inhibition by calmidazolium. Mol Pharmacol. 2003;63(3):624-31.

166. Wang H, Xu H, Wu LJ, Kim SS, Chen T, Koga K, Descalzi G, Gong B, Vadakkan KI, Zhang X, Kaang BK, Zhuo M. Identification of an adenylyl cyclase inhibitor for treating neuropathic and inflammatory pain. Sci Transl Med. 2011;3(65):65ra3.

167. Zhuo M. Neuronal mechanism for neuropathic pain. Mol Pain. 2007;3:14.

168. Hess KC, Jones BH, Marquez B, Chen Y, Ord TS, Kamenetsky M, Miyamoto C, Zippin JH, Kopf GS, Suarez SS, Levin LR, Williams CJ, Buck J, Moss SB. The "soluble" adenylyl cyclase in sperm mediates multiple signaling events required for fertilization. Dev Cell. 2005;9(2):249-59.

169. Levy DE, Bao M, Cherbavaz DB, Tomlinson JE, Sedlock DM, Homcy CJ, Scarborough RM. Metal coordination-based inhibitors of adenylyl cyclase: novel potent P-site antagonists. J Med Chem. 2003;46(11):2177-86.

170. Kikura M, Morita K, Sato S. Pharmacokinetics and a simulation model of colforsin daropate, new forskolin derivative inotropic vasodilator, in patients undergoing coronary artery bypass grafting. Pharmacol Res. 2004;49(3):275-81.

171. Yoneyama M, Sugiyama A, Satoh Y, Takahara A, Nakamura Y, Hashimoto K. Cardiovascular and adenylate cyclase stimulating effects of colforsin daropate, a water-soluble forskolin derivative, compared with those of isoproterenol, dopamine and dobutamine. Circ J. 2002;66(12):1150-4.

172. Onda T, Hashimoto Y, Nagai M, Kuramochi H, Saito S, Yamazaki H, Toya Y, Sakai I, Homcy CJ, Nishikawa K, Ishikawa Y. Type-specific regulation of adenylyl cyclase. Selective

pharmacological stimulation and inhibition of adenylyl cyclase isoforms. Journal of Biological Chemistry. 2001;276(51):47785.

173. Iwatsubo K, Minamisawa S, Tsunematsu T, Nakagome M, Toya Y, Tomlinson JE, Umemura S, Scarborough RM, Levy DE, Ishikawa Y. Direct inhibition of type 5 adenylyl cyclase prevents myocardial apoptosis without functional deterioration. J Biol Chem. 2004;279(39):40938-45.

174. Brand CS. CHARACTERIZATION OF SELECTIVE INHIBITION OF ADENYLYL CYCLASE ACTIVITY BY SMALL MOLECULE INHIBITOR NKY80. Digital Commons @ TMC. 2012.

175. Yan SZ, Beeler JA, Chen Y, Shelton RK, Tang WJ. The regulation of type 7 adenylyl cyclase by its C1b region and Escherichia coli peptidylprolyl isomerase, SlyD. J Biol Chem. 2001;276(11):8500-6.

176. Fagan KA, Mahey R, Cooper DM. Functional co-localization of transfected Ca(2+)-stimulable adenylyl cyclases with capacitative Ca2+ entry sites. J Biol Chem. 1996;271(21):12438-44.

177. Davis TL, Bonacci TM, Sprang SR, Smrcka AV. Structural and Molecular Characterization of a Preferred Protein Interaction Surface on G Protein beta gamma Subunits. Biochemistry. 2005;44(31):10593-604.

178. Bonacci TM, Ghosh M, Malik S, Smrcka AV. Regulatory Interactions between the Amino Terminus of G-protein beta-gamma Subunits and the Catalytic Domain of Phospholipase C beta 2. J Biol Chem. 2005;280(11):10174-81.

179. Grant BJ, Lukman S, Hocker HJ, Sayyah J, Brown JH, McCammon JA, Gorfe AA. Novel allosteric sites on Ras for lead generation. PLoS One. 2011;6(10):e25711.

180. Huey R, Morris GM, Olson AJ, Goodsell DS. A semiempirical free energy force field with charge-based desolvation. J Comput Chem. 2007;28(6):1145-52.

181. Morris GM, Huey R, Lindstrom W, Sanner MF, Belew RK, Goodsell DS, Olson AJ. AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. J Comput Chem. 2009;30(16):2785-91.

182. Humphrey W, Dalke A, Schulten K. VMD: visual molecular dynamics. Journal of molecular graphics. 1996;14(1):33-8, 27-8.

183. Whisnant RE, Gilman AG, Dessauer CW. Interaction of the two cytosolic domains of mammalian adenylyl cyclase. Proc Natl Acad Sci U S A. 1996;93(13):6621-5.

184. Sunahara RK, Dessauer CW, Whisnant RE, Kleuss C, Gilman AG. Interaction of G(s alpha) with the cytosolic domains of mammalian adenylyl cyclase. J Biol Chem. 1997;272(35):22265-71.

185. Salomon Y, Londos C, Rodbell M. A highly sensitive adenylate cyclase assay. Anals of Biochemistry. 1974;58:541.

186. Dessauer CW. Kinetic analysis of the action of P-site analogs. Methods in Enzymology. 2002;345:112-26.

187. Seifert R, Lushington GH, Mou TC, Gille A, Sprang SR. Inhibitors of membranous adenylyl cyclases. Trends Pharmacol Sci. 2012;33(2):64-78.

188. Whitley R, Alford C, Hess F, Buchanan R. Vidarabine: a preliminary review of its pharmacological properties and therapeutic use. Drugs. 1980;20(4):267-82.

189. Iwatsubo K, Bravo C, Uechi M, Baljinnyam E, Nakamura T, Umemura M, Lai L, Gao S, Yan L, Zhao X, Park M, Qiu H, Okumura S, Iwatsubo M, Vatner DE, Vatner SF, Ishikawa

Y. Prevention of heart failure in mice by an antiviral agent that inhibits type 5 cardiac adenylyl cyclase. Am J Physiol Heart Circ Physiol. 2012;302(12):H2622-8.

190. Torgan CE, Kraus WE. Regulation of type II adenylyl cyclase mRNA in rabbit skeletal muscle by chronic motor nerve pacing. Am J Physiol. 1996;271(2 Pt 1):E253-60.

191. Suzuki Y, Shen T, Poyard M, Best-Belpomme M, Hanoune J, Defer N. Expression of adenylyl cyclase mRNAs in the denervated and in the developing mouse skeletal muscle. Am J Physiol. 1998;274(6 Pt 1):C1674-85.

192. Berdeaux R, Stewart R. cAMP signaling in skeletal muscle adaptation: hypertrophy, metabolism, and regeneration. American journal of physiology Endocrinology and metabolism. 2012;303(1):E1-17.

193. Drozdov I, Svejda B, Gustafsson BI, Mane S, Pfragner R, Kidd M, Modlin IM. Gene network inference and biochemical assessment delineates GPCR pathways and CREB targets in small intestinal neuroendocrine neoplasia. PLoS One. 2011;6(8):e22457.

194. Duerr EM, Mizukami Y, Ng A, Xavier RJ, Kikuchi H, Deshpande V, Warshaw AL, Glickman J, Kulke MH, Chung DC. Defining molecular classifications and targets in gastroenteropancreatic neuroendocrine tumors through DNA microarray analysis. Endocrine-related cancer. 2008;15(1):243-56.

195. Yu SJ, Yu JK, Ge WT, Hu HG, Yuan Y, Zheng S. SPARCL1, Shp2, MSH2, E-cadherin, p53, ADCY-2 and MAPK are prognosis-related in colorectal cancer. World journal of gastroenterology : WJG. 2011;17(15):2028-36.

196. Bogard AS, Xu C, Ostrom RS. Human bronchial smooth muscle cells express adenylyl cyclase isoforms 2, 4, and 6 in distinct membrane microdomains. J Pharmacol Exp Ther. 2011;337(1):209-17.

197. Bogard AS, Adris P, Ostrom RS. Adenylyl cyclase 2 selectively couples to E prostanoid type 2 receptors, whereas adenylyl cyclase 3 is not receptor-regulated in airway smooth muscle. J Pharmacol Exp Ther. 2012;342(2):586-95.

198. Bogard AS, Birg AV, Ostrom RS. Non-raft adenylyl cyclase 2 defines a cAMP signaling compartment that selectively regulates IL-6 expression in airway smooth muscle cells: differential regulation of gene expression by AC isoforms. Naunyn Schmiedebergs Arch Pharmacol. 2014;387(4):329-39.

199. Ohlstein EH, Berkowitz BA. SCH 23390 and SK&F 83566 are antagonists at vascular dopamine and serotonin receptors. Eur J Pharmacol. 1985;108(2):205-8.

200. Pinto C, Papa D, Hubner M, Mou TC, Lushington GH, Seifert R. Activation and inhibition of adenylyl cyclase isoforms by forskolin analogs. J Pharmacol Exp Ther. 2008;325(1):27-36.

201. Shen JX, Wachten S, Halls ML, Everett KL, Cooper DM. Muscarinic receptors stimulate AC2 by novel phosphorylation sites, whereas Gbetagamma subunits exert opposing effects depending on the G-protein source. Biochem J. 2012;447(3):393-405.

202. Levitzki A. From epinephrine to cyclic AMP. Science. 1988;241:800.

203. Bar-Sinai A, Marbach I, Shorr RG, Levitzki A. The GppNHp-activated adenylyl cyclase complex from turkey erythrocyte membranes can be isolated with its beta gamma subunits. Eur J Biochem. 1992;207(2):703-8.

204. Yuan C, Sato M, Lanier SM, Smrcka AV. Signaling by a Non-dissociated Complex of G Protein beta-gamma and alpha Subunits Stimulated by a Receptor-independent Activator of G Protein Signaling, AGS8. J Biol Chem. 2007;282(27):19938-47.

205. Dell EJ, Connor J, Chen S, Stebbins EG, Skiba NP, Mochly-Rosen D, Hamm HE. The beta gamma Subunit of Heterotrimeric G Proteins Interacts with RACK1 and Two Other WD Repeat Proteins. J Biol Chem. 2002;277(51):49888-95.

206. Scott JK, Huang SF, Gangadhar BP, Samoriski GM, Clapp P, Gross RA, Taussig R, Smrcka AV. Evidence that a protein-protein interaction 'hot spot' on heterotrimeric G protein betagamma subunits is used for recognition of a subclass of effectors. EMBO J. 2001;20(4):767-76.

207. Bonacci TM, Mathews JL, Yuan C, Lehmann DM, Malik S, Wu D, Font JL, Bidlack JM, Smrcka AV. Differential targeting of Gbetagamma-subunit signaling with small molecules. Science. 2006;312(5772):443-6.

208. Mathews JL, Smrcka AV, Bidlack JM. A novel Gbetagamma-subunit inhibitor selectively modulates mu-opioid-dependent antinociception and attenuates acute morphine-induced antinociceptive tolerance and dependence. J Neurosci. 2008;28(47):12183-9.

209. Lehmann DM, Seneviratne AM, Smrcka AV. Small molecule disruption of G protein beta gamma subunit signaling inhibits neutrophil chemotaxis and inflammation. Mol Pharmacol. 2008;73(2):410-8.

210. Casey LM, Pistner AR, Belmonte SL, Migdalovich D, Stolpnik O, Nwakanma FE, Vorobiof G, Dunaevsky O, Matavel A, Lopes CM, Smrcka AV, Blaxall BC. Small molecule disruption of G beta gamma signaling inhibits the progression of heart failure. Circ Res. 2010;107(4):532-9.

211. Qin J, Xie Y, Wang B, Hoshino M, Wolff DW, Zhao J, Scofield MA, Dowd FJ, Lin MF, Tu Y. Upregulation of PIP3-dependent Rac exchanger 1 (P-Rex1) promotes prostate cancer metastasis. Oncogene. 2009;28(16):1853-63.

212. Zhao T, Nalbant P, Hoshino M, Dong X, Wu D, Bokoch GM. Signaling requirements for translocation of P-Rex1, a key Rac2 exchange factor involved in chemoattractant-stimulated human neutrophil function. J Leukoc Biol. 2007;81(4):1127-36.

213. Lin Y, Smrcka AV. Understanding molecular recognition by G protein betagamma subunits on the path to pharmacological targeting. Mol Pharmacol. 2011;80(4):551-7.

214. Seneviratne AM, Burroughs M, Giralt E, Smrcka AV. Direct-reversible binding of small molecules to G protein betagamma subunits. Biochim Biophys Acta. 2011;1814(9):1210-8.

215. Bai Y, Tsunematsu T, Jiao Q, Ohnuki Y, Mototani Y, Shiozawa K, Jin M, Cai W, Jin HL, Fujita T, Ichikawa Y, Suita K, Kurotani R, Yokoyama U, Sato M, Iwatsubo K, Ishikawa Y, Okumura S. Pharmacological stimulation of type 5 adenylyl cyclase stabilizes heart rate under both microgravity and hypergravity induced by parabolic flight. J Pharmacol Sci. 2012;119(4):381-9.

216. Braeunig JH, Schweda F, Han PL, Seifert R. Similarly potent inhibition of adenylyl cyclase by P-site inhibitors in hearts from wild type and AC5 knockout mice. PLoS One. 2013;8(7):e68009.

217. Ortiz-Capisano MC, Ortiz PA, Harding P, Garvin JL, Beierwaltes WH. Adenylyl cyclase isoform v mediates renin release from juxtaglomerular cells. Hypertension. 2007;49(3):618-24.

218. Aldehni F, Tang T, Madsen K, Plattner M, Schreiber A, Friis UG, Hammond HK, Han PL, Schweda F. Stimulation of renin secretion by catecholamines is dependent on adenylyl cyclases 5 and 6. Hypertension. 2011;57(3):460-8.

219. Pluznick JL, Zou DJ, Zhang X, Yan Q, Rodriguez-Gil DJ, Eisner C, Wells E, Greer CA, Wang T, Firestein S, Schnermann J, Caplan MJ. Functional expression of the olfactory signaling system in the kidney. Proc Natl Acad Sci U S A. 2009;106(6):2059-64.

220. Rieg T, Tang T, Murray F, Schroth J, Insel PA, Fenton RA, Hammond HK, Vallon V. Adenylate cyclase 6 determines cAMP formation and aquaporin-2 phosphorylation and trafficking in inner medulla. J Am Soc Nephrol. 2010;21(12):2059-68.

221. Musi N, Hayashi T, Fujii N, Hirshman MF, Witters LA, Goodyear LJ. AMP-activated protein kinase activity and glucose uptake in rat skeletal muscle. American journal of physiology Endocrinology and metabolism. 2001;280(5):E677-84.

222. Emery AC, Eiden MV, Eiden LE. A new site and mechanism of action for the widely used adenylate cyclase inhibitor SQ22,536. Mol Pharmacol. 2013;83(1):95-105.

223. Brunskole Hummel I, Reinartz MT, Kalble S, Burhenne H, Schwede F, Buschauer A, Seifert R. Dissociations in the effects of beta2-adrenergic receptor agonists on cAMP formation and superoxide production in human neutrophils: support for the concept of functional selectivity. PLoS One. 2013;8(5):e64556.

224. Vadakkan KI, Wang H, Ko SW, Zastepa E, Petrovic MJ, Sluka KA, Zhuo M. Genetic reduction of chronic muscle pain in mice lacking calcium/calmodulin-stimulated adenylyl cyclases. Mol Pain. 2006;2:7.

225. Wang H, Gong B, Vadakkan KI, Toyoda H, Kaang B-K, Zhuo M. Genetic Evidence for Adenylyl Cyclase 1 as a Target for Preventing Neuronal Excitotoxicity Mediated by N-Methyl-D-aspartate Receptors. J Biol Chem. 2007;282(2):1507-17.

226. Villacres EC, Wong ST, Chavkin C, Storm DR. Type I adenylyl cyclase mutant mice have impaired mossy fiber long-term potentiation. J Neurosci. 1998;18(9):3186-94.

227. Zhang MM, Liu SB, Chen T, Koga K, Zhang T, Li YQ, Zhuo M. Effects of NB001 and gabapentin on irritable bowel syndrome-induced behavioral anxiety and spontaneous pain. Molecular brain. 2014;7:47.

228. Chen T, O'Den G, Song Q, Koga K, Zhang MM, Zhuo M. Adenylyl cyclase subtype 1 is essential for late-phase long term potentiation and spatial propagation of synaptic responses in the anterior cingulate cortex of adult mice. Mol Pain. 2014;10:65.

229. Wayman GA, Wei J, Wong S, Storm DR. Regulation of type I adenylyl cyclase by calmodulin kinase IV in vivo. Mol Cell Biol. 1996;16(11):6075-82.

230. Bayewitch ML, Avidor-Reiss T, Levy R, Pfeuffer T, Nevo I, Simonds WF, Vogel Z. Inhibition of adenylyl cyclase isoforms V and VI by various Gbetagamma subunits. FASEB J. 1998;12(11):1019.

231. Neer EJ, Pulsifer L, Wolf LG. The amino terminus of G protein alpha subunits is required for interaction with beta gamma. J Biol Chem. 1988;263(18):8996-70.

232. Denker BM, Neer EJ, Schmidt CJ. Mutagenesis of the amino terminus of the alpha subunit of the G protein Go. In vitro characterization of alpha o beta gamma interactions. J Biol Chem. 1992;267(9):6272-7.

233. Dbouk HA, Vadas O, Shymanets A, Burke JE, Salamon RS, Khalil BD, Barrett MO, Waldo GL, Surve C, Hsueh C, Perisic O, Harteneck C, Shepherd PR, Harden TK, Smrcka AV, Taussig R, Bresnick AR, Nurnberg B, Williams RL, Backer JM. G protein-coupled receptor-mediated activation of p110beta by Gbetagamma is required for cellular transformation and invasiveness. Sci Signal. 2012;5(253):ra89.

234. Baragli A, Grieco ML, Trieu P, Villeneuve LR, Hebert TE. Heterodimers of adenylyl cyclases 2 and 5 show enhanced functional responses in the presence of Galpha s. Cell Signal. 2008;20(3):480-92.

235. Ding Q, Gros R, Chorazyczewski J, Ferguson SS, Feldman RD. Isoform-specific regulation of adenylyl cyclase function by disruption of membrane trafficking. Mol Pharmacol. 2005;67(2):564-71.

236. Sun Z, Smrcka AV, Chen S. WDR26 functions as a scaffolding protein to promote Gbetagamma-mediated phospholipase C beta2 (PLCbeta2) activation in leukocytes. J Biol Chem. 2013;288(23):16715-25.

237. Premont RT, Matsuoka I, Mattei M-G, Pouille Y, Defer N, Hanoune J. Identification and Characterization of a Widely Expressed Form of Adenylyl Cyclase. J Biol Chem. 1996;271(23):13900-7.

238. Hoshi N, Langeberg LK, Gould CM, Newton AC, Scott JD. Interaction with AKAP79 modifies the cellular pharmacology of PKC. Mol Cell. 2010;37(4):541-50.

239. Zhang J, Ma Y, Taylor SS, Tsien RY. Genetically encoded reporters of protein kinase A activity reveal impact of substrate tethering. Proc Natl Acad Sci U S A. 2001;98(26):14997-5002.

240. Willoughby D, Masada N, Crossthwaite AJ, Ciruela A, Cooper DM. Localized Na+/H+ exchanger 1 expression protects Ca2+-regulated adenylyl cyclases from changes in intracellular pH. J Biol Chem. 2005;280(35):30864-72.

241. Lader AS, Xiao YF, Ishikawa Y, Cui Y, Vatner DE, Vatner SF, Homcy CJ, Cantiello HF. Cardiac Gsalpha overexpression enhances L-type calcium channels through an adenylyl cyclase independent pathway. Proc Natl Acad Sci U S A. 1998;95(16):9669-74.

242. Ayling LJ, Briddon SJ, Halls ML, Hammond GR, Vaca L, Pacheco J, Hill SJ, Cooper DM. Adenylyl cyclase AC8 directly controls its micro-environment by recruiting the actin cytoskeleton in a cholesterol-rich milieu. J Cell Sci. 2012;125(Pt 4):869-86.

243. Ehrlich AT, Furuyashiki T, Kitaoka S, Kakizuka A, Narumiya S. Prostaglandin E receptor EP1 forms a complex with dopamine D1 receptor and directs D1-induced cAMP production to adenylyl cyclase 7 through mobilizing G(betagamma) subunits in human embryonic kidney 293T cells. Mol Pharmacol. 2013;84(3):476-86.

244. Davare MA, Avdonin V, Hall DD, Peden EM, Burette A, Weinberg RJ, Horne MC, Hoshi T, Hell JW. A beta2 adrenergic receptor signaling complex assembled with the Ca2+ channel Cav1.2. Science. 2001;293(5527):98-101.

245. Halls ML, Cooper DM. Sub-picomolar relaxin signalling by a pre-assembled RXFP1, AKAP79, AC2, beta-arrestin 2, PDE4D3 complex. EMBO J. 2010;29(16):2772-87.

246. Lavine N, Ethier N, Oak JN, Pei L, Liu F, Trieu P, Rebois RV, Bouvier M, Hebert TE, Van Tol HH. G protein-coupled receptors form stable complexes with inwardly rectifying potassium channels and adenylyl cyclase. J Biol Chem. 2002;277(48):46010.

247. Rebois RV, Robitaille M, Gales C, Dupre DJ, Baragli A, Trieu P, Ethier N, Bouvier M, Hebert TE. Heterotrimeric G proteins form stable complexes with adenylyl cyclase and Kir3.1 channels in living cells. J Cell Sci. 2006;119(Pt 13):2807-18.

248. Dupre DJ, Baragli A, Rebois RV, Ethier N, Hebert TE. Signalling complexes associated with adenylyl cyclase II are assembled during their biosynthesis. Cell Signal. 2007;19(3):481-9.

249. Takida S, Wedegaertner PB. Heterotrimer formation, together with isoprenylation, is required for plasma membrane targeting of Gbetagamma. J Biol Chem. 2003;278(19):17284-90.

250. Rebois RV, Maki K, Meeks JA, Fishman PH, Hebert TE, Northup JK. D2-like dopamine and beta-adrenergic receptors form a signaling complex that integrates Gs- and Gi-mediated regulation of adenylyl cyclase. Cell Signal. 2012;24(11):2051-60.

251. Efendiev R, Dessauer CW. A kinase-anchoring proteins and adenylyl cyclase in cardiovascular physiology and pathology. J Cardiovasc Pharmacol. 2011;58(4):339-44.

252. Timofeyev V, Myers RE, Kim HJ, Woltz RL, Sirish P, Heiserman JP, Li N, Singapuri A, Tang T, Yarov-Yarovoy V, Yamoah EN, Hammond HK, Chiamvimonvat N. Adenylyl cyclase subtype-specific compartmentalization: differential regulation of L-type Ca2+ current in ventricular myocytes. Circ Res. 2013;112(12):1567-76.

253. Houslay MD, Baillie GS, Maurice DH. cAMP-Specific phosphodiesterase-4 enzymes in the cardiovascular system: a molecular toolbox for generating compartmentalized cAMP signaling. Circ Res. 2007;100(7):950-66.

254. Boivin B, Lavoie C, Vaniotis G, Baragli A, Villeneuve LR, Ethier N, Trieu P, Allen BG, Hébert TE. Functional beta-adrenergic receptor signalling on nuclear membranes in adult rat and mouse ventricular cardiomyocytes. CardiovascRes. 2006;71(1):69-78.

255. Melsom CB, Orstavik O, Osnes JB, Skomedal T, Levy FO, Krobert KA. Gi proteins regulate adenylyl cyclase activity independent of receptor activation. PLoS One. 2014;9(9):e106608.

256. Ping P, Anzai T, Gao M, Hammond HK. Adenylyl cyclase and G protein receptor kinase expression during development of heart failure. American Journal of Physiology: Heart and Circulatory Physiology. 1997;273(2):H707-17.

257. Gao MH, Lai NC, Roth DM, Zhou J, Zhu J, Anzai T, Dalton N, Hammond HK. Adenylylcyclase increases responsiveness to catecholamine stimulation in transgenic mice. Circulation. 1999;99(12):1618-22.

258. Gao MH, Tang T, Guo T, Miyanohara A, Yajima T, Pestonjamasp K, Feramisco JR, Hammond HK. Adenylyl cyclase type VI increases Akt activity and phospholamban phosphorylation in cardiac myocytes. J Biol Chem. 2008;283(48):33527-35.

259. Gao MH, Tang T, Lai NC, Miyanohara A, Guo T, Tang R, Firth AL, Yuan JX, Hammond HK. Beneficial effects of adenylyl cyclase type 6 (AC6) expression persist using a catalytically inactive AC6 mutant. Mol Pharmacol. 2011;79(3):381-8.

260. Ostrom RS, Violin JD, Coleman S, Insel PA. Selective enhancement of beta-adrenergic receptor signaling by overexpression of adenylyl cyclase type 6: colocalization of receptor and adenylyl cyclase in caveolae of cardiac myocytes. Molecular Pharmacology. 2000;57(5):1075.

261. Ostrom RS, Gregorian C, Drenan RM, Xiang Y, Regan JW, Insel PA. Receptor number and caveolar co-localization determine receptor coupling efficiency to adenylyl cyclase. Journal of Biological Chemistry. 2001;276(45):42063.

262. Ostrom RS, Bundey RA, Insel PA. Nitric oxide inhibition of adenylyl cyclase type 6 activity is dependent upon lipid rafts and caveolin signaling complexes. J Biol Chem. 2004;279(19):19846-53.

263. Fagan KA, Smith KE, Cooper DM. Regulation of the Ca2+-inhibitable adenylyl cyclase type VI by capacitative Ca2+ entry requires localization in cholesterol-rich domains. J Biol Chem. 2000;275(34):26530.

264. Smith KE, Gu C, Fagan KA, Hu B, Cooper DM. Residence of adenylyl cyclase type 8 in caveolae is necessary but not sufficient for regulation by capacitative Ca(2+) entry. J Biol Chem. 2002;277(8):6025.

265. Patel HH, Tsutsumi YM, Head BP, Niesman IR, Jennings M, Horikawa Y, Huang D, Moreno AL, Patel PM, Insel PA, Roth DM. Mechanisms of cardiac protection from ischemia/reperfusion injury: a role for caveolae and caveolin-1. FASEB J. 2007;21(7):1565-74.

266. Hippe HJ, Ludde M, Schnoes K, Novakovic A, Lutz S, Katus HA, Niroomand F, Nurnberg B, Frey N, Wieland T. Competition for Gbetagamma dimers mediates a specific cross-talk between stimulatory and inhibitory G protein alpha subunits of the adenylyl cyclase in cardiomyocytes. Naunyn Schmiedebergs Arch Pharmacol. 2013;386(6):459-69.

267. Scherer NM, Toro MJ, Entman ML, Birnbaumer L. G-protein distribution in canine cardiac sarcoplasmic reticulum and sarcolemma: comparison to rabbit skeletal muscle membranes and to brain and erythrocyte G-proteins. Archives of biochemistry and biophysics. 1987;259(2):431-40.

268. Kamal FA, Smrcka AV, Blaxall BC. Taking the heart failure battle inside the cell: small molecule targeting of Gbetagamma subunits. J Mol Cell Cardiol. 2011;51(4):462-7.

269. Kamal FA, Mickelsen DM, Wegman KM, Travers JG, Moalem J, Hammes SR, Smrcka AV, Blaxall BC. Simultaneous adrenal and cardiac g-protein-coupled receptor-gbetagamma inhibition halts heart failure progression. J Am Coll Cardiol. 2014;63(23):2549-57.

270. Avidor-Reiss T, Nevo I, Saya D, Bayewitch M, Vogel Z. Opiate-induced Adenylyl Cyclase Superactivation Is Isozyme-specific. J Biol Chem. 1997;272(8):5040-7.

271. Avidor-Reiss T, Nevo I, Levy R, Pfeuffer T, Vogel Z. Chronic opioid treatment induces adenylyl cyclase V superactivation. Involvement of Gbetagamma. J Biol Chem. 1996;271(35):21309-15.

272. Ejendal KF, Dessauer CW, Hebert TE, Watts VJ. Dopamine D(2) Receptor-Mediated Heterologous Sensitization of AC5 Requires Signalosome Assembly. Journal of signal transduction. 2012;2012:210324.

273. Schmid MC, Schulein R, Dehio M, Denecker G, Carena I, Dehio C. The VirB type IV secretion system of Bartonella henselae mediates invasion, proinflammatory activation and antiapoptotic protection of endothelial cells. Molecular microbiology. 2004;52(1):81-92.

274. Schmid MC, Scheidegger F, Dehio M, Balmelle-Devaux N, Schulein R, Guye P, Chennakesava CS, Biedermann B, Dehio C. A translocated bacterial protein protects vascular endothelial cells from apoptosis. PLoS pathogens. 2006;2(11):e115.

275. Pulliainen AT, Pieles K, Brand CS, Hauert B, Bohm A, Quebatte M, Wepf A, Gstaiger M, Aebersold R, Dessauer CW, Dehio C. Bacterial effector binds host cell adenylyl cyclase to potentiate Galphas-dependent cAMP production. Proc Natl Acad Sci U S A. 2012;109(24):9581-6.

Vita

Cameron Servetus Brand was born in Houston, TX. He attended the University of Texas at Dallas, majoring in molecular biology. He graduated and received his Bachelor of Science degree in May 2009. The following fall he enrolled at the Graduate School of Biomedical Sciences at the University of Texas Health Science Center at Houston, joining the Cell and Regulatory Biology program. He received his Master of Science degree in Biomedical Sciences in December 2012, and will receive his Doctor of Philosophy degree in Cell and Regulatory Biology in May 2015.