Role of Gβγ for the Cellular Regulation of Adenylyl Cyclase Isoforms Type V and Type VI

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CONTENTS

ABBREVIATIONS

β-AR	β-adrenergic receptor
βARK	β-adrenergic receptor kinase
A_{260}	absorption at 260 nm
AA	amino acid
AC	adenylyl cyclase
Amp	ampicillin
AP	alkaline phosphatase
APS	ammonium persulfate
cAMP	cyclic adenosine-3',5'-monophosphate
cGMP	cyclic guanosine-3',5'-monophosphate
ATP	adenosine 5'-triphosphate
Bis	N,N'-methylene-bis-acrylamide
Bp	base pairs
BSA	bovine serum albumin
CaM	calmodulin
Ci	Curie
CK	creatine kinase
CMF-PB	calcium and magnesium free phosphate buffer
CP	creatine phosphate
Cpm	counts per minute
CSPD	disodium 3-(4-methoxyspiro{1,2-dioxethane-3,2'-(5'-chloro)tricyclo [3.3.1.1.^{3,7}]decan}-4-yl) phenyl phosphate
CTX	cholera toxin
Da	Dalton
DAG	diacylglycerine
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulfoxide
DNase	deoxyribonuclease
DTT	dithiothreitol
EDTA	ethylenediamine -N,N,N'N'-tetraaceticacid
EGTA	ethylene glycol-bis-(2-aminoethylether)-N,N,N'N'-tetraacetic acid
FCS	fetal calf serum

FITC	fluoroisothiocyanate
g	gravity
Gal	β-galactosidase
Gα	α-subunit of a heterotrimeric G-protein
Gβ	β-subunit of a heterotrimeric G-protein
Gγ	γ-subunit of a heterotrimeric G-protein
Gβγ	βγ-dimer of a heterotrimeric G-protein
G_i	inhibitory G-protein
G_s	stimulatory G-protein
G_t	transducin
GDP	guanosine-5'-diphosphate
G-protein	heterotrimeric guanine nucleotide binding protein
GTP	guanosine-5'-triphosphate
GTPγS	guanosine-5'-[γ-thio]-triphosphate
HEK 293 cells	human embryonic kidney cells
h	hour
IgG	immunoglobulin G
IM	incubation medium
IP_3	inositol-1,4,5,-triphosphate
kDa	kiloDalton
LB medium	Luria-Bertani medium
M	mole per liter
MEM	minimal essential medium
min	minute
MK	myokinase
MOPS	3-(N-morpholino) propane sulfonicacid
Nt	nucleotide
OD	optical density
PAA	polyacrylamide
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pH	logarithmic measure of hydrogen ion concentration
PI	phosphatidylinositol

PI-4,5-P_2	phosphatidylinositol-4,5-bisphosphate (PIP_2)
PI-3,4,5-P_3	phosphatidylinositol-3,4,5-triphosphate (PIP_3)
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PTX	pertussis toxin
PMSF	phenylmethylsulfonylfluoride
PVDF	polyvinylidene fluoride
RNase	ribonuclease
Rpm	rotations per minute
s	second
S.D.	standard deviation
SDS	sodium dodecyl sulfate
Sf9 cells	cells derived from the pupal ovary of *Spodoptera frugiperda*
SIN-1	3-morpholino-sydnonimine
STI	soybean trypsin inhibitor
TAE	tris-acetate-EDTA buffer solution
TCA	trichloroaceticacid
TEMED	N, N, N', N'-tetramethylethylenediamine
TLCK	N-p-tosyl-l-lysine chloromethyl ketone
TPCK	N-p-tosyl-l-phenylalanine chloromethyl ketone
TPA	12-O-tetradecanoyl phorbol-13-acetate
Tris	tris-(hydroxymethyl)-aminomethane
Tween 20	polyoxyethylene-(20)-monolaurate
U	unit for enzyme activity
UTP	uridine 5'-triphosphate
UV	ultraviolet
% (v/v)	volume/volume percent
% (w/v)	weight/volume percent
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
YT medium	yeast type medium

1 INTRODUCTION

1.1 Cellular Signal Transduction

Each cell division and differentiation, each immune response, each synaptic transmission throughtout development and through the life span of an individual is instigated by a biological signal communicated between cells. The receipt and intracellular propagation of such a signal and its interpretation by the cell and subsequent response, is referred to as signal transduction. A signaling molecule may be a protein, a small peptide, an amino acid, a nucleotide, a steroid, a retinoid, a fatty acid derivative, or a dissolved gas. The signaling molecule can be detected *via* specific receptors which trigger cellular responses that cause alterations in cellular activity and/or changes in gene expression.

Nonpolar signaling molecules, such as estrogens and other steroid hormones, which are able to diffuse through cell membranes due to their lipophilic properties, can bind to proteins that interact directly with DNA and modulate gene transcription (Albert, 2005). However, most of the signaling molecules are hydrophilic in nature and cannot pass through the cell membrane. Instead, signal transmission to the inside of the cell occurs *via* membrane-localized, cell surface receptors. These receptors are integral membrane proteins that undergo an alteration in their structure upon binding of the signal substance (the first messenger) on the outside of the membrane. This structural alteration triggers the activation, production, or release of a "secondary messenger" on the inside of the membrane. These transmembranous (TM) receptors are divided into three classes, based on the transduction mechanism used to propagate signals into the cell's interior (Scott *et al*., 2000).

I) Ligand-operated ion channels also known as ion-channel-coupled receptors, are involved in rapid synaptic signaling between electrically excitable cells. This type of signaling is mediated by a number of neurotransmitters that transiently open or close the ion channel formed by the protein to which they bind. The binding of the neurotransmitters briefly changes the ion permeability of the plasma membrane, and thereby the excitability of the postsynaptic cell (Figure 1a) (Kotzyba-Hibert *et al*., 1999; MacDermott *et al*., 1999; Whiting *et al*., 1999). Nicotinic receptors (Barry *et al*., 2005), 5-HT_3 receptors (Kawanabe *et al*., 2005), $GABA_A$ and $GABA_C$ receptors (Kneussel, 2005), glycine receptors (Jentsch *et al*., 2002), and some glutamate, histamine and serotonin activated anionic channels

(Hamill *et al*., 1996; Jordan, 2005) are examples of ion-channel-coupled receptors.

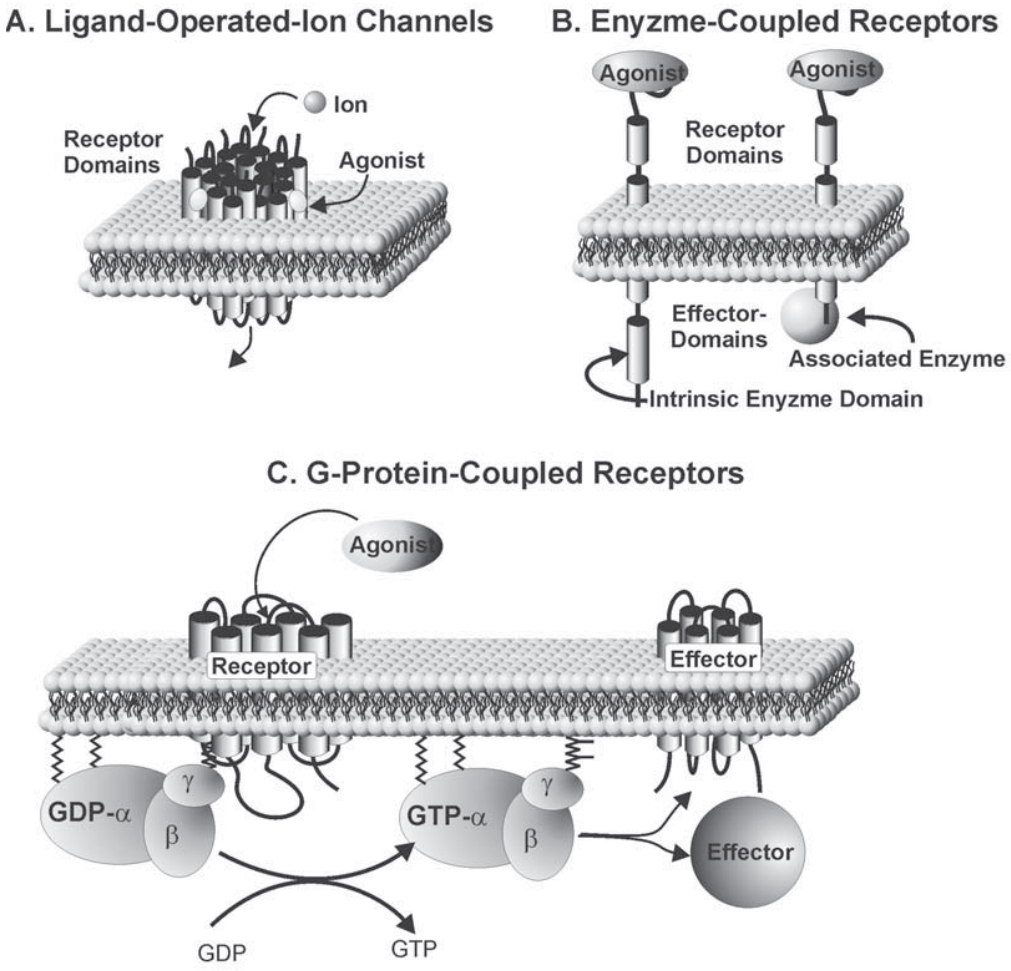


Figure 1: Principles of signal transduction through cell membrane receptors.

Extracellular stimulation can trigger intracellular effects *via* different receptor classes. A) Ligand-operated-ion channels form an extracellular receptor domain and a transmembraneous (TM) channel pore, which is controlled by the ligands. B) Enzyme-coupled receptors contain catalytic sites or associate with enzymes. C) The class of G-protein-coupled receptors transmits the signals to effectors *via* heterotrimeric G-proteins composed of an α-subunit and a βγ dimer. The effectors are either membrane-bound or located in the cytosol.

II) Enzyme-coupled receptors have an extracellular ligand-binding domain and a cytosolic domain that either contains intrinsic enzyme activity or is able to associate directly with an enzyme (Figure 1b). The enzyme-coupled receptors are classified based on the type of enzymatic activity they use to transduce the signal. For example some receptors (*e.g.*, the natriuretic peptide receptors) have guanylyl cyclase activity and generate cyclic guanosine monophosphate as an intracellular mediator (Schulz, 2005). Tyrosine kinase receptors

have tyrosine kinase activity or are associated with proteins containing tyrosine kinase activity, which propogate intracellular signals by phosphorylating specific tyrosine residues on intracellular proteins (Roskoski, Jr., 2005). The majority of growth factor receptors belong to the tyrosine kinase receptors. These include receptors for epidermal growth factor, platelet-derived growth factor, fibroblast growth factor, hepatocyte growth factor, insulin, insulin-like growth factor-1, vascular endothelial growth factor, and macrophage-colony stimulating factor. Serine/threonine kinase-coupled receptors have serine and threonine kinase activity (*e.g.*, receptors for the transforming growth factor-β/activin protein family) and can phosphorylate specific serine or threonine residues to transduce intracellular signals (Krieglstein *et al*., 2002). These growth factor receptors play crucial roles during normal development and tissue homeostasis (Itoh *et al*., 2000). In addition to receptors with kinase activity, there are receptors with tyrosine phosphatase activity. These receptor tyrosine phosphatases remove phosphate groups from tyrosine residues of specific intracellular signaling proteins (Huntington *et al*., 2004; Irie-Sasaki *et al*., 2003).

III) G-protein-coupled receptors (GPCRs) represent the largest group of cell surface receptors. GPCRs are characterized by a seven TM topology (Figure 1c). A diverse array of ligands binds to and activates GPCRs, including photons, odorants, neurotransmitters, hormones, glycoproteins, and chemokines, leading to diverse biological responses. GPCRs can be divided into three different families (A, B, C) with divergent primary sequences. Based on sequence homology, 89% of GPCRs fall into family A (Jastrzebska *et al*., 2006; Surgand *et al*., 2006; Thevenin *et al*., 2005), family B (Harmar, 2001) and family C (Pin *et al*., 2003) (Pin *et al*., 2004) constitute only 7% and 4% of GPCRs, respectively (Fredriksson *et al*., 2003; Menzaghi *et al*., 2002).

In contrast to ion-channel-coupled receptors and enzyme-coupled-receptors, G-protein-coupled receptors possess no effector domain. Instead, they activate a heterotrimeric G-protein that modulates the activity of effectors (Figure.1c). G-protein-regulated effectors can be TM proteins (*e.g.*, adenylyl cyclases), ion-channels, membrane-associated proteins (*e.g.*, phospholipases), or cytosolic proteins (*e.g.*, phosphatidylinositide-3-kinases). The modulation of these effectors results in the production of second messengers, such as cyclic adenosine monophosphate (cAMP) (Watts *et al*., 2005), Ca^{2+} (Landry *et al*., 2006), or inositol trisphosphate (IP_3) (Ohno-Shosaku *et al*., 2005). These second messengers are soluble and can diffuse to some extent into the cytoplasm; whereas second messengers such as diacylglycerol (DAG) and phosphatidylinositol-3,4,5-triphosphate are

membrane-associated lipids with limited diffusibility (Rhee, 2001). Second messengers control a multitude of biological responses, including neuronal transmission, cellular immune responses, sensory and tactile responses, stress, growth, and reproduction (Roberts *et al*., 2004). In the following paragraph, G-protein-mediated signal transduction will be described in more detail.

1.2 G-Protein-Mediated Signal Transduction

1.2.1 Heterotrimeric G-Proteins

Heterotrimeric G-proteins consist of an α-subunit and a βγ-dimer. They are membrane bound and are closely associated with the intracellular domains of GPCRs (Chen *et al*., 2001). G-proteins bind to and are activated by liganded GPCRs. The specific protein combination of α-, β-, and γ-subunits, in combination with other regulatory and scaffolding proteins, connects a particular GPCR to a specific effector (Milligan *et al*., 2006).

In mammals, there are at least 16 different Gα-subunits, 5 different Gβ-subunits, and 12 different Gγ-subunits, which associate to form various heterotrimeric G-proteins (McCudden *et al*., 2005). The variety of Gβγ-dimers that can be formed adds to the large diversity of G-proteins and may be another mechanism regulating receptor-G-protein specificity (Cabrera-Vera *et al*., 2003). The α-subunits typically have an apparent molecular weight of 39 - 45 kD (Nürnberg *et al*., 1995) and contain the GTPase domain of the heterotrimer, which can bind and hydrolyze GTP. The β-(~35 - 38 kD) and γ-(~ 5 - 8 kD) subunits are tightly associated with each other and are thus often referred to as a functional monomer (Myung *et al*., 2006). GDP-bound Gα-subunits bind tightly to the Gβγ heterodimer. This association localizes Gα to the plasma membrane and is essential for the functional coupling of G-proteins to GPCRs (Evanko *et al*., 2005). In addition, Gβγ binding to GDP-bound Gα slows the spontaneous rate of GDP release, thus acting as a guanine-nucleotide dissociation inhibitor (GDI) (Higashijima *et al*., 1987). Agonist-bound GPCRs act as guanine nucleotide exchange factors (GEFs), promoting the release of bound GDP from Gα. The transition state nucleotide-free Gα then binds GTP which is present at a significantly higher cellular concentration than GDP. The binding of GTP causes conformational changes within the three flexible switch regions of Gα (Wall *et al*., 1995), resulting in the dissociation of Gβγ. Both GTP-bound Gα and free Gβγ are capable of initiating signals by interacting with downstream effector proteins, such as protein kinases (*e.g.*, PKC), ion-channels (*e.g.*, K^+-channels), enzymes (*e.g.*, adenylyl cyclase), GEFs for

the GTPase RhoA ("RhoGEFs"), and other effector molecules (Forse, 2000), thus regulating a multitude of cellular functions. The intrinsic GTPase activity of the Gα-subunit causes the hydrolysis of GTP to GDP. Thus, the G-protein cycle is reset by the formation of "inactive" Gα-GDP which has a low affinity for effectors, but a high affinity for Gβγ and is capable once again of interacting with GPCRs (McCudden *et al*., 2005). The duration of the G-protein signal is determined by the lifetime of the GTP on the Gα-subunit, which can also be controlled by the regulators of G-protein signaling (RGS) proteins or by covalent modifications (Chen *et al*., 2001). RGS proteins are GTPase-accelerating proteins that increase the GTP hydrolysis rate of Gα and thus initiate the inactivation of the G-protein and consequently the termination of the GPCR signaling pathway (Berman *et al*., 1996).

1.2.1.1 Gα-Subunits and their Effectors

Gα-subunits can be grouped into four families: I) the G_s family (G_s and G_{olf}), which is best recognized as activators of adenylyl cyclases (AC), II) the large and functionally diverse G_i family of pertussis toxin (PTX)-sensitive G-proteins that inhibit ACs, III) the G_q family, whose members are activators of phospholipase C-β, and IV) the recently described G_{12} family that comprises cytoskeletal-regulatory G-proteins (Strathmann *et al*., 1991) (Table.1).

All four classes of Gα-subunits have well-established cellular targets. The first identified Gα-effector was adenylyl cyclase (AC) (see section 1.4), discovered by Sutherland and Rall (Sutherland *et al*., 1960; Rall, 1971). After the identification of AC as the enzyme responsible for the activation of the secondary messenger, cAMP, a GTP-binding-protein that stimulated AC was isolated, which is now referred to as $G\alpha_s$ (Ross *et al*., 1977).

Co-crystallization studies of $G\alpha_s$ together with the catalytic domains of AC have identified specific G-protein/AC contact sites at the α2-β4 and the α3-β5 loops of $G\alpha_s$. In addition, the α4-β6 loop of $G\alpha_s$ also plays an indirect role in AC activation (Grishina *et al*., 2000). Interestingly, it has been demonstrated that GDP-bound $G\alpha_s$ can also stimulate AC, albeit with a much lower potency than GTP-bound-$G\alpha_s$ (Sunahara *et al*., 1997). In addition to a stimulation of AC by $G\alpha_s$, an increased concentration of Gβγ-dimers can lead to a synergistic stimulation of AC, as has been demonstrated for AC II and AC IV (Weitmann *et al*., 2001). $G\alpha_{olf}$ is an olfactory-bulb-specific G-protein that is a member of the $G\alpha_s$ family and activates AC like $G\alpha_s$ (Olianas *et al*., 1999).

Table 1: Classification and functional properties of Gα-subunits (Nürnberg, 2004)

Family	Subunit	Tissue distribution	Effector / Role
G_s	$G\alpha_{s(s),\ s(l)}$[a]	ubiquitous	↑AC
	$G\alpha_{s(xl),\ s(xxl)}$[a]	neurons, neuroendocrine	↑AC
	$G\alpha_{olf}$	olfactory epithelium, brain, testes, pancreas	↑AC
G_i	$G\alpha_{i1}$	mostly neurons	↓ACI,V,VI, (↑GIRK, ↑PLCβ?)[b]
	$G\alpha_{i2}$	ubiquitous	↓ACI,V,VI, (↑GIRK,↑PLCβ,↑PI3K)[b]
	$G\alpha_{i3}$	mostly non-neuronal	↓ACI,V,VI, (↑GIRK,↑PLCβ,↑PI3K?)[b]
	$G\alpha_{o1,2,3}$[c]	neurons, neuroendocrine, cardiac myocytes?	↓AC?, ↓VDCC[b], ↑GIRK, ↑PLCβ?
	$G\alpha_{t(r)}$	retinal rods, taste cells	↑cGMP-PDE
	$G\alpha_{t(c)}$	retinal cones,	↑cGMP-PDE
	$G\alpha_{gust}$	taste cells, GI brush cells	↑PDE?
	$G\alpha_{z}$	neurons, endocrine, platelets	↓ACI, ACV, (↑GIRK, ↓VDCC)[b]
G_q	$G\alpha_{q}$	ubiquitous	↑PLC-β, Rho-GEF
	$G\alpha_{11}$	widely, not platelets	↑PLC-β, Rho-GEF
	$G\alpha_{14}$	testis, hematopoietic cells and tissues	↑ PLC-β
	$G\alpha_{15/16}$[d]	hematopoietic cells and tissues	↑ PLC-β
G_{12}	$G\alpha_{12}$	ubiquitous	↑Rho-GEF, ↑Btk, ↑Gap1[m], cadherin
	$G\alpha_{13}$	ubiquitous	↑Rho-GEF, radixin

a: (s) and (l) indicate short and long splice variants of $G\alpha_s$; (xl) and (xxl) indicate additional (extra)-long splice variants of $G\alpha_s$. Receptor coupling of $G\alpha_s$ (xl)(xxl) has not been demonstrated so far. b: Regulation of the effector presumably depends on direct interaction with Gβγ-dimers released from PTX-sensitive heterotrimeric G-protein. c: $G\alpha_{o3}$ corresponds to deamidated $G\alpha_{o1}$ (346Asn→Asp), representing 30% of total $G\alpha_o$ in brain. d: $G\alpha_{15}$ and $G\alpha_{16}$ are the mouse and human homologues of the GNA 15 gene product, respectively. ↑: stimulation; ↓: inhibition; AC: adenylyl cyclase, α_1-AR: α_1-adrenergic, α_2AR: α_2-adrenergic receptor, β-AR: β-adrenergic receptor, Btk: Bruton´s tyrosine kinase, cGMP-PDE: cGMP-phosphodiesterase, Gap1m: Ras GTPase-activating protein, GI: gastrointestinal; GIRK: G-protein-regulated inward rectifier K^+-channel, met-enk: met-enkephalin, M_1Cho: M_1-muscarinic cholinergic, M_2Cho: M_2-muscarinic cholinergic, PLC-β: phospholipase C-β, PI3K: phosphatidylinositol-3-kinase, Rho-GEF: guanine nucleotide exchange factor of the monomeric GTPase Rho, VDCC: voltage-dependant Ca^{2+}-channel.

Certain bacterial toxins have become important pharmacological tools for the investigation of G-protein-mediated signal transduction, *e.g.,* cholera toxin (CTX) and pertussis toxin (PTX). Cholera toxin is an enzyme released by *Vibrio cholerae* that attaches the adenine diphosphate (ADP)-ribose moiety of an intracellular nicotinamide adenine dinucleotide to the side chain of an arginine residue in human $G\alpha_s$ (at position 201). The side chain of this arginine accelerates GTP hydrolysis by orienting an oxygen atom of the γ-phosphate in a

position that is optimal for catalysis. The attachment of ADP-ribose to the side chain markedly slows GTP hydrolysis and locks $G\alpha_s$ in its active, GTP-bound form (Merritt *et al*., 1995).

The family of G_i-proteins includes Gαi-isoforms and the related $G\alpha_o$-isoforms. The G_i-protein family is characterized by its sensitivity to pertussis toxin (PTX) (Wu *et al*., 2005). PTX, the pathogenic toxin of *Bordetella pertussis,* is similar to CTX in that it catalyzes the attachment of ADP-ribose to the side chain of a cysteine, which is located in the C-terminal tail of the Gα-subunit of the G_i-family. This modification prevents the interaction of G-proteins with their receptors (Sprang, 1997). $G\alpha_i$-, $G\alpha_o$-, and $G\alpha_z$-proteins have an inhibitory effect on AC (Watts *et al*., 2005). The closely related $G\alpha_{i2}$ and $G\alpha_{i3}$ can be distinguished by their effects on AC: $G\alpha_{i2}$ inhibits forskolin-stimulated AC activity, whereas $G\alpha_{i3}$ inhibits $G\alpha_s$-stimulated AC activity (Obadiah *et al*., 1999). Members of the $G\alpha_o$ subfamily inhibit the voltage-dependant Ca^{2+}-channels (Clark *et al*., 2004). In addition, $G\alpha_i$ proteins are also found on endomembranes and hence may function as universal switches of a broad range of cellular processes (Gohla *et al*., 2007; Holtje *et al*., 2000; Nürnberg *et al*., 1996).

Transducin is a retina-specific G-protein which activates a cGMP-dependent phosphodiesterase. $G\alpha_{gust}$ (or gustducin), a taste bud-specific, transducin-like G-protein, activates a cAMP-degrading phosphodiesterase (cAMP-PDE) (Caicedo *et al*., 2003; Yan *et al*., 2001). Similarly, vision is dependent on GPCR-mediated phototransduction, a unique signaling cascade that utilizes $G\alpha_t$ dissociated from rhodopsin and Gβγ, and activates cGMP-phosphodiesterase (cGMP-PDE). This active PDE hydrolyses cGMP which controls the conductivity of cyclic GMP-gated Na^+/Ca^{2+}-channels in the plasma membrane (Kasahara *et al*., 2000; Wada *et al*., 2000). $G\alpha_{t(r)}$ and $G\alpha_{t(c)}$ are present in the rod and cone, respectively (Arshavsky *et al*., 2002).

The Gq-family together with the related G-proteins ($G\alpha_{11}$, $G\alpha_{14}$, and $G\alpha_{15/16}$) activate phosphoinositide-specific phospholipase C (PI-PLC-β) isozymes (Rhee *et al*., 2000). PI-PLCs hydrolyze the phosphoester bond of the plasma membrane lipid phosphatidylinositol 4,5-biphosphate (PIP_2), generating the ubiquitous second messengers inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) (Rhee, 2001). The hydrophilic Ins-1,4,5-P_3 can translocate to the endoplasmic reticulum (ER), and stimulate the release of Ca^{2+} from the ER by binding to the IP_3 receptors. The lipophilic DAG, on the other hand, remains in the membrane and activates protein kinase C, which, in the presence of Ca^{2+}-ions, can phosphorylate serine and threonine residues of various proteins. Intracellular Ca^{2+} is

increased by two distinct mechanisms that are G_q dependent: I) $G\alpha_q$ may activate Ptd Ins-4,5-P_2 hydrolysis and induce release of Ca^{2+} from the ER, II) $G\alpha_{11}$ may enhance the Ca^{2+}-activated Ca^{2+}-influx that replenishes intracellular Ca^{2+}-stores (Macrez-Lepretre *et al*., 1997). $G\alpha_q$ also activates Bruton´s tyrosine kinase (Btk) *in vivo* and *in vitro*, and this activation is required for receptor-mediated stimulation of the p38 mitogen-activted protein kinase (MAPK) (Jiang *et al*., 1998).

Among the various G-proteins identified so far, $G\alpha_{15}$ is unique with respect to its promiscuous receptor-coupling properties. G_{15} is found exclusively in the murine hematopoietic cell lineage, shares the closest sequence similarity with $G\alpha_q$, and likewise activates PLCβs. Using a heterologous expression system, Offermanns and collegues demonstrated an indiscriminate coupling of G_{15} to any tested GPCR, including those that do not naturally stimulate PLCβ (Offermanns *et al*, 2001). $G\alpha_{15}$ and its human counterpart $G\alpha_{16}$ share 85% amino-acid sequence identity. However, the functional characterization of $G\alpha_{16}$ has revealed that although $G\alpha_{16}$ couples to many GPCRs similar to $G\alpha_{15}$, it is not as indiscriminating as $G\alpha_{15}$ (Blahos *et al*., 2001).

The family of G_{12}-proteins is ubiquitously expressed. The two members of this G-protein family, $G\alpha_{12}$ and $G\alpha_{13}$, has been implicated in events such as stress fiber formation (Buhl *et al*., 1995; Gohla *et al*., 1999a), cellular transformation (Cvejic *et al*., 2000), regulation of Na^+/H^+ (Dhanasekaran *et al*., 1994), and modulation of inducible nitric oxide synthase expression and regulation of Erk and c-jun-N-terminal kinase activity (Arai *et al*., 2003). $G\alpha_{12/13}$ proteins can regulate the small G-protein RhoA *via* effectors that possess Dbl-homology (DB) and pleckstrin-homology (PH) domains characteristic of Rho-family guanine nucleotide exchange factors (RhoGEFs) (Kristelly *et al*., 2004). It was shown that the GEFs for Rho, p115RhoGEF, PDZ-RhoGEF and LARG are effectors of $G\alpha_{12}$ and $G\alpha_{13}$ (Booden *et al*., 2002; Gohla *et al*., 2000; Kelly *et al*., 2006). In addition, various other proteins like Btk, Ras GTPase-activating protein (Gap1m), cadherin or radixin have been shown to interact with $G\alpha_{12}$ and $G\alpha_{13}$ (Meigs *et al*., 2002).

1.2.1.2 Gβ-Subunits and Gγ-Subunits

Contrary to the Gα-subunits, Gβγ-complexes possess no intrinsic enzymatic activity and are much more lipophilic. Despite the fact that Gβγ consists of two polypeptide chains, Gβ (β_1-β_5: 340-353 amino acids) and Gγ (γ_1-γ_{14} 68-75 amino acids), Gβγ is generally considered as a functional monomer because Gβγ is a tightly complexed dimer which

dissociates only under denaturing conditions (Figure 2).

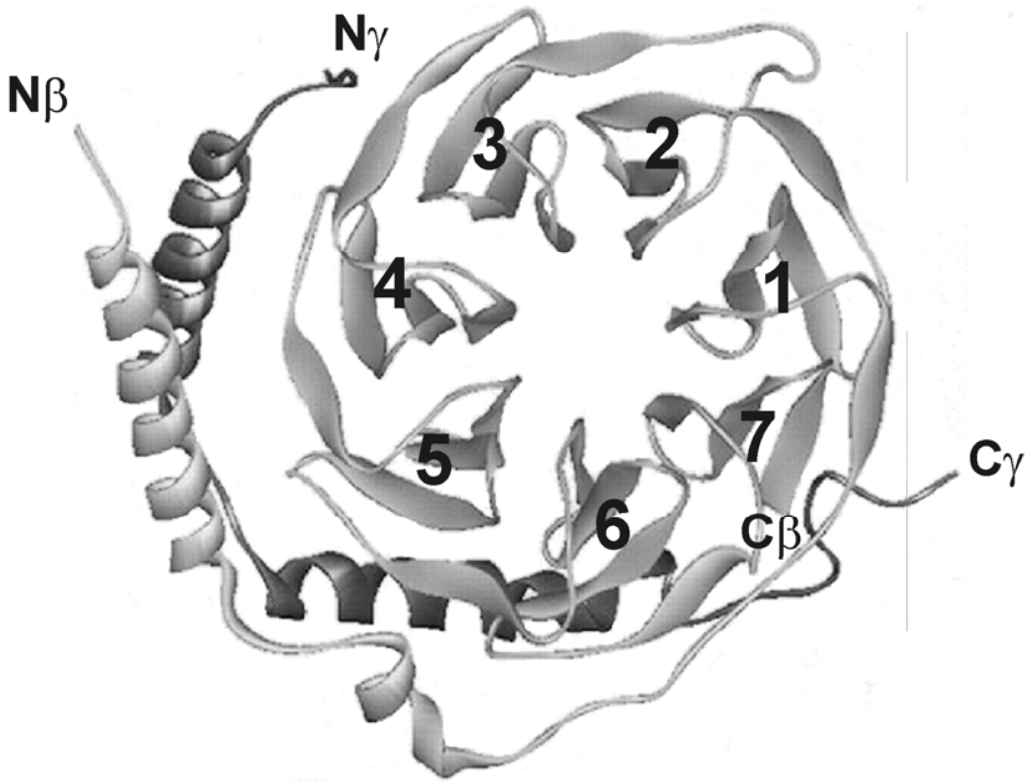


Figure 2: Structure of Gβγ.

The structure of the Gβγ-dimer shows that Gβ (light grey) forms a seven-blade propeller consisting of seven WD-40 repeats. The blades of $G\beta_1$ are numbered so that the first WD repeat occurs in blade 1. Gγ (dark grey) forms two α-helices that bind to the single α-helix of Gβ and to several of the WD-40 blades (Lambright *et al*., 1996; Wall *et al*., 1995).

Gβ-subunits contain seven repeating units of approximately 43 amino acids each, as well as an amino-terminal region that forms an amphipathic α-helix. These repeating units are examples of tryptophan-aspartic acid (WD) repeats, a motif found in a variety of proteins. The WD repeats consist of approximately 40 amino acids, typically bracketed by glycine-histidine (GH) and tryptophan-aspartic acid (WD) and a variable region of seven to eleven amino acids (Garcia-Higuera *et al*., 1998). Crystal structure analysis indicates that the WD repeats of the Gβ-subunit define the stereochemistry of the overall structure by forming a β-propeller of seven β-sheets. Each sheet contains four antiparallel strands radiating outward from a central core (Springer, 1997). At the end of the fourth strand, a loop connects the periphery of one sheet with the central strand of the next sheet, and the structure repeats, forming a seven member β-propeller. The entire structure, including the seven membered β-propeller, is called a β-super-barrel (Wall *et al*., 1995).

The γ-subunit is nestled in a shallow groove formed on one side by the extended amino terminus of the β-portion and on the other by polypeptide loops that connect to β-blades five, six, and seven. The γ-chain runs parallel to the β-amino-terminus, and it is composed of simple α-helices that form a coiled-coil region. A coiled-coil is formed when two or more α-helices wrap around each other to form a left-handed supercoil. Throughout the signaling cycle, as the βγ dimer interacts with Gα, receptors and effectors, the β- and γ-subunits remain in a tightly bound state (Pellegrino *et al*., 1997).

Table 2: Classification and expression patterns of Gβ- and Gγ-subunits (Nürnberg, 2004)

Subtype	**Gβ-subunit**	**Tissue distribution**
Family I:	$G\beta_1$	widely
	$G\beta_2$[a]	widely
	$G\beta_3$[b]	widely, cone and taste cells
	$G\beta_{3(s)}$	?
	$G\beta_4$	neurons, lung, placenta
Family II:	$G\beta_5$[b, c]	neurons, lung, germ cells, lymph, ovary
	$G\beta_{5(s)}$	retina
Subtype	Gγ-subunit	Tissue distribution
Family I:	$G\gamma_1$[a], $G\gamma_{rod}$	retinal rods, neurons
	$G\gamma_9$ ($G\gamma_{cone}$, $G\gamma_{14}$)	retinal cones, neurons
	$G\gamma_{11}$	widely, non-neuronal cells/tissues
Family II:	$G\gamma_5$	widely, placenta, liver
	$G\gamma_{10}$	widely, brain, placenta
Family III:	$G\gamma_2$ ($G\gamma_6$)	widely, neurons
	$G\gamma_3$	widely, neurons, haematopoietic cells
	$G\gamma_4$	widely
	$G\gamma_7$	widely, brain, thymus, eye
	$G\gamma_8$ ($G\gamma_{olf}$)	neurons, olfactory epithelia
	$G\gamma_{12}$	ubiquitous
Family IV: (proposed)	$G\gamma_{13}$	widely, lingual and olfactory epithelium, neurons

a: $G\beta_2$ cannot form dimers with $G\gamma_1$. b: Splice variants exist. c: $G\beta_5$ forms dimers with certain regulators of G-protein signaling (RGS6,7,9,11); dimerization of $G\beta_5$ with Gγ has been observed following recombinant expression of $G\beta_5$ and Gγ. Besides being widely expressed at low levels, Gβ5 is more abundanly found in the indicated tissues.

Similar to the Gα-subunits, the Gβ- and Gγ-subunits have been classified on the basis of their amino acid sequences (Table 2). The molecular weight of the Gβ-subunits varies

from 35 to 39 kDa (340-353 amino acids); whereas, the Gγ-subunits are small proteins variing from 6 to 8 kDa (68-75 amino acids). The Gβ family consists of seven members ($G\beta_1$-$G\beta_5$ and the short (s) and long (l) splice variant of $G\beta_{3(s/l)}$ and $G\beta_{5(s/l)}$), and twelve Gγ-subunits are known (Garcia-Higuera *et al*., 1996; Schwindinger *et al*., 2001; Sondek *et al*., 1996) (Table 2).

The amino acid sequence identity of the $G\beta_{1\text{-}4}$ isoforms vary from 78%-88%, and this high degree of sequence homology may correspond to the similarity of their functional properties. Gβ- and Gγ-subunits are grouped into subfamilies (Table 2). For the Gβ I family members, no significant differences in the interaction with effectors such as the Gα-subunit or receptors have been reported (Gautam *et al*., 1998). The Gβ II-family consists only of $G\beta_5$ and has only 51%-53% similarity with Gβ I-family in the amino acid sequence. $G\beta_5$ has an extension of 13 additional amino acid residues (Brunk *et al*., 1999; Jones *et al*., 2004; Watson *et al*., 1996a). Interestingly, the association of $G\beta_5$ with the Gγ-subunit is weaker than in other Gβγ-dimers, as demonstrated by the higher detergent lability of $G\beta_5\gamma_2$ dimers (Jones *et al*., 1999; Lukov *et al*., 2004).

The Gγ-subunits show a higher structural diversity than the Gα- and Gβ-subunits, ranging from 24%-76% identity in the amino acid sequence (Gautam *et al*., 1998). They are classified into three subfamilies, based on the amino acid identity and differences in post-translational modification. The recently discovered $G\gamma_{13}$ shows only 24-32% amino acid identity to all known isoforms and could thus be the prototype of a 4th subfamily (Huang *et al*., 2003). Gγ-subunits are modified by an isoprenylation of a cysteine residue within the so-called CaaX motif (Cysteine-aliphatic-aliphatic-X, where X is either leucine or serine) in the C-terminus of the protein (Casey *et al*., 1996; Fukada *et al*., 1990). Most of the Gγ-subunits posess a leucine at the C-terminal amino acid of the CaaX motif. In this case, the cysteine lying within the motif is modified with a C20-geranyl-geranyl residue. If the last amino acid is a serine, as is the case with the $G\gamma_1$-, $G\gamma_9$-, and $G\gamma_{11}$-isoforms, then the Gγ-subunit is C15-farnesylated (Chen *et al*., 2004; De Gunzburg, 1991; Parish *et al*., 2000). The isoprenylation of the Gγ-subunit results in the proteolytic clevage of the aaX residue and the methylation of the C-terminal cysteine (Newman *et al*., 1991). Isoprenylation is prevented when cysteine is substituted with another amino acid (for example $G\gamma_{2(C68S)}$). The isoprenylation is responsible for the anchoring of the Gβγ-dimer to the cytoplasmic membrane. In addition, the isoprenylation of the Gγ-subunit appears to play a role in the interaction of the Gβγ-dimer with receptors and effectors, even though it

is not necessary for the association with Gβ (Myung *et al*., 2000; Yasuda *et al*., 1998).

1.2.2 Gβγ-Regulated Effectors

For a long period of time, it was thought that the only function of mammalian Gβγ was to stabilize the Gα-subunit in its GDP-bound inactive state and to anchor it to the membrane (Florio *et al*., 1985; Roof *et al*., 1985). The first proof that Gβγ was an active regulator, was the demonstration that the muscarinic K^+-channels of the heart can be stimulated by the Gβγ-dimer (Breitwieser *et al*., 1985; Koyrakh *et al*., 2005; Mirshahi *et al*., 2003). Since then, Gβγ-dimers have been shown to mediate signal transduction with numerous other proteins in the cell, including Gα-subunits, GPCRs, GRKs, phosducin, pleckstrin, and various effectors (Ivanova-Nikolova *et al*., 1997). Gβγ-dimers participate in receptor activation, the GTPase cycle, receptor desensitization, and effector activation (McCudden *et al*., 2005) (Table 3).

Gβγ-dimers bind directly to GPCRs and enhance the binding of Gα-subunits to GPCRs. The interaction of Gβγ-dimers with the Gα-subunit covers the effector-interacting surfaces of both proteins. Gβγ-dimers function as GDP-release inhibiting factors for Gα-subunits. Gβγ-dimers are required for phosphorylation of receptors by some GRKs. Phosducin, pleckstrin, and possibly other proteins with pleckstrin homology domains bind Gβγ-dimers and inhibit Gβγ-mediated signaling. Various effectors have been shown to be regulated by Gβγ-dimers, including AC, PLCβ (Watson *et al*., 1994; Wu *et al*., 1993), GIRK (Rishal *et al*., 2003), and N-and P/Q-type Ca^{2+}-channels (Kaneko *et al*., 1999). Gβγ-dimers also activate the MAP kinase pathway (Yamauchi *et al*., 1999) and stimulate both PI3Kγ and PI3Kβ (Maier *et al*., 2000). Gβγ has been shown to regulate various AC isoforms both positively and negatively. In cells expressing AC II and AC IV, Gβγ stimulates cAMP production, provided that $G\alpha_s$ is simultaneously activated through a $G\alpha_s$-coupled receptor (Gao *et al*., 1991; Tang *et al*., 1991). AC I, AC III and AC VIII are inhibited by Gβγ (Diel *et al*., 2006; Steiner *et al*., 2006). Gβγ's inhibition of AC I is not due to sequestration of $G\alpha_s$, but rather appears to be mediated through an independent binding site for Gβγ on AC I (Diel *et al*., 2008).

Table 3: Gβγ-regulated effectors (Nürnberg, 2004)

Effector	Response to Gβγ
AC I, AC VIII	↓
AC II[a], AC IV[a], AC VII[a]	↑
G-protein-regulated inward rectifier K^+-channels (GIRK1-4)	↑
G-protein-coupled-receptor-kinase (GRK 2, 3)	↑
N-, P/Q-, R-, T-type voltage-dependant Ca^{2+}-channels (VDCC)	↓
Phosphatidylinositol-3-kinase-β, -γ (PI3K-β[b],-γ)	↑
Phospholipase C-β1-3	↑
Phospholipase A2	↑
Raf-1 protein kinase	↑
p140Ras-GEF	↑
P-Rex1	↑
Bruton´s-tyrosine kinase (Btk)	↑
Interleukin-2(IL-2)-inducible tyrosine kinase (Tsk)	↑
Phosducin and phosducin-like proteins	(-)

a: AC activity is superactivated by Gβγ only if coactivated by $G\alpha_s$. b: Stimulation has been demonstrated under *in vitro* conditions only. P-Rex1: a guanine-nucleotide-exchange factor of the Rac GTPase that is synergistically activated by phosphatidylinositol-3,4,5-trisphosphate and Gβγ. Phosducin and phosphoducin-like proteins regulate G-protein-mediated signaling by binding to Gβγ and removing the dimer from the cell membranes. p140Ras-GEF: guanine-nucleotide exchange factor of the Ras GTPase, Raf-1: member of the raf subfamily of serine-theonine protein kinases. ↑: stimulation; ↓: inhibition; (-): no effect.

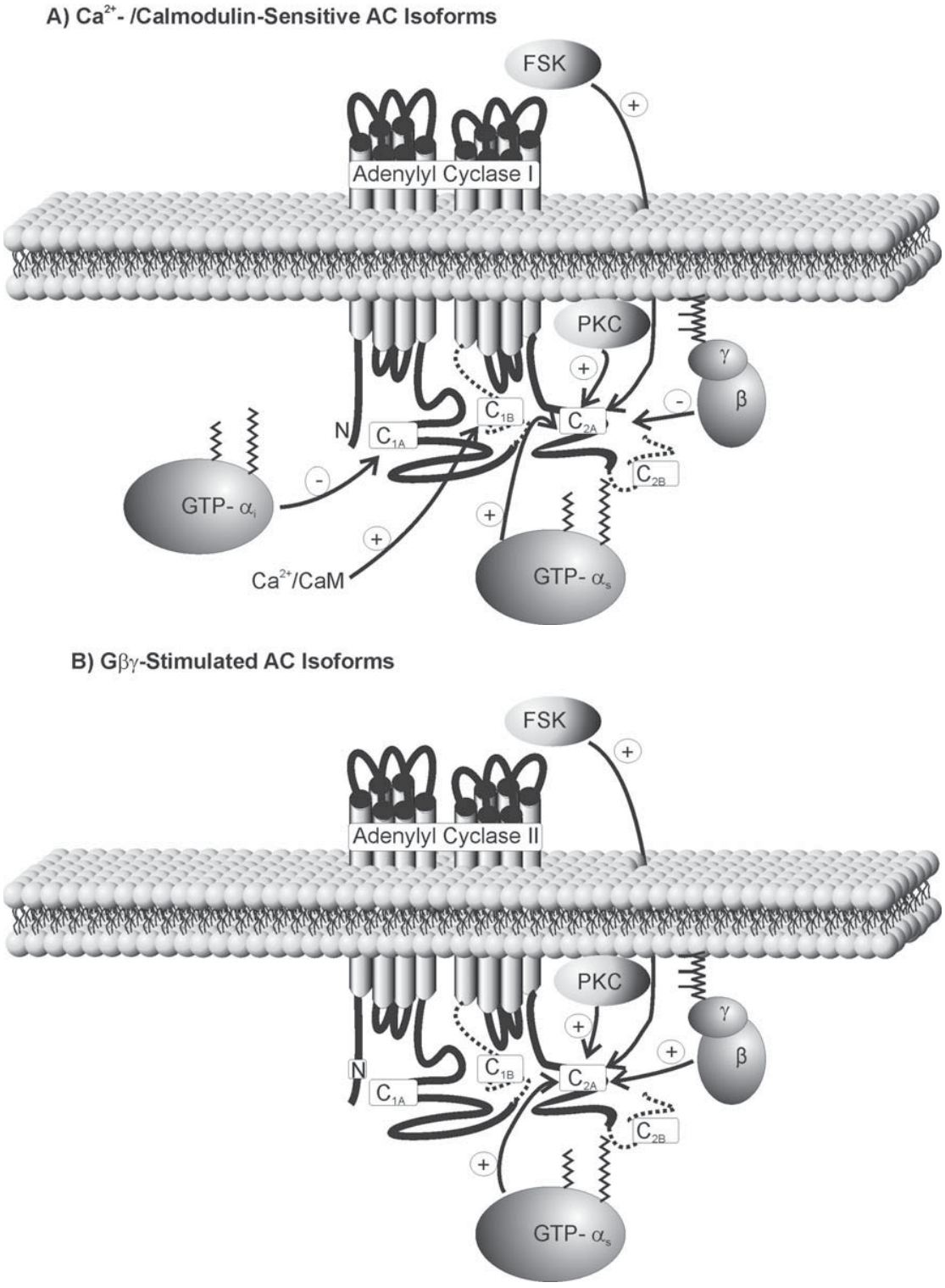
The region needed for Gβγ inhibition of AC I appears to lie in the C1a domain of the enzyme (the amino terminal part of the first large cytoplasmic domain) (Chen *et al*., 1997). Recent attention has turned toward examining the mechanisms responsible for Gβγ-specific signaling. In general, most Gβ-subunits can dimerize with most Gγ-subunits. There are some exceptions though; for example, $G\beta_2$ dimerizes with $G\gamma_2$ but not with $G\gamma_1$, and $G\beta_3$ does not dimerize with either $G\gamma_1$- or $G\gamma_2$; whereas, $G\beta_1$ dimerizes with $G\gamma_1$ as well as $G\gamma_2$-subtypes. Although Gβγ-dimers of varying compostion may form *in vivo* as well, Gβγ-dimer combinations may also exhibit cell type or tissue specificities. For example, the primary Gβγ-dimer in the retina is $G\beta_1\gamma_1$; whereas, $G\beta_1\gamma_2$ is the most common dimer formed in the brain. In some tissues, $G\beta_5$ is also localized in the cytoplasm of the cells (Watson *et al*., 1996b) and forms heterodimeric complexes with specific RGS proteins by interacting with the GGL-domain (G-protein γ-subunit-like) of the RGS proteins (Levay *et al*., 1999).

1.3 Adenylyl Cyclase as a Prototypical Effector of G-Proteins

Adenylyl cyclase (AC) is one of the many effectors that play a vital role in various signal transduction processes. It has been known for over twenty years that different types of cAMP-generating proteins exist. They are found in all mammals and play diverse roles in cell regulation. Before molecular cloning, only 3 species of mammalian adenylyl cyclases were identified: I) a brain-specific, $G\alpha_s$ and Ca^{2+}/CaM-sensitive enzyme; II) a ubiquitously expressed, $G\alpha_s$-stimulated cyclase (Brostrom *et al*., 1975); and III) a Ca^{2+}/CaM and $G\alpha_s$-independent soluble enzyme found only in the testes (Braun *et al*., 1975).

In the 1970s, a diterpene refered to as forskolin or Makandi was isolated from the roots of the herb *Coleus forskohlii*. The first known adenylyl cyclases were shown to be activated by forskolin (Seamon *et al*., 1981). The characterization of additional adenylyl cyclases advanced with the introduction of forskolin sepharose affinity chromatography (Pfeuffer *et al*., 1983). Based on this method, the G_s- and Ca^{2+}/CaM-sensitive isoforms were isolated from bovine brain, and the Ca^{2+}/CaM-insensitive isoforms were isolated from rabbit heart and later from olfactory cilium (Pfeuffer *et al*., 1985; Pfeuffer *et al*., 1989). The activation of AC, resulting in the intracellular production of cyclic adenosine-3′,5′-monophosphate (cAMP) is initiated by the binding of hormones to cell surface receptors. Epinephrine, dopamine, prostaglandin PGE2, adenosine, and glucagons are a few examples of the many hormones that activate AC through membrane-bound receptors (Coppe *et al*., 1978).

It is now recognized that mammalian ACs are a large family of enzymes encoded by at least ten independent genes. Most of the AC isoforms are expressed in only a limited number of tissues. Determination of the precise expression pattern of each of the ten isoforms was difficult, due to the low expression levels and the lack of specific high-affinity antibodies. Thus, molecular cloning techniques have identified nine mammalian genes that encode membrane-bound ACs and one gene encoding a soluble AC isoform. These genes do not tend to cluster within the genome, but rather are distributed among different chromosomes (Sunahara *et al*., 1996). The ten AC isoforms can be divided into five distinct families based on their amino acid sequence similarity and functional attributes. Figure 3 schematically shows the first three discovered AC-families together with simplified signaling cascades.


A) Ca2+- /Calmodulin-Sensitive AC Isoforms
FSK
+
Adenylyl Cyclase I
PKC
+
γ
β
-
C1B
C2A
N
C1A
C2B
GTP- αi
-
+
+
Ca2+/CaM
GTP- αs
B) Gβγ-Stimulated AC Isoforms
FSK
+
Adenylyl Cyclase II
PKC
+
γ
β
+
C1B
C2A
N
C1A
C2B
+
GTP- αs

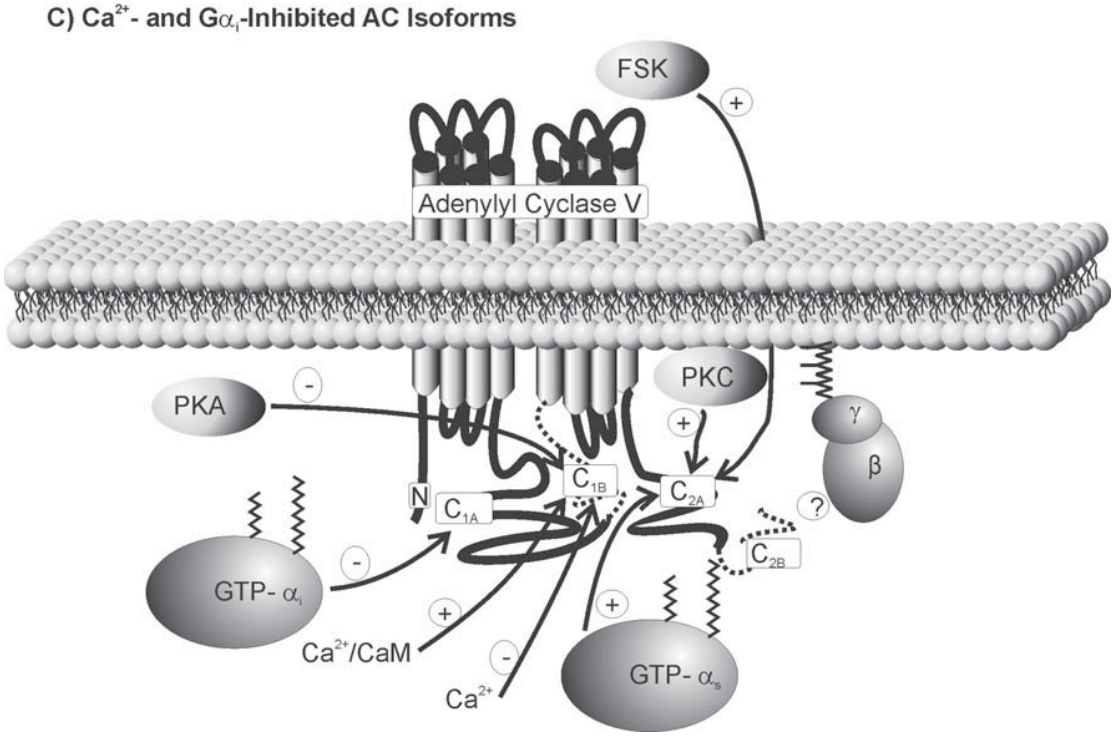


Figure 3: Main classification of adenylyl cyclase isoforms.

AC I, AC III and AC VIII are Ca^{2+}/CaM-sensitive isoforms and are represent in Figure 3A. AC II, AC IV and AC VII are Gβγ-stimulated isoforms and are represented in Figure 3B. AC V and AC VI are Ca^{2+} and $G\alpha_i$-inhibited isoforms and are represented in Figure 3C. A, B and C illustrate the details on the regulation of Ca^{2+}/CaM-sensitive isoforms, Gβγ-stimulated isoforms and Ca^{2+} and $G\alpha_i$-inhibited isoforms, respectively. Agonists like isoproterenol activate β_2-adrenergic receptors that couple to $G\alpha_s$. Upon activation, $G\alpha_s$ as well as Gβγ are released to activiate their effectors such as adenylyl cyclases. Gβγ-regulation of AC II is dependent on $G\alpha_s$ co-activation and does not activate AC by itself. α_1-adrenergic receptors couple to the $G\alpha_i$. (+: stimulatory; -: inhibitory; FSK: Forskolin; PKA: protein kinase A; PKC: protein kinase C; CaM: calmodulin)

The Ca^{2+}/CaM-sensitive forms are AC I, AC III, and AC VIII. The Gβγ-stimulated forms are AC II, AC IV, and AC VII. AC V and AC VI are distinguished by their sensitivity to inhibition by both Ca^{2+} and $G\alpha_i$-isoforms ($G\alpha_o$, $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, and $G\alpha_z$) (Asboth *et al*., 2001) (Figure 3). AC IX is the most divergent of the membrane-bound family and is insensitive to forskolin (Sosunov *et al*., 2001). The tenth isoform, soluble AC (sAC), is the most divergent of all mammalian adenylyl cyclases and is similar to adenylyl cyclases found in cyanobacteria (Fraser *et al*., 2005).

The distribution of these AC isoforms in RNAs is summarized in Table 4. In general, all membrane-bound AC isoforms are found in, but are not limited to, excitable tissues such as neurons and muscles. Within the brain, AC isoforms localize to different, discrete brain regions. Furthermore, AC II and AC VII are similar in structure yet show very different patterns of expression throughout the brain (Hellevuo *et al*., 1995). They have also been

detected in the lungs as well as in the olfactory epithelium (Levy *et al*., 1991). Strong mRNA hybridization of AC type II is seen in the cerebellum, hippocampus, and cortex. AC VII is mainly expressed within the cerebellar-granular cell layer and is only weakly expressed in any other part of the brain.

Table 4: Tissue distribution of mammalian adenylyl cyclases

AC Isoform	Tissue Distribution	Reference
AC I	brain (neurons), adrenal gland (medulla)	Krupinski *et al*., 1989
AC II	lung, brain, skeletal muscle	Feinstein *et al*., 1991
AC III	brain, olfactory epithelium, pancreas, male germ cells, brown adipose tissue	Bakalyar *et al*., 1990
AC IV	brain	Gao *et al*., 1991
AC V	heart, brain (striatum)	Ishikawa *et al*., 1992
AC VI	ubiquitous	Yoshimura *et al*., 1992
AC VII	brain, platelets	Krupinski *et al*., 1992
AC VIII	brain, lung	Cali *et al*., 1994
AC IX	brain, skeletal muscle	Paterson *et al*., 1995
AC X	testis	Buck *et al*., 1999

Underlined tissues express the respective isoform at high levels. The indicated expression patterns are based on the mRNA abundance.

Moderate levels of AC I and AC VIII are found in the neurons, particularly in neurons in brain regions that are associated with learning and memory (Cali *et al*., 1994; Xia *et al*., 1992). AC I is synthesized at moderate levels in the neocortex and olfactory system. AC III is found in the cilia of the immature neuroepithelium olfactory receptor cells throughout development, in distinct parts of these cilia in mature cells, and also at low levels in the brain, heart, adrenal medulla, lung, and retina (Buck *et al*., 1999). AC IV, AC VII, and AC IX are expressed in a variety of tissues, including brain, heart, kidney, and liver (Gao *et al*., 1991; Hellevuo *et al*., 1995). The tenth mammalian AC isoform, which is molecularly and biochemically distinct from the TM ACs, is the soluble adenylyl cyclase (sAC). It was originally purified from the cytosolic fraction of rat testes (Buck *et al*., 1999).

AC VI was first isolated from the canine cardiac cDNA library. It is considerably more homologous to AC V than any other adenylyl cyclase type. AC VI is most abundant in the heart and the brain. Unlike AC V though, it is also found in low levels in a variety of other tissues (Katsushika *et al*., 1992). The two cyclases AC V and VI are expressed in the mammalian heart. The relative amounts of AC V and VI in fetal, neonatal, and adult rat

hearts were determined using a sensitive ribonuclease protection assay. It has thus been shown that their expression is differentially regulated during ontogenic development. Results demonstrated that AC V accumulates in the rat heart and becomes the highly predominant isoform after birth leading to an increase in cardiac adenylyl cyclase activity (Espinasse *et al*., 1995).

1.3.1 Structure of Adenylyl Cyclases

The structure of AC is highly reminiscent of the structures of certain channels and ATP-dependent transporters, particularly the P-glycoprotein and the cystic fibrosis transmembrane conductance regulator (CFTR). This relationship prompted speculation that adenylyl cyclase might also serve as channels or transporters, but so far there is very little evidence to support this conjecture (Schultz *et al*., 1992).

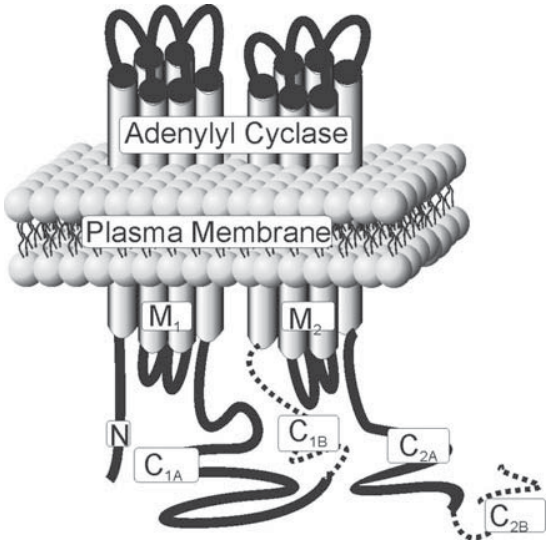


Figure 4: Structure of adenylyl cyclases.

N: amino terminal region, M_1: 1st transmembrane region with transmembrane domains 1-6, M_2: 2nd transmembrane region with transmembrane domains 7-12, C_{1A} + C_{1B}: 1st cytosolic loop, C_{2A} + C_{2B}: 2nd cytosolic loop (Taussig *et al*., 1994).

The nine cloned isoforms of TM mammalian AC have molecular weights of roughly 200-250 kDa (range of 1064 – 1248 amino acid residues); whereas, the non-TM, soluble sAC has a molecular weight of 187 kDa. The TM-ACs share a primary structure consisting of two TM regions, M_1 and M_2, and three cytoplasmic regions, N, C_1, and C_2 (Figure 4).

The TM regions each contain six predicted membrane-spanning helices. The function of M_1 and M_2, aside from membrane localization, is unknown, despite their topological

analogy to transporters. The C_1 and C_2 regions are subdivided into C_{1A} and C_{1B} (containing 360-390 amino acids) and C_{2A} and C_{2B}, (containing 255-330 amino acids), respectively. In general, the sequence homology of the different adenylyl cyclases is approximately 60%. The sequence identity within the cytoplasmic domains of the different adenylyl cyclases ranges from 50% to 90%. The most highly conserved sequences are in the C_{1A} and C_{2A} domains. Moreover, C_{1A} and C_{2A} resemble each other in each adenylyl cyclase (roughly 50% similar and 25% identical) (Tang *et al*., 1995). C_{1A}- and C_{2A}-domains heterodimerize with each other in solution (Whisnant *et al*., 1996; Yan *et al*., 1996). The C_{1B} region is large (15 kDa), variable, and contains several regulatory sites. The C_{2B} region is vanishingly short in some isoforms and lacks identified functions; hence C_2 and $C_{2A/2B}$ are sometimes referred to interchangeably. The construction of fused C_1-C_2 domains and individual soluble domains results in activities characteristic of the full-length membrane-bound AC forms in terms of modulation by G-protein, Gα-subunit, forskolin, substrate inhibitors, and P-site inhibitors. The utilization of the recombinant, soluble domains of AC has facilitated the biophysical characterization of enzyme function (Cooper, 2003).

1.3.2 Regulation of Adenylyl Cyclases

The best understood pathways for the regulation of adenylyl cyclase activity are those involving the indirect stimulation of the enzyme by GPCR-ligands such as β-adrenergic agonists. Binding of the agonist (*e.g.*, isoproterenol) to an appropriate receptor (in this case β-adrenergic receptor) causes activation of a G-protein, $G\alpha_s$ which in turn stimulates adenylyl cyclase. There are G-protein such as $G\alpha_i$-isoforms that inhibit adenylyl cyclase directly as well as more complex mechanisms that lead to both stimulation and inhibition (Table 5). The various regulatory effects on adenylyl cyclases, specifically those that belong to the family of Type I, Type II and Type V are graphically represented in Figure 3.

Table 5: Regulation and regulatory properties of mammalian adenylyl cyclases (Hanoune *et al.*, 2001)

AC isoform	$G\alpha_s$	$G\alpha_i$	$G\beta\gamma$	Forskolin	Ca^{2+}/CaM	Protein kinases
AC I	↑	↓Ca^{2+}/CaM-stimulated	↓	↑	↑	↑PKC (weak) ↓ CaM kinase IV
AC II	↑		↑	↑		↑ PKC
AC III	↑	↓	↓	↑	↑ (*in vitro*)	↑ PKC (weak)
AC IV	↑		↑	↑		↑ PKC
AC V	↑	↓		↑	↓ <1µM	↓ PKA ↑PKCα/ζ
AC VI	↑	↓		↑	↓ <1µM	↓ PKA, PKC
AC VII	↑		↑	↑		↑ PKC
ACVIII	↑	↓Ca^{2+}		↑	↓	→PKC
AC IX	↑	↓		→	↓calcineurin	
sAC	→	→		→		

↑ positive regulatory response; ↓ negative regulatory response; → no regulatory response

1.3.2.1 Regulation of Adenylyl Cyclases by Forskolin

The stimulation of cAMP-formation by forskolin appears to involve both direct activation of adenylyl cyclase and facilitation and / or enhancement of receptor-mediated activation of the enzyme. The hydrophobicity of forskolin directs its interactions with the membrane-spanning regions of all adenylyl cyclases, except AC IX (Iyengar, 1993). AC IX is non-responsive to forskolin because of a Ser^{942}→ Ala^{1112} and a Leu^{912}→ Tyr^{1082} change in the binding pocket of AC II. When these changes are reversed by site-directed mutagenesis, the resulting AC IX mutant can be activated by forskolin. Forskolin binds to the catalytic core at the opposite end of the same ventral cleft that contains the active site. It activates AC by connecting together the two domains in the core using a combination of hydrophobic and hydrogen-bonding interactions that are distributed equally between the two domains. The forskolin binding pocket is a narrow hydrophobic crevice that almost completely buries the forskolin molecule once it is bound. The pocket residues are absolutely conserved in AC I-VIII and differ only subtly in AC IX. The forskolin-dependent activation of AC II, AC IV, AC V, AC VI, and AC VII is synergistic with $G\alpha_s$-mediated coactivation; whereas, activation by forskolin and $G\alpha_s$ is additive for AC I, AC III, and AC VIII. $G\alpha_s$ binds similarly between the two domains but at a location on the

perimeter of the catalytic core (Insel *et al*., 2003).

1.3.2.2 Regulation of Adenylyl Cyclases by Gα-Subunits

Under physiological conditions, all mammalian ACs are potently activated by GTP-$G\alpha_s$. This activation is synergistic or additive, but not competitive with respect to forskolin. Crystallographic evidence suggests that the main contact between $G\alpha_s$ and AC occurs through a α-helix that is highly mobile throughout the GTPase cycle of G-proteins (Feldman *et al*., 2002). GTP-$G\alpha_s$ binds to a crevice on the outside of the wreath formed by the α2' and α3' of C_2 and by the N-terminal portion of C1. GTP-$G\alpha_s$ is capable of dimerizing C_1 and C_2 as does forskolin, although this cannot be its only function as suggested by mutational analysis. If C_1 contact is abolished, activation can be partially rescued when forskolin is used to dimerize C_1 and C_2. Therefore, there must also be a regulatory role for GTP-$G\alpha_s$. This role is probably to induce a conformational change that allosterically stimulates catalysis. The 7° rotation of C_1, which moves the catalytic residues into their proper positions, is probably the result of torque applied by GTP-$G\alpha_s$ as it pushes the C_1 away from its binding site (Zhang *et al*., 1997).

Multiple splice variants of $G\alpha_s$ have been identified: $G\alpha_{s\text{-short}}$, $G\alpha_{s\text{-long}}$, and $G\alpha_{sxl}$. $G\alpha_{sxl}$ is a relatively new member and is less well characterized. The long and short splice variants are biochemically indistinguishable in their capacity to directly activate AC, but the behavior of the hormone-receptor-stimulated ACs varies considerably. $G\alpha_{sxl}$ can activate ACs directly, but no hormone-receptor-mediated effects through $G\alpha_{sxl}$ have been demonstrated yet (Cherfils *et al*., 2003). GTP-$G\alpha_s$ displays a tenfold greater affinity for activating AC compared to the GDP-bound-$G\alpha_s$.

Members of the $G\alpha_i$ family inhibit AC V and AC VI. Interestingly, their mode of inhibition is not through direct competition with $G\alpha_s$, because forskolin-stimulated activity is also inhibited. Symmetry and sequence homology arguments led to the suggestion that $G\alpha_i$ binds to catalytic core on a groove pseudosymmetrically related to the $G\alpha_s$ binding groove. Mutational analysis confirmed that the groove formed by α2' and α3' of C_1 is the primary site for the binding of $G\alpha_i$ to AC V. The inhibitory mechanism postulates a rotation of the C_1 in the opposite direction as that induced by $G\alpha_s$ (Wittpoth *et al*., 1999).

The highly expressed $G\alpha_o$ can inhibit AC 1 and AC VIII, although it is not as potent as the other $G\alpha_i$-subunits on AC V and AC VI. The $G\alpha_i$-subunits are posttranslationally modified by long-chain acyl (myristoyl) and thioacyl (palmitoyl) moieties; myrystoylation is

required for $G\alpha_i$-mediated inhibition of AC. It is also important to note that ACII, AC IV, and AC VII are not directly modulated by $G\alpha_i$ (Evanko *et al*., 2005).

1.3.2.3 Regulation of Adenylyl Cyclases by Gβγ-Dimers

Gβγ-dimers are strong modulators of AC activity, which can be either stimulatory, as in the case of AC II, AC IV, and AC VII, or inhibitory, as for AC I, AC III, and AC VIII. Gβγ acts to stimulate the AC activity of AC II, AC IV, and AC VII, albeit only when $G\alpha_s$ is co-activated. The putative binding site for Gβγ on the Gβγ-stimulated family of ACs has been mapped in the C2 domain by amino acids 956 to 982 of AC II. Despite the high degree of sequence conservation among AC catalytic domains, this sequence is not found in other AC isoforms not modulated by Gβγ. Indeed, this sequence in the C2 domain contains a short putative Gβγ binding motif, QXXER. The consensus for this is based on GRK2 that requires Gβγ for activation, as well as Gβγ-activated inwardly rectifying K^+-channels, the activated PLCβ isoforms, and the Gβγ-inhibited AC I. Disruption of the consensus QXXER motif in any of these instances abrogates all Gβγ effects. Recently, a hexapeptide, NAAIRS, which had been established as an appropriate tool for substitution experiments, revealed that the Gβγ stimulation site in C1b, which is mediated by amino acids, is located in the stretch of amino acids 490-509. This region was confined to the PFAHL motif, which is present in AC II, AC IV, and AC VII, but not in the other ACs, and which also serves as a general mediator of Gβγ stimulation. This stimulation takes place only when $G\alpha_s$ is bound to the AC. The Gβγ site is adjacent to the $G\alpha_s$ site but does not overlap with it (Chakrabarti *et al*., 2003).

Gβγ-dimers are among the most potent of all negative regulators of AC I and AC VIII and can markedly inhibit the effects of forskolin, $G\alpha_s$, and Ca^{2+}/CaM on AC activities. A peptide generated from the catalytic region of AC I and analogous to the region containing the QXXER motif in the AC II sequence also displays dramatic effects on the Gβγ regulation of AC activity. This peptide could reverse both Gβγ-dependent inhibition of AC I activity and Gβγ-dependent superactivation of $G\alpha_s$-stimulated AC II, suggesting that this region of AC I also serves for binding Gβγ.

1.3.2.4 Regulation of Adenylyl Cyclases by Ca^{2+}/Calmodulin

Ca^{2+}/CaM activates AC I, AC VIII, and AC III. Specifically, intracellular Ca^{2+} released from IP_3-sensitive stores is unable to affect these Ca^{2+}-sensitive AC isoforms; whereas,

activation of Ca^{2+}-entry through voltage-gated Ca^{2+}-channels or through capacitative entry is effective at activating these isoforms. The calmodulin binding site has been identified in the C_{1b} domain of AC I. Stimulation by $G\alpha_s$ is synergistically enhanced by Ca^{2+}/CaM. AC VIII has two calmodulin binding sites. One is located at the N-terminus and the other at the C_{2b} domain of this cyclase (Baker *et al*., 1998; Cali *et al*., 1994; Wayman *et al*., 1994).

Although millimolar concentrations of Ca^{2+} inhibit all AC isoforms, AC V and AC VI are already inhibited by micromolar concentrations. Ca^{2+}-ions derived from the capacitive entry are thought to be the sole physiological source of Ca^{2+} to inhibit AC V and AC VI. These AC isoforms, which are mostly found in the heart, are involved in the regulation of the intensity and frequency of the heart muscle contraction through a negative backward coupling mechanism *via* cAMP, which is produced when Ca^{2+} enters the cell (Kume *et al*., 2000).

The Ca^{2+}/CaM-stimulated phosphoprotein-phosphatase calcineurin has an inhibitory effect on AC IX that is probably based upon activation by a yet unknown protein kinase whose specific effect can be reversed by calcineurin (Paterson *et al*., 2000). Ca^{2+}/CaM, which normally activates AC I, AC III, and AC VIII, can also inhibit AC I and AC III indirectly through phosphorylation by CaM kinases II and IV, respectively. CaM kinase II phosphorylates AC I in its C1b domain and disables Ca^{2+}/CaM activation by interfering with the calmodulin binding site (Wayman *et al*., 1996). CaM kinase IV inhibits AC III by phosphorylating it at Ser-1076 (Wayman *et al*., 1995).

2 AIM OF STUDY

The theoretical background of this work originated from previous findings of our group who postulated that the adenylyl cyclase (AC) isolated from rabbit myocardial membranes differs from AC isolated from bovine brain cortex (Pfeuffer *et al*., unpublished). Although isolated from non-activated myocardial membranes, the purified AC was coupled to a $G\alpha_s$ monomer. The $G\alpha_s$ was in a non-activated state (bound to GDP). Purification and isolation of adenylyl cyclase showed that the complex was devoid of Gβγ-dimers. The stability of AC V-GDP-bound-$G\alpha_s$ complex in the absence of $G\alpha_s$ activators suggested that it may be found in intact cells. If indeed this GDP-bound-$G\alpha_s$-adenylyl cyclase complex (devoid of Gβγ) exists *in vivo*, what would the Gβγ effect be on this complex when added to it? It has been known since 1992 that AC V and AC VI are present in myocardial tissues (Ishikawa *et al*., 1992). Using membranes of COS-1 cells after transient expression of AC V or AC VI together with $G\gamma_2$ and various Gβ-subunits, it has been reported that these two adenylyl cyclase isoforms are markedly inhibited by Gβγ (Bayewitch *et al*., 1998). Bayewitch *et al*. is the only research group that has shown a Gβγ effect on AC V and AC VI in cell membranes. Many other groups have not been able to show this Gβγ-inhibition on AC V, and rather state that Gβγ has no effect on AC V (Tang *et al*., 1991). Recently, another group showed that Gβγ has a stimulatory effect on AC V (Gao *et al*., 2007). However, it has not yet been analyzed whether this effect also occurs in intact cells. Therefore, the aim of this research was to investigate if Gβγ inhibits or stimulates AC V in intact cells.

3 MATERIALS

3.1 List of Manufacturers and Distributors

(1) Aldrich, Steinheim, Germany
(2) Amersham, Braunschweig, Germany
(3) Bio101 / Dianova, Hamburg, Germany
(4) Biognostik, Göttingen, Germany
(5) Bio-Rad Laboratories, Richmond, CA, USA
(6) Boehringer, Mannheim, Germany
(7) Calbiochem-Novabiochem, Bad Soden, Germany
(8) Carl Roth, Karlsruhe, Germany
(9) Falcon, Heidelberg, Germany
(10) Fluka Chemie, Buch, Switzerland
(11) Gibco BRL Life Technologies, Eggenstein, Germany
(12) ICN Biomedicals, Eschwege, Germany
(13) Invitrogen, Leek, Netherlands
(14) Konica, Tokyo, Japan
(15) Kodak, Berlin, Germany
(16) MBI, Fermentas, St. Leon-Rot, Germany
(17) Merck, Darmstadt, Germany
(18) Millipore, Neu-Isenburg, Germany
(19) Pharma Waldorf, Düsseldorf, Germany
(20) Pharmacia Biotech, Freiburg, Germany
(21) Promega, Madison, WI, USA
(22) RBI, Natick, MA, USA
(23) Roche, Mannheim, Germany
(24) Serva, Heidelberg, Germany
(25) Sigma-Aldrich, Deisenhofen, Germany
(26) Tropix / Serva, Heidelberg, Germany
(27) Prof. Dr. P. Gierschik, Ulm, Germany
(28) Prof. Dr. H. Lemoine, Düsseldorf, Germany
(29) Prof. Dr. D. Palm, Würzburg, Germany
(30) Prof. Dr. G. Schultz, Berlin, Germany

(31) Prof. Dr. Z. Vogel, Rehovot, Israel

(32) Dr. G. Böl, Düsseldorf, Germany

(33) Dr. S. Feinstein, Baltimore, MD, U.S.A

(34) Dr. P. Ferreira, Düsseldorf, Germany

(35) Dr. A. Hülster, Düsseldorf, Germany

(36) Dr. F. Kluxen, Düsseldorf, Germany

(37) Dr. H. Metzger, Frankfurt , Germany

(38) Dr. S. Mollner, Düsseldorf, Germany

(39) Dr. R. Simmoteit, Würzburg, Germany

(40) Dr. M. Staufenbiel, Sandoz, Basel, Switzerland

(41) Dr. J. Wallach, Düsseldorf, Germany

3.2 Chemicals

Acetic acid	17
Acrylamide	8
Agarose	15
Aluminium choride	25
Aluminium oxide	17
Ammonium persulfate	5
Angiotensin	7
Adenosine 5'-triphosphate (ATP)	25
Benzamidine hydrochloride	1
Benzethonium chloride	25
Bupranolol	28
Bovine serum albumine (BSA)	24
Bromophenol blue	24
Calmodulin (CaM)	25
Cyclic adenosine-3',5'-monophosphate (cAMP)	19
Cholera toxin (CTX)	25
Chloroquin	25
Coomassie brilliant blue G-250	21
Creatine phosphate (CP)	6
Disodium 3-(4-methoxyspiro{1,2-dioxethane-3,2'-(5'-chloro)tricyclo[3.3.1.1.^{3,7}] decan}-4-yl) phenyl phosphate (CSPD)	26

Diethanolamine	26
Dimethylsulfoxide (DMSO)	10
Dowex 50x8 (H^+)	5
Dithiothreitol (DTT)	17
Ethylene glycol-bis (2-aminoethylether)-N,N,N'N'-tetraaceticacid (EGTA)	17
Forskolin	37
Glycerine	11
Guanosine-5'-triphosphate γS (GTPγS)	6
N-(2-Hydroxyethyl) piperazine-N'-(2-ethanesulfonicacid) (HEPES)	17
I-Light block	26
Imidazole	8
Isoproterenol	24
Magnesium chloride	25
Manganese chloride	25
β-Mercaptoethanol (β-ME)	25
Methoxamine	22
N, N'- Methylene-bis-acrylamide (bisacrylamide)	24
3-(N-morpholino) propane sulfonicacid (MOPS)	24
Nitroblock	26
Pansobin	7
Phenylephrine	22
Pilocarpine	12
Phenylmethylsulfonylfluoride (PMSF)	25
Ponceau S	17
Prasozin	12
Rontiszint 2211	8
Sephadex G-25 (NAP10; PD10)	20
3-Morpholino sydnonimine hydrochloride (Sin-1)	7
Sodium dodecyl sulfate (SDS)	25
Somatostatin	7
Staurosporin	7
Suramin	7
N-p-tosyl-l-lysine chloromethyl ketone (TLCK)	25
N-p-tosyl-l-phenylalanine chloromethyl ketone (TPCK)	25

Tris-(hydroxymethyl)-aminomethane (Tris)	24
Trypsin-ethylenediamine)-N,N,N'N'-tetraceticacid (Trypsin-EDTA)	11
Tween 20	25
Phospholipase C inhibitor (U73122)	7
Xanomeline	25

3.3 Enzymes, Proteins and Other Biologically Active Substances

Alkaline phosphatase	23
Creatine kinase	23
Myokinase	25
DNase I	23
RNase A	24
Soybohnen trypsin inhibitor	6
Restriction enzymes	20, 23

3.4 Non-Radioactively Labeled Nucleotides

Adenosine-5'-triphosphate (ATP)	25
Guanosine-5'-diphosphate (GDP)	25
Guanosine-5'-triphosphate (GTP)	25
Uridine 5'-triphosphate (UTP)	25

3.5 Radioactively Labeled Nucleotides

[2, 8-^{3}H] adenine, 1.63 TBq/mmol	12
[α-^{32}P] ATP, 29.6 TBq/mmol	12
[8-^{3}H] cAMP, 0.7-1.1 TBq/mmol	12

3.6 Cell Culture, Cell Culture Media and Supplements

Bacteriological culture plates	9
Cell culture material	9
Dulbecco's modified eagle's medium (DMEM)	11
Dulbeco's phosphate buffered saline (PBS)	11
Fetal calf serum (FCS)	6
Geneticin® (G418)	6

Gentamicin11	25
Human embryonic kidney cells, wild type (HEK 293 cells)	40
Human embryonic kidney ddenylyl cyclase type II cells (HEK AC II cells)	35
Human embryonic kidney adenylyl cyclase type V cells (HEK AC V cells)	32
Human lymphoma cells, wild type (S49 cells)	30
Human lymphoma cells, $G\alpha_s$ deficient (S49cyc$^-$ cells)	30
Luria-Bertani (LB) medium (capsules)	3
Minimal essential medium (MEM)	11
Monkey kidney cells, wild type (COS-1 cells)	40
Penicillin / Streptomycin (10,000 U/10,000µg/ml)	3
2x Yeast type medium (capsules)	3

3.7 Vectors

pcDNA3	13
pXMD1	36

3.8 cDNA Clones

pcDNA3 ACII	33
pcDNA3 AC V	41
pcDNA3 ACVI	31
pcDNA3 $G\alpha_s$	27
pcDNA3 $G\beta_1$	27
pcDNA3 $G\gamma_2$	27
pcDNA3 $G\alpha_t$	27
pcXMD-gal	36

3.9 Protein Markers and DNA Standards

Rainbow marker	2
DNA markers	16
Lambda-DNA, Hind III	20
1kb DNA ladder	16

3.10 G-Proteins, Antibodies and Antisense Oligonucleotides

Gβγ	39
$G\alpha_{s(l)}$	38
Anti-Gβ-antiserum	39
Anti-rabbit-IgG-AP	6
Antisense $G\alpha_s$ oligonucleotide	4
BBC-1 (against adenylyl cyclase)	38
BBC-2 (against adenylyl cyclase)	38
BBC-2 AP	38
FITC-$G\alpha_s$-oligonucleotide	4
Membranes of human platelets	34

3.11 Blotting Membranes and Films

Immobilon polyvinyliden difluoride membrane (PVDF-membrane)	18
X-Ray film HR-10 medical	14
X-ray film cassette (Number 553)	14

4 EXPERIMENTAL PROCEDURES

4.1 Standard Biochemical Methods

4.1.1 Measurement of Protein Concentration

Different methods were used to measure protein concentration. All of these methods were based on a specific protein-dependent color-complex formation.

For protein preparations containing high concentrations of detergent, the method by Lowry (Lowry *et al*., 1951) modified by Peterson (Peterson, 1983) was used. This method is based on reactivity of the peptide nitrogen[s] with Cu^{2+} under alkaline conditions and reduction of Folin-Coicalteu phosphomolybdiophosphotungstic acid to heteropolymolybdenum blue by copper-catalyzed oxidation of aromatic acids. The Lowry-method is sensitive to protein concentration ranging from 0.005 – 0.1 mg/ml.

The protein concentration determination according to Bradford was used for protein concentration ranging from 5-25µg (Bradford, 1976). This assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible color change. This assay is useful since the extinction coefficient of a dye-albumin complex solution is constant over a 10-fold concentration range.

Another method was detection of proteins in sodium dodecyl sulfate-polyacryamide gel electrophoresis (SDS-PAGE) following staining with Coomassie Brilliant Blue (see sections 4.1.2 and 4.1.3). The images of each gel were digitalized using a transmitted-light scanner (Diana-III, Raytest) and densitometrically evaluated using "Aida" software (Raytest). The working detection range for this method was 50 – 1000 ng/lane. For the methods described above, bovine serum albumin was used as a protein concentration standard.

4.1.2 SDS-Polyacrylamide Gel Electrophoresis

SDS-PAGE offers a rapid and relatively accurate way to determine relative protein molecular weights using a discontinuous polyacrylamide gel as a support medium and sodium dodecyl sulfate (SDS) to which the polypeptide binds in proportion to its relative molecular mass (Laemmli, 1970). Furthermore, SDS-PAGE is useful to follow the

progress of a purification procedure and to determine the purity of proteins in the fractions from purification. This method was also suitable for the quantification of protein concentrations (see section 4.1.1).

The protein samples were mixed with Laemmli buffer [62.5mM Tris/HCl, 10% (v/v) glycerine, 5% (v/v) β-mercaptoethanol (β-ME), 2% (w/v) SDS, and 0.02% (w/v) bromphenolblue]. SDS is an anionic detergent and confers a negative charge on the polypeptide in proportion to its length. β-mercaptoethanol is a reducing agent which prevents formation of disulfide bonds. Disulfide bonding is covalent and is not disrupted by SDS. The electrophoresis was carried out in running buffer 25mM Tris (pH 8.3 - 8.7), 192mM glycine, and 0.1% (w/v) SDS. Proteins were run at 100 V in the stacking gel to concentrate them, and then they are separated in the running gel at 150 V. The "block" gel prevents the proteins from running off the bottom of the gel.

Table 6: Preparation of various gels for SDS PAGE

	STACKING GEL	RUNNING GEL	"BLOCK" GEL
Acrylamide/Bisacrylamide (30%/0.8%)	4.5% (w/v)	10% (w/v)*	30% (w/v)
SDS	0.1% (w/v)	0.1% (w/v)	
APS	0.1% (w/v)	0.1% (w/v)	1.5% (w/v)
TEMED	0.1% (w/v)	0.1% (w/v)	1.5% (w/v)
Tris/HCl	125mM, pH 6.8	375mM, pH 8.8	

* For better resolution between 7 - 30 kDa, a 15% running gel was used.

4.1.3 Coomassie Staining of Polyacrylamide Gels

Coomassie Blue staining is based on the nonspecific binding of the dye Coomassie Brilliant Blue R250 to proteins. The gel was soaked in a solution of the dye (1% (w/v) Coomassie Brilliant Blue in 45% (v/v) methanol and 10% (v/v) acetic acid) for at least 30 minutes. The gel was then destained using a mixture of 5% methanol and 10% acetic acid to remove the background color prior to analysis of the gel.

4.1.4 Electrotransfer of Proteins onto PVDF Membranes

In this method the proteins were separated in polyacrylamide gels as described in section 4.1.2, and were transferred to a PVDF membrane (Towbin *et al*., 1979; Burnette, 1981). A prestained molecular weight marker (Rainbow marker) was used as a protein standard. The rainbow marker on the gel acts as a control for the transfer of proteins from the gel to

the PVDF membrane. This was followed by detection of the proteins with specific antibodies.

After gel electrophoresis, the gel was removed, and the stacking gel was separated from the resolving gel. The resolving gel was equilibrated in the blot transfer buffer (200ml methanol, 100ml 25mM Tris, 192mM glycine, 700ml H_2O) for 10 minutes. The PVDF membrane was also equilibrated in the blot transfer buffer. The PVDF membrane was placed on the gel, avoiding the trapping of air bubbles in between the membrane and the gel. This gel-membrane was placed, sandwich-style between two filter papers and two sponges, into a cassette. This cassette was then placed in a blot transfer cell filled with blot transfer buffer. The blot transfer took place at 250 mA, 70V for 2 h.

4.1.5 Immunodetection of Proteins by Chemiluminescence

This process is a quick and extremely sensitive method for the detection of transferred proteins blotted onto membranes. It is based on the principle that CSPD substrates produce a luminescent signal when acted upon by alkaline phosphatase, which dephophorylates the substrate and yields anions that ultimately decompose, resulting in light emission (Bronstein *et al*., 1992). Immobilized proteins are probed with specific antibodies to identify and quantify any antigens present.

Specifically, after the transfer of proteins from the gel to the PVDF membrane, the membrane was washed two times for 5 minutes with PBS and followed by two times washing for 10 minutes with PBS/Tween 20 (1l PBS, 3 g Tween 20). To saturate the unspecific binding sites, the membrane was incubated in I-light block [1 g light block, 100ml PBS, 5 g Tween 20 (0.1% final), 1 g Na-azide]. The solution was stirred for 30 minutes at 70°C and filtered for 1 h, followed by washing for 5 minutes with PBS/Tween 20 twice. This was followed by incubation of the membrane for 1 hour with the required antibody (diluted in I-light block 1:20000). The unbound antibody was removed by four 10 minutes washes with PBS/Tween 20. If the antibody was not coupled with the appropriate alkaline phosphatase, then the second incubation was done for 1 hour with the alkaline-phosphatase-enzyme-coupled antibody (dilution 1:20000).

After the incubation with AP-enzyme-coupled antibody, the membrane was washed two times with assay buffer (0.1 M diethanolamine, 1mM $MgCl_2$, 0.02% Na-azide) for 5 minutes each time. It was followed by transferring the membrane to a different dish for incubation in nitroblock solution (2.5ml nitroblock, 47.5ml assay buffer) for 5 minutes.

After two more 5 minutes washes in assay buffer, the membrane was incubated in CSPD (400µl CSPD, 50ml assay buffer) for 5 minutes. All the incubation and wash steps were carried out in glass or plastic dishes at room temperature on a shaker. The membrane was placed in a cassette on filter paper soaked in assay buffer. The cassette and filter paper were placed into a plastic cover. This whole package was then exposed to film. The exposure lasted from five seconds to 30 minutes, depending on the strength of the signal.

4.1.6 Stripping and Reprobing of Membranes

PVDF membranes can be reprobed by stripping bound primary and secondary antibodies and incubating with different antibodies. The nitrocellulose membrane was submerged in stripping buffer [62.5mM Tris/HCl (pH 6.7), 100mM β-ME, 2% SDS] and incubated at 50°C for 30 minutes with occasional agitation. This was followed by washing the membrane 3 times in large volumes of TBST-buffer for 10 minutes each. Following the wash steps the membrane was immersed in a 10% solution of Roti-Block for 1 hour, and the nitrocellulose membrane was reprobed for immunodetection (see section 4.1.5). Alternatively, the blot could be washed in PBS and stored at 4°C in PBS.

4.1.7 Staining of Western Blots with Ponceau Red

Following the electrotransfer of proteins (see section 1.1.4) and chemiluminesence (see section 1.1.5), the membrane was incubated in Ponceau Red solution [0.2% (w/v) Ponceau S, 3% (w/v) sulfosalicylic acid, 3% (w/v) trichloro acetic acid] for 5 minutes in a shaker. The color was removed by several washes with deionised H_2O (dH_2O) (Kohn, 1958).

4.1.8 Determination of Adenylyl Cyclase Activity in vivo

[^{3}H]-Adenine assay was carried out to measure the activity of adenylyl cyclase in living cells. The assay is based on analyzing the by the conversion of ATP to cAMP (Federman *et al*., 1992). A sequential double chromatography method was used for the separation of [^{32}P]-cAMP (generated from [α-^{32}P]-ATP) from other radioactively labeled adenine nucleotides (Salomon *et al*., 1974). The first column was the Dowex AG50-X12 which is a H^+-exchange resin and the second column was aluminium oxide which isolates [α-^{32}P]-cAMP from other labeled adenine nucleotides. [^{3}H]-cAMP was used as a standard in order

to compensate for any loss of [α-^{32}P]-cAMP from the columns.

4.1.8.1 [^{3}H]-Adenine Assay

The adenylyl cyclase activity was determined in living cells grown in 24-well plates. Twenty four hours following seeding or transfection, the cells were labelled with 2µCi [^{3}H]-adenine/500µl medium, in a 24-well plate for 90 minutes at 37°C and 5% CO_2, leading to the build-up of [^{3}H]-ATP. Excess [^{3}H]-adenine was removed by washing the cells with fresh medium containing no [^{3}H]-adenine. The cells were then stimulated with forskolin or isoproterenol. To inhibit phosphodiesterases, the cells were incubated with phosphodiesterase inhibitors in 1mM IBMX for 10 minutes at 37°C and 5% CO_2. The reaction was stopped by adding 100µl of the stop solution [(30% (v/v) TCA, 10mM cAMP, 10mM ATP, 5000 cpm [^{32}P]-ATP, 5000 cpm [^{32}P]-cAMP)].

The [^{32}P]-cAMP that formed was separated from the remaining [^{32}P]-ATP, using the sequential double method (Salomon, 1974). The sample was first loaded onto a Dowex AG50-X12 column that had been conditioned with 0.1 M HCl and washed with H_2O. Each column was washed with 2ml of H_2O to remove the majority of [^{32}P]-ATP, and the Dowex column was placed over a neutral alumina column (activity grade super 1, Type WN-6). The sample was eluted from the Dowex onto the alumina column with 5ml H_2O. Once the eluate from the Dowex was drained through the alumina column, the cAMP was eluated from the alumina columns with 6ml of 0.1 M imidazole into the scintillation vials containing 12.5ml scintillation solution. The samples were counted for both [^{3}H] and [^{32}P], and the counts were converted to measure the conversion of ATP to cAMP.

The first eluate from the Dowex column contained [^{3}H]-ATP (formed in the cells from [^{3}H]-adenine), and [^{32}P]-ATP which is derived from the stop mix and which was used as an internal standard. About 20% of the ATP that was not retrieved, got lost during elution; therefore, the [^{32}P]-ATP standard ([^{32}P]-ATP std) was used to correct this error. The following equation is used to determine the amount of ATP:

[^{3}H]-ATP x [^{32}P]-ATP(std)/[^{32}P]-ATP = ATP (in cpm)

Similarly, eluates from the alumina column contained [^{3}H]-cAMP converted from [^{3}H]-ATP after stimulating the cells and [^{32}P]-cAMP from the stop mix. About 50%-60% of the amount of cAMP was lost from these columns, so this was corrected using the [^{32}P]-cAMP(std). The following equation was used to calculate the amount of cAMP:

[^{3}H]-cAMP x [^{32}P]-cAMP (std)/[^{32}P]-cAMP = cAMP (in cpm)

The conversion of ATP to cAMP was calculated as the ratio of the total amount of cAMP to the total amount of nucleotides, which was: cAMP/(ATP + cAMP).

4.1.8.2 Generation of [^{32}P]-cAMP

[^{32}P]-cAMP was synthesized with the help of adenylyl cyclase located in human platelet membranes. [^{32}P]-cAMP is required for the [^{3}H]-adenine assay. This assay was used to determine the adenylyl cyclase activity described in section 4.1.8.

The reaction was started by incubating 20µg platelet membranes with 750µl of ATP mix (7.5µl of 33mM ATP, 75µCi [α-^{32}P]-ATP, 375µl of creatine phosphate buffer (800µl creatine solution, 1.6µl of 0.5 M DTE), 37.5µl of creatine kinase buffer (5 mg creatine kinase, 500µl creatine phosphate buffer), 7.5µl H_2O) in a water bath at 30°C for 30 minutes. Adenylyl cyclase was stimulated with 15µl of 10mM forskolin. The reaction was deactivated at 95°C for 10 minutes in a heating block. The Eppendorf tubes were centrifuged at 10000 rpm at room temperature for 5 minutes. The suspension was poured onto an alumina column preequilibrated with 40mM Tris (pH 7.4) and eluted with ten times with 500µl of 40mM Tris (pH 7.4). Each 500µl aliquot of the eluted 40mM Tris (pH 7.4) suspension was collected into vials and the radioactivity was counted. The [^{32}P]-cAMP positive fractions were pooled in one vial and frozen at -20°C.

4.1.9 Determination of Adenylyl Cyclase Activity in vitro

In contrast to the determination of adenylyl cyclase *in vivo*, the *in vitro* tests were carried out using immunoprecipitated adenylyl cyclase or adenylyl cyclase purified from membranes. Since the membranes do not have the capability to convert adenine to ATP, ATP mix was added to the membranes. During this enzymatic assay, ATP is broken down by membrane bound ATPase. Hence, to keep the concentration of ATP constant, an ATP-regenerating system consisting of creatine kinase and creatine phosphate was added to the ATP mix.

For the adenylyl cyclase assay, the respective agonists (30µl) were added to glass test tubes on ice. Thirty µl of the ATP mixture [1µl of 0.01mM GTP, 5µl of creatine phosphokinase solution (15 U/ml creatine phosphokinase, 0.1 mg/ml BSA), 5µl of myokinase solution (9.8 U/ml myokinase, 0.1 mg/ml BSA), 5µl of incubation medium (25mM Tris-HCl pH 7.4, 1mM cAMP), 10µl of creatine phosphate solution (20mM creatine phosphate, 75mM Tris-HCl pH 7.4), 1µl of ATP solution (0.1mM ATP, 2mM $MgCl_2$), 1µl of 2mM $MgCl_2$,

2µl of [^{32}P]-ATP (H_2O ,105 cpm ATP*)] was added to the test tubes. The protein concentration of the membrane solution was estimated. Thirty µl of the membrane solution was added to each test tube. The test tubes were then mixed well, and the reaction was started by placing them in a 30°C water bath for 20 minutes. The reaction was stopped by pipetting 75µl of the stop solution (40mM ATP, 10mM cAMP, 1% SDS, 104 dpm/50µl [^{3}H]-cAMP) into each test tube.

A day before the test was carried out, the alumina columns were regenerated with 6ml of imidazole and the Dowex columns were regenerated with 10ml of water. The test mixtures were then loaded onto the Dowex columns. When the probes were loaded onto the columns, two washes were done with 1ml of H_2O each time. The Dowex columns were then mounted on the alumina columns, and 5ml of H_2O was added to the Dowex columns and allowed to pass through the column by gravity. Three ml of imidazole was added to the alumina columns, and the eluate was collected in scintillation vials containing 12.5ml of scintillator solution. The cpm of the eluated cAMP was quantified from the relation of [^{32}P]-cAMP/[^{3}H]-cAMP in a scintillation counter. The specific activity is calculated as:

Amount of cAMP (pmole/mg/min) =

{cpm ([^{32}P]-cAMP/[^{3}H]-cAMP) x ATP_{std}}/{cpm [^{32}P]-ATP_{std} x mg Protein x min}

4.2 Molecular Biological Methods

4.2.1 Transformation of E.coli by Electroporation

Electroporation serves as a technique to introduce foreign DNA into cells. For electroporation, cells were grown to mid-log phase and were then washed extensively with water to eliminate all salts. Usually, glycerol was added to the water to a final concentration of 10% so that the cells could be stored frozen and saved for future experiments. To electroporate DNA into cells, washed *E.coli* were mixed with the DNA to be electroporated and then pipetted into a plastic cuvette containing electrodes. A short electric pulse of approximately about 2400 volts/cm, was applied to the cells causing smalls holes in the membrane through which the DNA enters. The cells were then incubated with broth before plating.

4.2.1.1 Generation of Electrocompetent Cells

Before starting the electroporation, electrocompetent *E.coli* cells were prepared. A frozen

glycerol stock of bacterial cells were streaked on to LB plates and grown over night. A single colony was selected for the starter culture and inoculated into 200ml of LB medium. Cells were allowed to grow at 37 ºC by shaking at 250-300 rpm, until density reached OD_{600} of 0.6-0.9. Two hundred ml of LB culture medium was split into 4 equal parts by pouring 50ml of culture medium into each chilled 50ml tubes. The tubes were centrifuged at 4000 rpm for 15 minutes at 4°C. The tubes were then placed on ice. The supernatant was immediately removed as the pellet lifts off quickly. The pellets were each resuspended in 25ml ice-cold distilled H_2O. The tubes were centrifuged for the second time at 4000 rpm for 15 minutes at 4°C. The tubes were then placed on ice. The supernatant was removed. The pellets were each resuspended again in 25ml ice-cold distilled H_2O. The tubes were centrifuged for the third time at 4000 rpm for 10 minutes at 4°C. The tubes were places on ice and the supernatant removed. This time each pellet was resuspended in 400µl ice-cold dH_2O in 10% glycerol in tubes. Aliquots of 100µl the suspended culture were distributed in tubes and frozen in liquid N_2 and stored at -80°C.

4.2.1.2 Transformation by Electroporation

For electroporation, the settings for Bishop Lab electroporator (BioRad Gene Pulser) for *E.coli* transformation were set as follows: Resistance: 200 Ω, Capacitance: 25 µFD, Volts for 0.1mM cuvettes: 1.8V. One µl of DNA plasmids (0.5µg) was added to the tubes containing fresh or thawed cells (on ice). The cells were mixed by tapping the tubes with a pipette tip. The DNA and cells were transferred into chilled cuvettes and incubated for 5 minutes on ice. Cuvette was placed into sample chamber and pulse was applied. The cuvette was removed, and immediately 1 ml of SOC medium was added. With a Pasteur pipette the cells were transferred to a sterile culture tube. The culture was further incubated for 30 to 60 minutes with moderate shaking at 37 ºC. The transformation culture was plated on LB plates containing appropriate antibiotics.

4.2.2 Amplification and Purification of Plasmids

Amplifications and purifications of plasmids were for the transfection of plasmids into cells as described in section 4.2.4. An *E.coli* colony was selected, immersed into 250ml LB medium, and incubated for 12 hours at 220 rpm at 37°C upto an OD_{600} varying from 0.9-1.5. DNA purification was performed according to the manufacturer's instructions using plasmid maxiprep kits from Gibco.

4.2.3 Analysis of Nucleic Acids by Gel Electrophoresis

Agarose gel electrophoresis is the easiest and commonest way of separating and analyzing DNA. The DNA is visualized in the gel by addition of the fluorescent dye ethidium bromide which binds strongly to DNA by intercalating between the bases.

4.2.3.1 Preparation of Agarose Gels

For the purpose of this study, most agarose gels contained between 0.7% – 2% of agarose. Typically, 0.7% gel shows good separation (resolution) of large DNA fragments (5-10 kb) and a 2% gel shows good resolution of small DNA fragments (0.2–1 kb).
In this case, 1% agarose was prepared from 3.5 g of agarose and 350ml of TAE (40mM Tris-acetate (pH 8.0), 1mM EDTA). Agarose was added into an Erlenmeyer flask containing 1xTAE. It was then heated in a microwave until the contents were dissolved. The amount of water lost through boiling was supplemented. The flask was then cooled to 50°C in a water bath. The agar was subsequently poured into the electrophoresis chambers, very carefully in order to avoid bubbles. The gel was allowed to polymerize and was either used right away to separate DNA or stored in 1xTAE buffer at 4°C.
The gel chamber was filled with 1xTAE buffer. The wells of the gel were loaded with 10µl of required samples and DNA marker. For comparison, a negative sample was also loaded onto the gel. The voltage was set at 70 V and the gel was run for approximately 60 minutes. In order to observe the DNA under a UV lamp at 260 nm, the gel was stained with ethidium bromide solution (10mM Tris-HCl, 1mM EDTA, 1 mg/ml ethidium bromide) for 10 minutes and then washed in water to remove excess ethidium bromide. The bands were then observed under the UV light and photographed.

4.2.4 Transfection of HEK 293 Cells and COS-1 Cells

The lipofectin, Dosper, was used for the transfection of different types of adenylyl cyclase in HEK 293 or COS-1 cells. Due to its cationic nature, Dosper binds to the negatively charged DNA and enables efficient transfection of DNA into eukaryotic cells. Dosper has the additional advantage of combining high transfection efficiencies with low cytotoxicity.
Twenty four hours prior to transfection, the cells were plated at a density of $4x10^5$/well in 24-well plates. On the day of transfection, DMEM was aspirated and replaced with 300µl DMEM without FCS. The transfection solution consisting of solution A (0.45µg DNA to

7.2µl DMEM/well) and solution B (3.7µl Dosper to 7.2µl DMEM/well) were mixed and incubated at room temperature for 20 minutes to form Dosper-DNA complexes. The transfection solution was then added to the wells. The cells were incubated at 37°C and 5% CO_2. After 6 h, 300µl of 20% FCS in DMEM was added to the transfection solution in the wells. 24 hours after transfection, the medium was replaced with fresh medium containing 10% FCS, and the cells were incubated for another 24 hours.

The level of adenylyl cyclase activity was assessed in transfected cells in the presence of different activators with a [^{3}H]-adenine test. After preparing the membranes, the detection of the presence of the transfected protein was determined by Western blots (see section 4.1.2.)

4.2.5 X-gal Staining of pXMD-gal-Transfected Cells

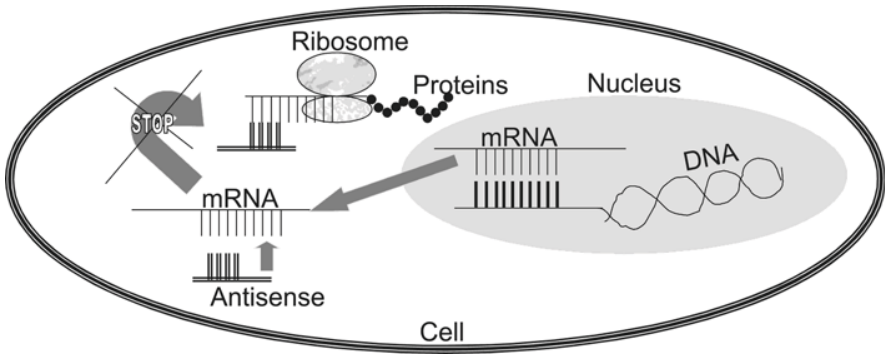
The transfection efficiency was measured by the amount of β-Galactosidase expressed in pXMD-gal-transfected cells (Sanes *et al*., 1986). The cells were transfected with pXMD-gal according to the procedure described in the section 4.2.4. Fourty eight hours later, the cells were washed with Ca^{2+}- and Mg^{2+}-free phosphate buffer solution (CMF-PBS pH 7.4), (137mM NaCl, 2.7mM KCl, 4.3mM Na_2HPO_4, 1.4mM KH_2PO_4) and then fixed with 2% (v/v) formaldehyde and 0.2% glutaraldehyde. Cells were then washed four more times with 0.1 M CMF-PBS. The β-galactosidase activity was visualized by incubating the cells in staining solution (5.13mM of K_3[Fe(CN)6], 5.13mM of K_4 [$Fe(CN)_6$], 2.05mM of $MgCl_2$ and 1 mg/ml of X-gal in PBS) for 30 to 120 minutes at 37°C. The number of blue-colored cells was counted. The transfection efficiency was calculated as the ratio the total number of blue colored cells to the total number of cells in the well.

4.2.6 Fluorescent-Oligonucleotide Uptake in Adherent Cells

Antisense oligonucleotides are designed to hybridize to their specific mRNA. This hybrid formation causes a steric or conformational obstacle for the translation of the targeted proteins. As a result, the production of a specific protein is inhibited without affecting the translation of ot

her genes (Brysch *et al*., 1994). In this study, the target protein to be down-regulated was $G\alpha_s$. A fluorescense-oligonucleotide was used to monitor the time required for antisense oligonucleotide uptake into the cell.

Figure 5: Principle of antisense-mediated target gene knock-down



There is current excitement in the RNA inhibition field because it is now possible to synthesize compounds with two-to-three orders of magnitude greater RNA-binding affinity. These third-generation antisense drugs fall into two categories: double-stranded short interfering RNA (siRNA) and single stranded DNA oligonucleotides. In the presence of transfection reagents in cell cultures, both of these third generation compounds produce significant reductions in target mRNA and proteins at concentrations below one nanomolar. This is dramatically higher than any previous antisense. Most experts in the field agree that the first-generation antisense drugs were simply not potent enough to achieve statistically robust efficacies (Juliano *et al*., 2008).

The conditions for the knock-down of the expression of a specific target gene needed to be empirically determined, depending on the cell type and on the target protein. The parameters varied in terms of time for antisense oligonucleotide uptake, optimum amount of the oligonucleotide, amount of cells and the time required for the natural down-regulation of the protein. Therefore, to optimize the process, a fluoroisothiocyanate (FITC)-coupled oligonucleotide was used and was later substituted with the required anti $G\alpha_s$ oligonucleotide. The factors taken into consideration for optimum uptake of oligonucleotide into a cell were as follows: Time required for the oligonucleotides uptake into COS-1 cells, concentration of the oligonucleotide and number of cells per well. These factors played critical roles in determining the optimal uptake of $G\alpha_s$ antisense oligonucleotide into the cell. The optimum conditions were applied to the required anti $G\alpha_s$ oligonucleotide.

4.2.7 Transfection of Adherent Cells with Antisense Oligonucleotides

For the optimum results, the following was carried out. For all the parameters, i.e:% conversion, transfection efficiency (0.3µg DNA/well is used with 3.7µl

Dosper/well), for immunoblot analysis (20µg could be loaded from the well onto the gel), for the uptake of antisense (the concentration had to be 2µM per well) and the cells were grown at 4 x 10^5 per well. The antisense oligonucleotides were added 24 hours after the transfection. Optimum conditions were set; anti $G\alpha_s$ antisense oligonucleotide was used 24 hours after the co-expression of $G\beta\gamma$ and $G\alpha_t$ on transient COS-1 AC V cells. This was done in parallel because it took the same amount of time to build up protein as it took to down-regulate it. Subsequently, optimal conditions using marked control $G\alpha_s$ oligonucleotide were established and applied to the $G\alpha_s$ antisense oligonucleotides, which were added to the COS-1 cells. These conditions were applied for the antisense oligonucleotide to down-regulate $G\alpha_s$. The down-regulation of protein expression was measured by either Western blots or [^{3}H]-adenine assays (see section 4.1.2 and 4.1.8.1).

4.2.7.1 Transfection and Effect of Antisense Oligonucleotides against $G\alpha_s$ in COS-1 Cells

On the first day, the wells were prewashed with 200µl 10% FCS DMEM and 4 x 10^5 cells/well were plated into the multiwell chamber slide with approximately 300µl medium. The cells were allowed to settle for at least 1 hour. The oligonucleotides were added at different time points. The time labeled to each well indicated the total time the cells were incubated with the oligonucleotide, i.e: from addition of the oligonucleotide to the cells to the removal of the oligonucleotide from the cells. Hence, each well was labeled 48 hour well, 24 hour well, 8 hour well, 4 hour well, 2 hour well and 1 hour well. 6µl of 100µM FITC control (fluorescence-labeled-sequence phosphorothioate oligonucleotide) was added directly to the 48 hours well to make a 2µM final concentration solution. On the second day, 6µl of 100µM FITC control was added to the 24 hours well. On the third day, 6µl of 100µM FITC control was added to the respective wells at the time points 8 hours, 4 hours, 2 hours, and 1 hour before removal of the oligonucleotide from these wells accordingly. One hour after the last addition of FITC control, the wells were washed with a medium containing no FCS. The medium was drained and replaced with 4% paraformaldehyde with minimal medium to fix the cells for 5 minutes. The medium was drained, and the cells were washed with minimal medium two times for 2 minutes each. The medium was drained off, and the cell culture slide was dehydrated with a graded series of alcohol from 70% to 100%, each time for 1 minute and air dried. To examine the fluorescence, a few drops of immersion oil were added and observed at 495 nm under a fluorescence microscope.

4.3 Expression of AC II and AC V in Mammalian Cells

4.3.1 Cultivation of HEK 293, COS-1, S49, and S49cyc$^-$ Cells

For the heterological expression of adenylyl cyclase and G-proteins in eukaryotic cells, the following cells were used.

HEK 293:	human embryonal kidney cells (wild type, adherent) ATCC: CRL1573
HEK 293 AC II:	human embryonal kidney cells (adherent), stably expressing AC II
HEK 293 AC V:	human embryonal kidney cells (adherent), stably expressing AC V
COS-1:	monkey kidney cells (wild type, adherent) ATCC: CRL1650
COS-1 AC II:	monkey kidney cells (adherent), stably expressing AC II
COS-1 AC V:	monkey kidney cells (adherent), stably expressing AC V
S49:	human lymphoma cells (wild type, non-adherent) ATCC: TIB 28
S49cyc$^-$:	mutated human lymphoma cells (lacking α_s, non-adherent)

4.3.1.1 HEK 293 Cell Lines

HEK 293 epithelial cells were generated by transformation of Human Embryonic Kidney cell cultures (hence HEK) with sheared adenovirus 5 DNA, and are first described in 1977 (Graham *et al*., 1977). Although an earlier report suggested that the cells contained adenovirus 5 DNA from both the right and left ends of the viral genome [RF32764], it is now clear that only left end sequences are present.

In culture, healthy HEK 293 cells attach to the bottom of the plate forming a monolayer. These cells double every twenty four hours. HEK 293 wild type cells were grown in MEM with 10% FCS at 37°C and 5% CO_2. Every 2 or 3 days, the cells were washed with 1mM EDTA in Ca^{2+}/Mg^{2+} free PBS solution, trypsinized, and diluted 1:4 or 1:7 in a fresh medium.

HEK 293 cells stably expressing AC II or AC V were grown in MEM with 0.5 mg/ml G418 and 10% FCS at 37°C and 5% CO_2. Every 2 or 3 days the cells were washed with 1mM EDTA in PBS solution, trypsinized, and diluted 1:2 or 1:3 in a fresh medium.

4.3.1.2 COS-1 Cell Lines

COS-1 fibroblast-like cells have been generated from an African green monkey kidney and are suitable for transfection with vectors expressing SV40 T antigen. This line contains

T antigen, retains complete permissiveness for lytic growth of SV40, supports the replication of temperature-sensitive A209 virus at 40°C, and supports the replication of pure populations of SV40 mutants with deletions in the early region. The line is derived from the CV-1 cell line (ATCC CCL-70) by transformation with an origin defective mutant of SV40 which codes for wild type T antigen.

In culture, healthy COS-1 cells attach to the bottom of the plate forming a monolayer. These cells double every twenty four hours. COS-1 wild type cells were grown in DMEM with 10% FCS at 37°C and 5% CO_2. Every 2 or 3 days, the cells were washed with 1mM EDTA in PBS solution, trypsinized, and diluted 1:4 to 1:7 in fresh medium.

COS-1 cells stably expressing AC II or AC V were grown in DMEM with 0.5 mg/ml G418 and 10% FCS at 37°C and 5% CO_2. Every 2 or 3 days, the cells were washed with 1mM EDTA in PBS solution, trypsinized, and diluted 1:2 or 1:3 in a fresh medium.

4.3.1.3 S49 and S49cyc⁻ Cell Lines

The S49 cell line has been derived from mouse lymphoma cells. The S49cyc⁻ cell line is a mutant of S49 mouse lymphoma cells deficient in $G\alpha_s$. Both these cell lines are non-adherent and have a fibroblast-like morphology. They were grown in DMEM with 10% FCS at 37 °C and 5% CO_2. Every 2-3 days, the cells were further diluted 1:4 to 1:6 in fresh medium.

4.3.2 Membrane Preparation of HEK 293 and COS-1 Cells

In order to adequately study the interaction of these various interactions of G-proteins in the plasma membrane with the enzyme adenylyl cyclase, a preparation of intact plasma membranes still containing the adenylyl cyclase activity is desirable. In this case, HEK 293 cells expressing AC or COS-1 cells expressing AC were prepared to test their adenylyl cyclase activity.

The cells were washed once with CMF-PBS solution. Five ml EDTA was added to the cells, cells were scraped off with a rubber policeman, and transferred into falcon tubes. They were then centrifuged for 5 minutes at 600 g at 4°C. The pellet was resuspended into 1ml of lysis buffer (10mM Tris-HCl pH 7.4, 1mM EGTA) containing protease inhibitors (0.5mM DTT, 0.5mM PMSF, 0.1mM benzethonium chloride, 1mM benzamidine, 3.2µg/ml STI, 88µg/ml TPCK, 22µg/ml TLCK, 2.8µg/ml trasylol) were incubated on ice for 10 minutes. Lysis of the cells was done using the freeze/thaw method (freezing in

liquid nitrogen and defrosting in a 30°C water bath) in three cycles. The cell nuclei and unbroken cells were separated by centrifugation (40 minutes, 400 g, 4°C), and the membrane pellet was resuspended in membrane buffer (25mM Tris-HCl pH 7.4, 1mM EGTA, 1mM $MgCl_2$, 0.5mM DTT, 50 ng/µl BSA, 10% glycerol). The membranes were frozen in liquid nitrogen and stored at -70°C. Membranes were analyzed by Western blots (see section 4.1.2). Adenylyl cyclase activity was estimated *in vitro* (as described in section 4.1.9).

4.3.3 Generation of COS-1 Cell Lines Stably Expressing AC II and AC V

By testing the adenylyl cyclase activity of the membranes, it could be confirmed that the required cells were transfected with the specific AC. The cells were washed twice with CMF-PBS, 48-72 hours after the transfection. Next they were trypsinized and diluted 1:20 with new 10% (v/v) FCS in DMEM medium including 0.5 mg/ml G418. The cells then were incubated overnight at 37°C and 5% CO_2. G418 was used for the selection of plasmid containing cells. Thereafter, the medium was changed every third day. In order to derive a monoclonal cell line, the cells were further diluted so that each second well in a 24-well plate theoretically contained one cell. They were further cultivated, until the wells contained about 3000 cells. Then they were plated in 10 cm petri dishes. The activity of adenylyl cyclase was tested with the [^{3}H]-adenine test and Western blots as described in section 4.1.8.1 and 4.1.2.

5 RESULTS

5.1 Gβγ-Regulation of AC-Activity in HEK 293 Cells Expressing AC II and AC V

5.1.1 Generation of HEK 293 Cells Expressing AC II and AC V

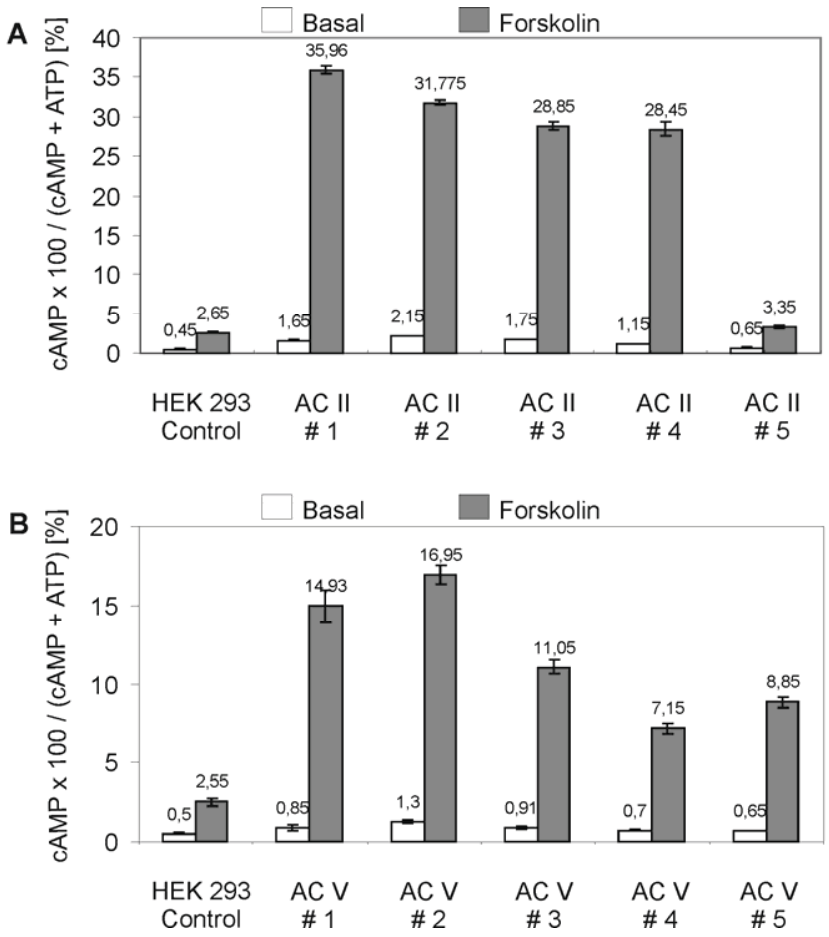


Figure 6: AC activity in HEK 293 cells stably expressing AC II (A) and AC V (B)

A) HEK 293 cells were transfected with AC II and selected with G-418 (0.8mg/ml). B) HEK 293 cells were transfected with AC V and selected with 0.8mg/ml G-418. AC II HEK 293 cells and AC V HEK 293 cells were maintained with 0.4mg/ml G-418 after seeding one cell per well. To analyze AC activity, cells were labeled with [^{3}H]-adenine for 60 min and stimulated with and without forskolin (10µl; 10min). cAMP formation was measured as described in section 4. Indicated are mean values ± S.D. of three independent experiments.

HEK 293 cells stably expressing AC II (Feinstein *et al*., 1991) or AC V (Feinstein *et al*., 1991) were established. Monoclonal cell lines were obtained by selection with 0.8mg/ml G-418 and were maintained using 0.4mg/ml G-418. The expression of AC II and AC V in HEK 293 cells was compared to wild type HEK 293 cells by Western blot analysis as well

as by AC activity assays. Subsequent to isolation of AC transfectants, a series of preliminary experiments was carried out to characterize the enzyme activity in different clonal cell lines.

Figure 6 shows the basal (white bars) and forskolin-stimulated (grey bars) activity of adenylyl cyclase in HEK 293 cells. Basal and forskolin-stimulated AC activity (AC activity) in HEK 293 cells stably expressing AC II (AC II HEK 293 cells) or stably expressing AC V (AC V HEK 293 cells) was compared to that of control vector-transfected cells. The forskolin-stimulated AC activity was five fold higher as compared to the basal AC activity in wild type cells (control). The basal AC activity of AC II HEK 293 cells ranged from three fold to four fold compared to the basal AC activity of wild type HEK 293 cells. The forskolin-stimulated AC activity in AC II HEK 293 cells (specifically AC II #2) was six fold higher than the forskolin-stimulated AC activity in wild type HEK 293 cells (control). Similarly the forskolin-stimulated AC activity in AC V HEK 293 cells (specifically AC V #1) showed 13.5 fold stimulation compared to the forskolin-stimulated AC activity in wild type HEK 293 cells (control). This increase in AC activity clearly showed that AC II and AC V have been stably expressed into HEK 293 cells. These specific clonal cell lines were further analyzed by Western blots.

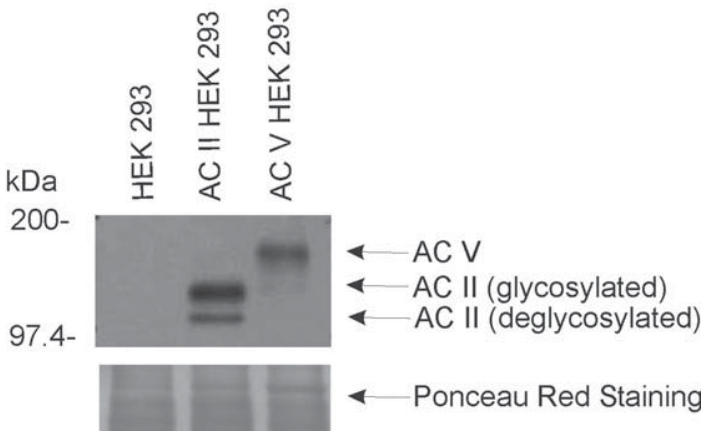


Figure 7: Detection of HEK 293 cells stably expressing AC II and AC V by Western blot analysis.

Membranes from HEK 293 cells (wild type, 25µg of protein), AC II HEK 293 cells (AC II #2, 25µg of protein) and AC V HEK 293 cells (AC V #1, 25µg of protein) were lyzed and separated by SDS-PAGE, blotted onto PVDF membrane and visualized by antibody BBC-2-AP and chemiluminescence as described in section 4. Vertical numbers indicate molecular mass (kDa) of iodinated standard proteins (upper panel). Equal loading was confirmed by staining the membranes with Ponceau Red (bottom panel).

As shown in Figure 7, membranes were prepared from wild type HEK 293 cells, AC II HEK 293 cells (AC II 2 from Figure7) and AC V HEK 293 cells (AC V 1 from Figure 6B)

to detect the expression of endogenous AC and stably expressed AC in HEK 293 cells. As can be clearly seen, there was strong expression of AC II in stably expressed AC II HEK 293 cells and strong expression of AC V in stably expressed AC V HEK cells, compared to the endogenous adenylyl cyclase present in wild-type HEK 293 cells. The lower, weaker band represents the unglycosylated form of AC; while, the higher, stronger band represents the glycosylated form (Bol *et al*., 1997).

The results of these experiments indicated that the respective AC were stably expressed in HEK 293 cells, therefore which were used for detailed further experimental analysis. In particular, these studies used the AC II HEK 293 clonal cell lines (AC II 2 from Figure 6a) and AC V HEK 293 clonal cell lines (AC V1 from Figure 6b).

5.1.2 Establishment of an Experimental System to Investigate the Regulation of AC II and AC V by Gβγ-Dimers in Intact Cells

As shown schematically in Figure 8, a system was set, to investigate the Gβγ-mediated effect on the specific adenylyl cyclase isoforms. The idea is to release the endogenous Gβγ present in HEK 293 cells from the $P2Y_2$ receptors. HEK 293 cells endogenous express$P2Y_2$ receptors (Gao *et al*., 1999) which are coupled to $G\alpha_q$. UTP was used to stimulate $P2Y_2$ receptors. Stimulation of $P2Y_2$ receptors leads to the release of $G\alpha_q$ and Gβγ. $G\alpha_q$ has no direct effect on ACs but can indirectly regulate AC through PKC. Staurosporin, an inhibitor of PKC, was used to inhibit the PKC effect on ACs, thus enabling to research the regulation of Gβγ released from $P2Y_2$ receptor on AC II and AC V. For this purpose, AC II and ACV were expressed in HEK 293 cells to establish HEK 293 cells stably expressing AC II and AC V HEK 293 cells. These cells were stimulated with forskolin and isoproterenol. Forskolin has a direct effect on all ACs, whereas isoproterenol stimulates ACs *via* $G\alpha_s$. AC II was used as a positive control. Release of Gβγ in the presence of $G\alpha_s$ had a synergistic effect on AC II. This effect was not seen if AC II was stimulated directly with forskolin.

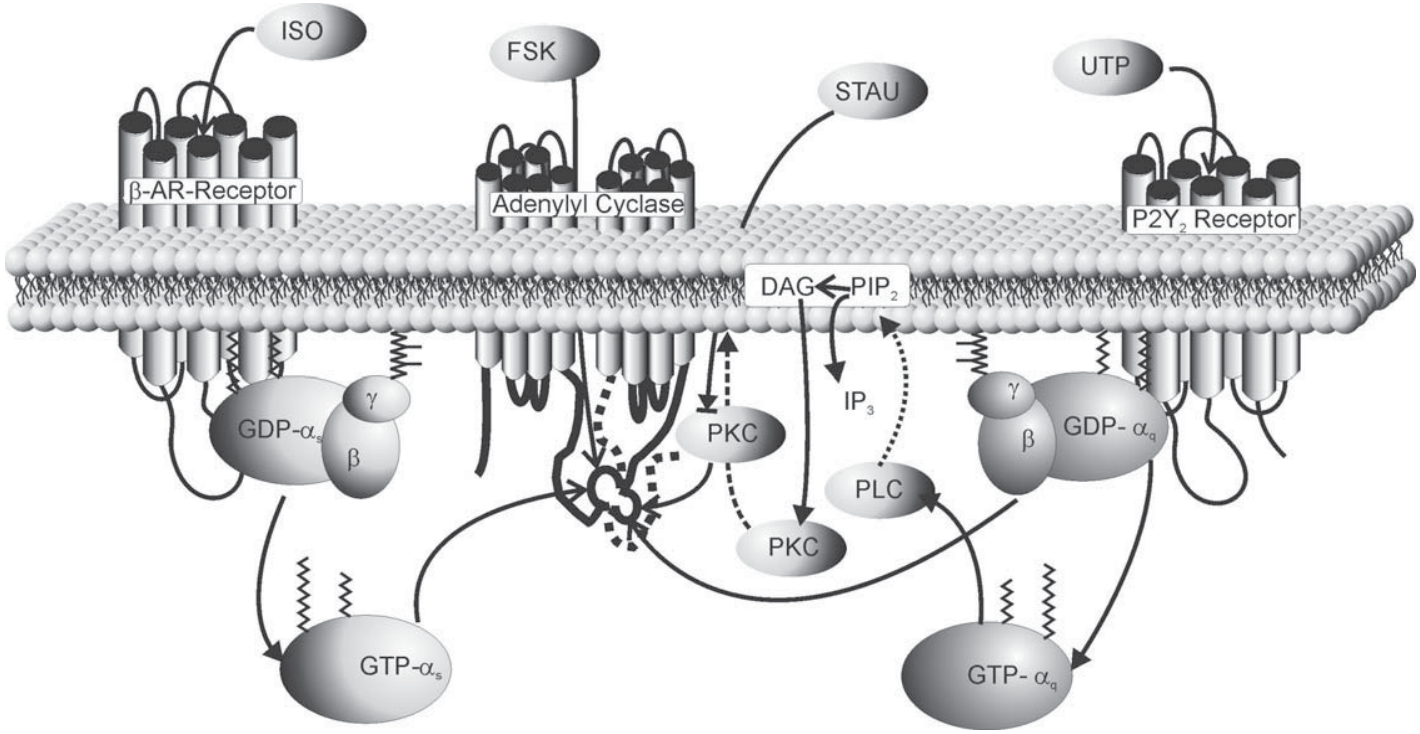


Figure 8: Schematic overview of the relevant molecular signaling pathways studied in this work

$G\alpha_s\beta\gamma$ is bound to the β-ARs. Isoproterenol is used to trigger the release of $G\alpha_s$ and $G\beta\gamma$. Forskolin is hydrophobic and therefore passes through the membrane and binds to the catalytic core of adenylyl cyclase directly. $G\alpha_q\beta\gamma$ is bound to the $P2Y_2$ receptor. UTP, an agonist for $P2Y_2$ receptors, is used to trigger the release of $G\alpha_q$ and $G\beta\gamma$. PLC isoforms are stimulated by $G\alpha_q$ as well as $G\beta\gamma$ where $G\alpha_q$ has a stronger affinity. On stimulation of PLC, PLC translocates to the membrane. Its substrate PIP_2 is hydrolyzed to DAG and IP_3. The hydrophilic IP_3 moves to the endoplasmic reticulum, where IP_3 stmulates the release of Ca^{2+} from intracellular stores. The lipophilic DAG remains in the membrane and activates PKC, which in turn activates adenylyl cyclase isoforms in an isoform-specific manner. Staurosporin is used to inhibit the PKC activity. The idea is to observe the effect of endogenous $G\beta\gamma$ (released from β-AR and $P2Y_2$ receptors) on AC II and AC V. FSK: Forskolin, ISO: isoproterenol, β-AR: β-adrenergic receptor, UTP: uridine triphosphate, Stau: staurosporin, PLC: phospholipase C, PKC: protein kinase C, PIP_2: phosphatidylinositol bisphosphate, IP_3: inositol1,4,5-trisphosphate, DAG: diacylglycerol, GDP: guanosine diphosphate, GTP: guanosine triphosphate.

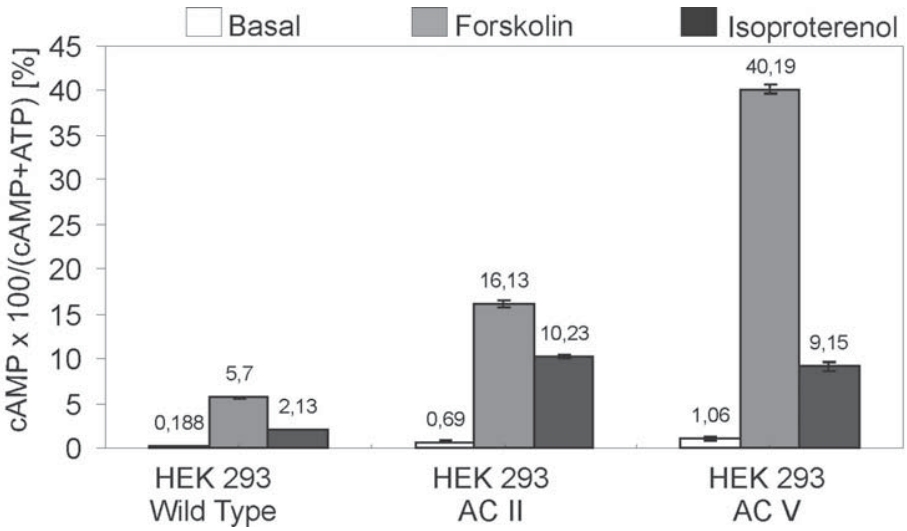


Figure 9: Effect of forskolin and isoproterenol on HEK 293 cells stably expressing AC II and AC V.

Each cell type was observed under the following conditions: basal activity (white bars), forskolin-stimulated AC activity (grey bars), and isoproterenol-stimulated AC activity (black bars). Cells were labeled with [^{3}H]-adenine for 60 min. and stimulated with forskolin (10µl; 10min) and isoproterenol (1µM; 10min). cAMP formation was measured as described in section 4. Indicated are mean values ± S.D. of three independent experiments.

As shown in Figure 9, the formation of cAMP reflected AC activity for each of the three cell types *i.e.*: wild type HEK 293, stably expressing AC II HEK 293 cells and stably expressing AC V HEK 293 cells under three conditions: basal condition (control), forskolin-stimulated condition (grey bars) and isoproterenol-stimulated (black bars). The AC activity in wild type HEK 293 cells was compared to the AC activity of AC II and AC V stably expressed in HEK 293 cells. Under forskolin-stimulated condition, the cAMP formation from ATP increased by factor of one in wild-type cells, three in AC II HEK 293 cells, and seven in AC V HEK 293 cells, and isoproterenol-stimulated condition increased the cAMP formation from ATP by factors of one in wild-type cells, four in AC II HEK 293 cells, and three in AC V HEK 293 cells. The conditions set throughout the whole research were that these cells will be stimulated directly with forskolin as well as with isoproterenol which stimulate the adenylyl cyclases *via* $G\alpha_s$. AC II HEK 293 cells were used as positive control since it is proven that $G\beta\gamma$ has a synergistic effect on AC II in the presence of $G\alpha_s$.

5.1.3 *Effect of Gβγ on HEK 293 Cells Stably Expressing AC II and AC V*

UTP, an agonist for $P2Y_2$ receptors, was used to trigger the release of endogenous Gβγ from trimer $G\alpha_q\beta\gamma$ which is bound to the $P2Y_2$ receptor (Figure 10 and 12). It is also known that $G\alpha_q$ is released and that $G\alpha_q$ stimulates PKC, which in turn activates AC II and AC V (Nowak *et al*., 1999). Staurosporin was used to inhibit this process (Kawabe *et al*., 1996), thus enabling observation of the effect of Gβγ solely on AC II HEK 293 cells (Figure 10) or AC V HEK 293 cells (Figure 11)

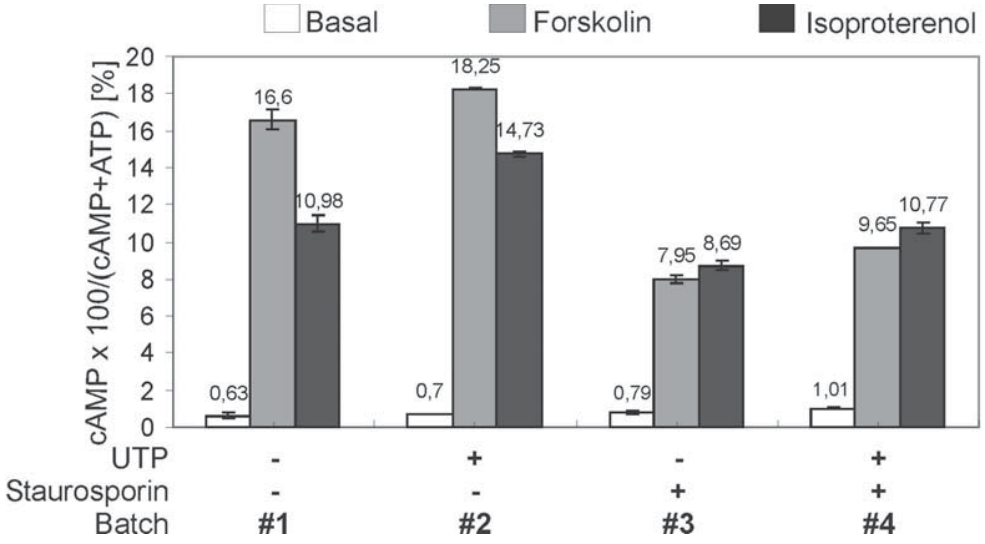


Figure 10: Effect of UTP on HEK 293 cells stably expressing AC II.

AC II HEK 293 cells were labeled with [^{3}H]-adenine for 60 minutes. These cells were stimulated with forskolin (10µl; 10min) and isoproterenol (1µM; 10min). The three bars together represent the basal activity (white bars), forskolin-stimulated AC activity (grey bars), and isoproterenol-stimulated AC activity (black bars) and are referred to in the text as a Batch. Each Batch was pretreated with Staurosporin (100nM) or UTP (20µM) or both or neither. cAMP formation was measured as described in section 4. Indicated are mean values ± S.D. of three independent experiments.

In Figure 10 and Figure 11, Batch 1 is a control which represents the AC activity of AC II HEK 293 cells and AC V HEK 293 cells respectively. Batch 2 shows the activity of AC II HEK 293 cells or AC V HEK 293 cells in the presence of UTP. Batch 3 shows the AC activity of AC II HEK 293 cells or AC V HEK 293 cells when pretreated with staurosporin, and Batch 4 shows the AC activity of AC II HEK 293 cells or AC V HEK 293 cells when pretreated with staurosporin and stimulated with UTP.

When comparing Batch 2 and Batch 1, there was 11.1%, 9.9% and 34% increase in cAMP level in the presence of UTP for each of the three conditions (basal, forskolin and isoproterenol), respectively (Figure 10). The stimulated effect of PKC was seen under all

three conditions. Comparing the PKC stimulated effect in Batch 2, it was seen that isoproterenol-stimulated effect was three fold higher compared to the basal and forskolin-stimulated effect. This increase in AC activity when stimulated with isoproterenol could be due to the release of Gβγ (released from stimulation with UTP) and its synergistic effect with $G\alpha_s$ (released from stimulation with isoproterenol) on AC II. The AC activity under basal and forskolin-stimulation was solely the effect of PKC on AC II.

Staurosporin was used to eliminate the PKC stimulation of AC II (Batch 3), in order to specifically observe the effect of Gβγ on AC II. Comparing Batch 3 to Batch 1, a clear reduction in AC activity was seen by 53% for the forskolin stimulation on AC II and 21% for the isoproterenol stimulation on AC II. This indicated that PKC had a high basal activity in AC II HEK 293 cells even before stimulation with UTP. A similar response was found in an experiment performed by Jacobowitz's group, where the stimulation by PKC on AC II in Sf9 cells was active before even being stimulated with UTP or other agonists (Jacobowitz *et al*., 1993). Comparing the basal PKC activity on AC II in Batch 3 to Batch 1 there was no reduction in AC activity in the presence of staurosporin.

When staurosporin was added to UTP-pretreated AC II HEK 293 cells (Batch 4 *versus* Batch 1), the AC activity reduced by 42% for forskolin-stimulated AC II cells and no change in AC activity was observed for isoproterenol-stimulated AC II cells. This indicated PKC activity overrules the forskolin stimulation on AC II, whereas the isoproterenol stimulation on AC II overrules PKC activity on AC II (isoproterenol stimulation in Batch one was similar to isoproterenol stimulation in Batch 4). Hence, the stimulation seen in Batch 2 when stimulated with isoproterenol was solely due to the release of Gβγ.

We therefore carried out a similar experiment to investigate the regulation of Gβγ on HEK 293 cells stably expressing AC V. Figure 11 represents the AC activity of AC V in HEK 293 cells. The cells were pretreated with staurosporin and stimulated with UTP.

Comparing the basal, forskolin-stimulated and isoproterenol-stimulated AC activity in AC V HEK 293 cells in Batches 1 and Batch 2, there was a 25%, 3%, and 2% increase in AC activity. Comparing Batch 1 to Batch 3, there was 26% and 11% decrease in AC activity for the basal and forskolin-stimulated AC V, respectively and no change in the isoproterenol-stimulated AC V activity. Comparing Batch 4 to Batch 1, there was a 17% and 9% decrease in AC activity only for the basal and forskolin-stimulated AC V, respectively and a 11% increase in AC activity for the isoproterenol-stimulated AC V

activity.

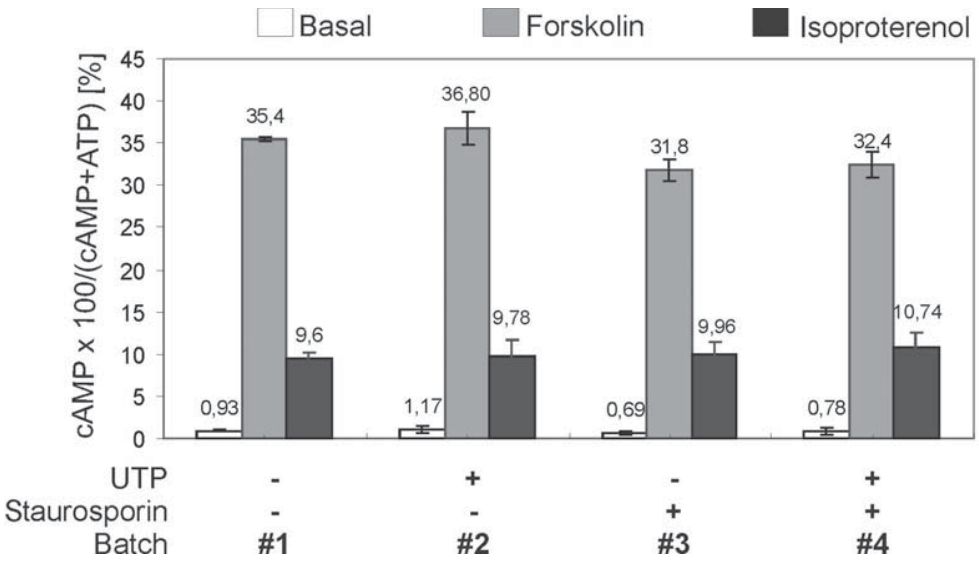


Figure 11: Effect of UTP on HEK 293 cells stably expressing AC V.

AC V HEK 293 cells were labeled with [^{3}H]-adenine for 60 minutes. These cells were stimulated with forskolin (10µl; 10min) and isoproterenol (1µM; 10min). The three bars together represent the basal activity (white bars), forskolin-stimulated AC activity (grey bars), and isoproterenol-stimulated AC activity (black bars) and are referred in the text to as a Batch. Each Batch was pretreated with Staurosporin (100nM) or UTP (20µM) or both or neither. cAMP formation was measured as described in section 4. Indicated are mean values ± S.D. of three independent experiments.

One can conclude that isoproterenol stimulation on AC V activity was the same in all cases irrespective of the pretreatment with or with out UTP or staurosporin (Figure 11). As for forskolin-stimulated AC V activity on stimulation with UTP there was a 3% increase and in the presence of staurosporin there was a reduction of 11%. One can also conclude that PKC basal activity was present in AC V HEK 293 cells but not as high as in AC II HEK 293 cells. It is important to note that staurosporin was added in order to inhibit the PKC activity on AC, so solely the activity of Gβγ on AC V could be observed. Only in its basal states, stimulation with UTP showed a 26% increase in AC activity (comparing Batch 1 to Batch 2), which was the PKC effect on AC V (not strongly seen when stimulated with forskolin). Comparing the basal activity in Batch 3 and Batch 4 to Batch 1, there was a 26% and 17% decrease in AC activity. According to Jacobwitz, PKC is active in its basal state for AC II (Jacobowitz *et al*., 1993), but whether the same holds true for AC V is not yet known. Thus it was not clear if this decrease (Batch 3 and Batch 4 compared to Batch 1) was due to inhibition of PKC activity or Gβγ effect. Therefore it was decided to coexpress Gβγ (specifically $G\beta_1\gamma_2$) into HEK 293 cells stably expressing

AC II or AC V (*i.e.* AC II HEK 293 and AC V HEK 293 cells).

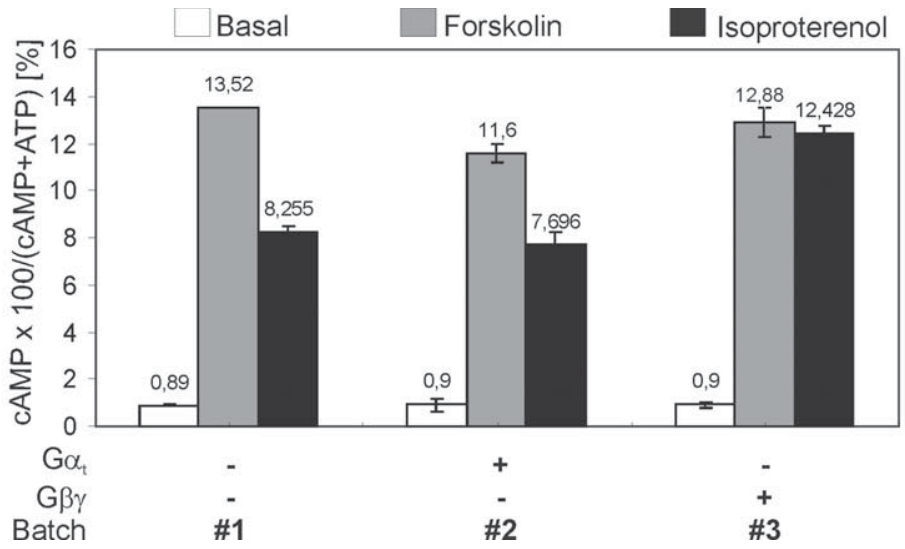


Figure 12: Effect of Gβγ and Gα$_t$ on HEK 293 stably expressing AC II.

AC II HEK 293 cells were transfected with Gα$_t$ (Batch 2) or Gβγ (Batch 3). 48 h after transfection, cotransfected AC II HEK 293 cells were labeled with [^{3}H]-adenine for 60 minutes. These cells were stimulated with forskolin (10µl; 10min) and isoproterenol (1µM; 10min). cAMP formation was measured as described in section 4. Indicated are mean values ± S.D. of three independent experiments.

HEK 293 cells stably expressing AC II were transfected together with Gβγ or Gα$_t$. Gα$_t$ was coexpressed and used as a negative control; as it scavenges free endogenous Gβγ, thus indirectly showing the effect of Gβγ (Batch 2). In Batch 3, AC II HEK 293 cells were transfected with Gβγ (Figure 12).

Comparing the Gα$_t$ columns (Batch 2) to the control columns (Batch 1) in Figure 12, the basal activity showed an increase of 10% whereas forskolin-stimulated and isoproterenol-stimulated cells showed a decrease of 15% and 7% in AC activity, respectively. Comparing the Gβγ columns (Batch 3) to the control columns (Batch 1) in Figure 12, the basal and forskolin-stimulated cells showed no difference in AC activity, but AC II HEK 293 cells stimulated by isoproterenol showed a 50% increase in AC activity. Gβγ stimulated AC II only in the presence of activated Gα$_s$, which was released when AC II was stimulated with isoproterenol (comparing Batch 1 to Batch 3). The synergistic effect of Gβγ and Gα$_s$ on AC II in Batch 3 confirmed the transfection of Gβγ into these cells; hence the same procedure was carried out on AC V HEK 293 cells.

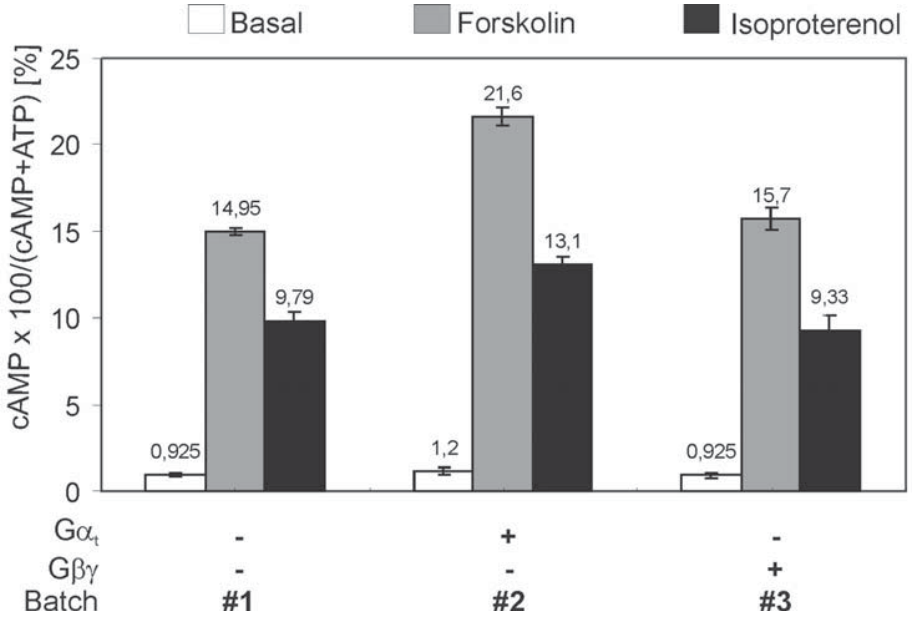


Figure 13: Effect of Gβγ and Gα$_t$ on HEK 293 stably expressing AC V.

AC V HEK 293 cells were transfected with Gα$_t$ (Batch 2) or Gβγ (Batch 3). 48 h after transfection, cotransfected AC V HEK 293 cells were labeled with [^{3}H]-adenine for 60 minutes. These cells were stimulated with forskolin (10µl; 10min) and isoproterenol (1µM; 10min). cAMP formation was measured as described in section 4. Indicated are mean values ± S.D. of three independent experiments.

As mentioned earlier, Gα$_t$ was used to sequester free endogenous Gβγ (Lustig *et al*., 1993), hence making it possible to indirectly observe the effect of Gβγ on AC V. Interestingly, an elevation of cAMP formation was observed with the basal, forskolin-stimulated, and isoproterenol-stimulated AC activity by 23%, 35% and 26%, respectively. The stimulation for basal and forskolin-stimulated AC activity was due to elimination of the effect of endogenous Gβγ on AC V, which could be an indirect inhibitory effect. The effect of isoproterenol on AC V could also be due to stimulated Gα$_s$ available from the sequestering of Gβγ by Gα$_t$. Comparing Batch 3 to Batch 1 in Figure 13, in the presence of Gβγ the basal, forskolin-stimulated as well as the isoproterenol-stimulated AC activity showed no change in AC activity. With increase in concentration of Gβγ (Batch 3), one would expect that the cAMP formation would decrease even more compared to the control. On the contrary, it remained the same. From this experiment it was clear that addition of Gα$_t$ showed stimulation in basal, forskolin- and isoproterenol-stimulated AC activity which was due to the capturing of Gβγ. Hence upon capturing Gβγ, its effect was eliminated, thus indirectly showing that Gβγ could have an inhibitory effect on AC V. To see if this difference was due to a specific effect on the amount or activity of AC in HEK 293 cells stably expressing AC II/AC V.

5.1.4 *Effect of Gβγ on HEK 293 Cells Transiently Expressing AC II and AC V*

It was decided to carry out the same experiments in HEK 293 cells transiently expressing AC II or AC V. Hence HEK 293 cells transiently expressing AC II were transfected with Gβγ or $G\alpha_t$ and stimulated with forskolin and isoproterenol to observe the effect of Gβγ on AC II.

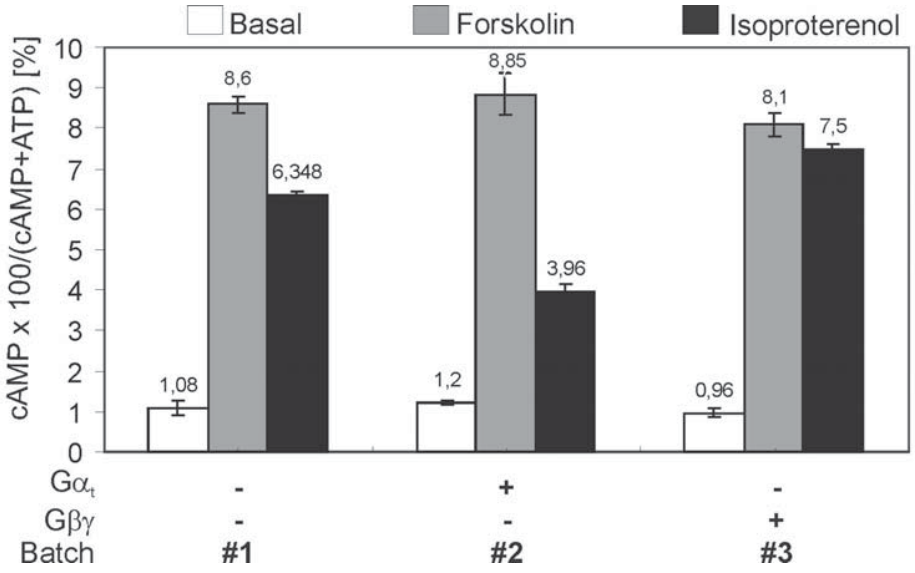


Figure 14: Effect of Gβγ and $G\alpha_t$ on HEK 293 cells transiently expressing AC II

HEK 293 cells transiently expressing AC II were transfected together with $G\alpha_t$ (Batch 2) or Gβγ (Batch 3). 48 h after transfection, cotransfected AC II HEK 293 cells were labeled with [^{3}H]-adenine for 60 minutes. These cells were stimulated with forskolin (10µl; 10min) and isoproterenol (1µM; 10min). cAMP formation was measured as described in section 4. Indicated are mean values ± S.D. of three independent experiments.

Figure 14 depicts the effect of Gβγ and $G\alpha_t$ on HEK 293 cells transiently expressing AC II. Figure 12 depicts the effect of Gβγ and $G\alpha_t$ on HEK 293 cells stably expressing AC II. Comparing Batch 1 to Batch 2, in the presence of $G\alpha_t$ AC activity increased by 10% and 3% for the basal and forskolin-stimulated AC activity and isoproterenol-stimulated AC activity decreased by 40%. In the presence of Gβγ (comparing Batch 3 to Batch 1), the basal and forskolin-stimulated activity decreased by 12% and 6%, respectively. The isoproterenol-stimulated AC activity increased by 16%. The first observation comparing Figure 12 and Figure 14 was that the AC activity is higher in the stably expressing AC II in HEK 293 cells compared to the transiently expressed AC II in HEK 293 cells. Table 7 and 8 gives a comparison of AC activity in the stably expressing AC II in HEK 293 cells and

transiently expressing AC II in HEK 293 cells.

Table 7: AC activity of AC II stably expressed in HEK 293 cells cotransfected with $G\alpha_t$ and $G\beta\gamma$

	Basal	Forskolin-stimulated	Isoproterenol-stimulated
Control → $G\alpha_t$	-	15%↓	7%↓
Control → $G\beta\gamma$	-	-	50%↑

Table 8: AC activity of AC II transiently expressed in HEK 293 cells cotransfected with $G\alpha_t$ and $G\beta\gamma$

	Basal	Forskolin-stimulated	Isoproterenol-stimulated
Control → $G\alpha_t$	10%↓	3%↓	40%↓
Control → $G\beta\gamma$	12%↓	6%↓	16%↑

In HEK 293 cells stably expressing AC II, the change in AC activity for the basal and forskolin-stimulated AC II was negligible. Most interesting to note was that addition of Gβγ to AC II was the 50% stimulation in AC activity in the presence of isoproterenol. This was seen directly (50% increase, see Figure 12) and indirectly (40% decrease, see Figure 14). An explanation for the 7% decrease (Figure 12) compared to the 40% decrease (Figure 14) in AC activity could be that there was a high amount of endogenous Gβγ already present due to stably expressing AC II in HEK 293 cells and the 16% increase (Figure 14) to the 50% increase could be due to the lower amount of Gβγ transiently expressed in AC II HEK 293 cells. In this case, elimination of Gβγ by the Gβγ-scavenger $G\alpha_t$ indirectly reveals how Gβγ and $G\alpha_s$ have a stimulating effect on AC II.

As a positive control, this experiment showed that Gβγ is present endogenously as well as by coexpression and that the expected AC activity with AC II did take place. Therefore, the same conditions were applied to HEK 293 cells transiently expressing AC V (Figure 15).

Comparing Batch 1 to Batch 2 in the presence of $G\alpha_t$, there was a 34%, 45% and 53% increase in basal, forskolin- and isoproterenol-stimulated AC activity, respectively. Comparing the AC activity in the presence of Gβγ to the control, there is a negligible increase for the basal, forskolin- and isoproterenol-stimulated AC activity. These changes in AC activity were minimal and therefore it can be concluded that no direct effect of Gβγ could be seen. However, $G\alpha_t$ effect on AC V induced a clear increase in AC activity for all three cases tested (basal, forskolin- and isoproterenol-stimulated). These results imply an indirect inhibitory effect of Gβγ on AC V.

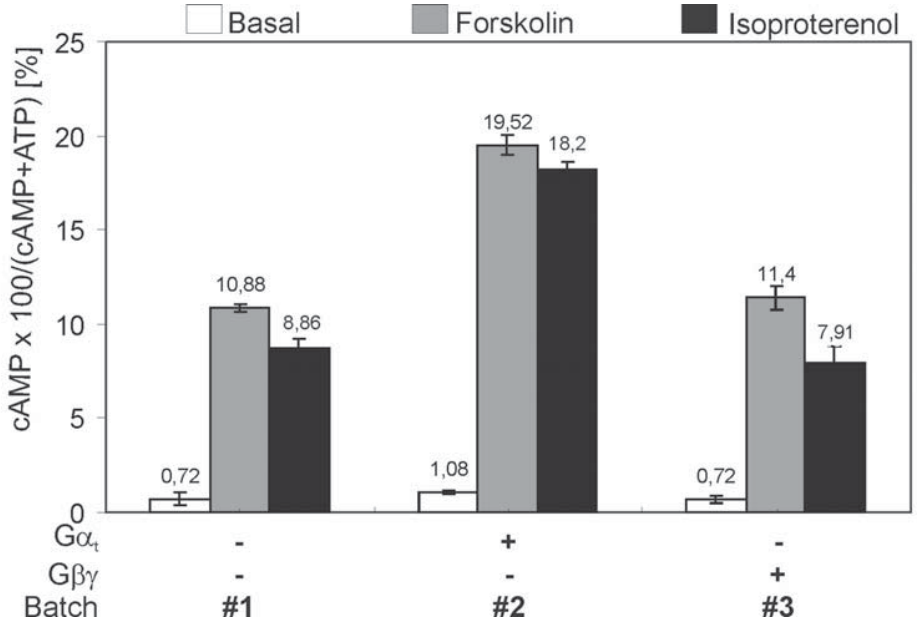


Figure 15: Effect of Gβγ and Gα$_t$ on HEK 293 cells transiently expressing AC V

HEK 293 cells transiently expressing AC V were transfected together with Gα$_t$ (Batch 2) or Gβγ (Batch 3). 48 h after transfection, cotransfected AC V HEK 293 cells were labeled with [^{3}H]-adenine for 60 minutes. These cells were stimulated with forskolin (10µl; 10min) and isoproterenol (1µM; 10min). cAMP formation was measured as described in section 4. Indicated are mean values ± S.D. of three independent experiments.

Table 9: AC activity of AC V stably expressed in HEK 293 cells cotransfected with Gα$_t$ and Gβγ

	Basal	Forskolin-stimulated	Isoproterenol-stimulated
Control → Gα$_t$	23%↑	35%↑	26%↑
Control → Gβγ	-	-	-

Table 10: AC activity of AC V transiently expressed in HEK 293 cells cotransfected with Gα$_t$ and Gβγ

	Basal	Forskolin-stimulated	Isoproterenol-stimulated
Control → Gα$_t$	34%↑	45%↑	53%↑
Control → Gβγ	-	-	-

Similar to AC activity of stable AC II HEK 293 lines, the AC activity was higher in HEK 293 cells stably expressing AC V compared to HEK 293 cells transiently expressing AC V (Table 9 and 10). It is possible that endogenous Gβγ was so high in HEK 293 cells that AC V was already at its maximum inhibition and addition of more Gβγ to the cell showed no effect. However, the stimulatory effect was seen when Gα$_t$ was expressed in HEK 293 cells either transiently or stably expressing AC V. This stimulatory effect was likely due to the fact that Gα$_t$ scavenges endogenous Gβγ, thus indirectly eliminating the inhibitory effect of Gβγ on AC V. These results pointed to two possible explanations for

why the inhibition was not seen directly upon addition of Gβγ. One possible explanation was that endogenous Gβγ in HEK 293 cells involved in the regulation of AC activity may be different from the coexpressed $G\beta_1\gamma_2$ dimer, so the inhibition was seen only indirectly *via* $G\alpha_t$. Another possible explanation is that endogenous Gβγ levels were already saturating with respect to AC regulation, and therefore the addition of Gβγ did not make any difference in the cAMP level.

5.2 *Gβγ-Regulation of AC-Activity in COS-1 Cells Expressing AC II and AC V*

Vogel's group could show the Gβγ effect on AC V *in vitro* in COS-1 membranes (Bayewitch *et al*., 1998). Therefore it was decided to investigate the regulation of Gβγ in COS-1 cells expressing AC V or AC II (positive control). Although immunoblot analysis cannot accurately quantitate the amount of endogenous Gβγ present in HEK 293 cells and COS-1 cells, the possibility that Gβγ is present in excess in HEK 293 cells comparatively to COS-1 cells had to be investigated.

5.2.1 *Detection of Gβ in COS-1 Cells*

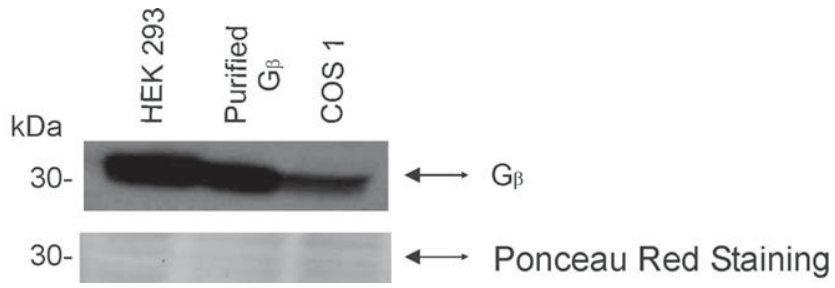


Figure 16: Detection of Gβ in HEK 293 and COS-1 cells by immunoblot analysis.

Membranes from HEK 293 cells (lane 1, wild type, 25µg), purified Gβ (lane 2, 5µg) and COS-1 cells (lane 3, wild type, 25µg) were lyzed. All lysates were separated by SDS-PAGE, blotted onto PVDF membrane and visualized by Gβ antibody and chemiluminescence as described in section 4. Equal loading was confirmed by staining the membranes with Ponceau Red (bottom panel).

Figure 16 depicits the presence of cellular Gβ in HEK 293 cells and COS-1 cells. The Gβ-signals were more intense in HEK 293 cells than in COS-1 cells, for the same amount of cellular protein. The band intensity was evaluated densitometrically using "Aida" software It clearly showed that 25µg of HEK 293 membrane cells had a 50% stronger band intensity compared to band intensity of 25µg of COS-1 membrane cells.

It was further decided to test the effect of Gβγ on AC V in a different cellular system.

$G\beta_1\gamma_2$ was transfected together with AC V in COS-1 cells. Bayewitch *et al*. have shown an inhibition of $G\beta_1\gamma_2$ on transiently expressing AC V in COS-1 membranes (Bayewitch *et al*., 1998).

5.2.2 Effect of Gβγ on COS-1 Cells Transiently Expressing AC II and AC V

COS-1 cells transiently expressing AC II or AC V were transfected together with $G\alpha_t$ or Gβγ. As mentioned above, $G\alpha_t$ was used to scavenge free endogenous Gβγ, thus eliminating the effect of Gβγ. In order to verify that Gβγ plays a role, AC II COS-1 were used as a positive control with coexpression of Gβγ, as it is known that Gβγ has a stimulatory effect on AC II in the presence of activated $G\alpha_s$.

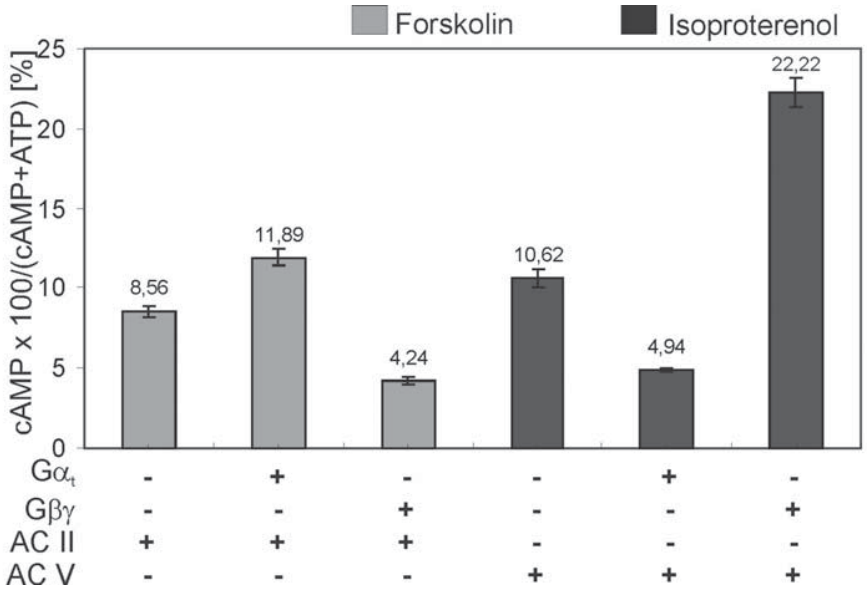


Figure 17: Effect of Gβγ and $G\alpha_t$ on COS-1 cells transiently expressing AC II and AC V

COS-1 cells transiently expressing AC V or AC II were transfected together with $G\alpha_t$ or Gβγ. 48 h after transfection, cotransfected AC II HEK 293 cells and AC V HEK 293 cells were labeled with [^{3}H]-adenine for 60 minutes. AC II HEK 293 cells were stimulated with isoproterenol (1µM; 10min) and AC V HEK 293 cells were stimulated with forskolin (10µl; 10min) cAMP formation was measured as described in section 4. Indicated are mean values ± S.D. of three independent experiments.

COS-1 cells were transiently transfected with AC II (AC II COS-1 cells) together with $G\alpha_t$ or Gβγ. Similarly, COS-1 cells were also transiently expressed with AC V (AC V COS-1 cells) together with $G\alpha_t$ or Gβγ. The AC II COS-1 cells were stimulated only with isoproterenol, in order to see the synergistic stimulation (used as a positive control). Conversely, the AC V COS-1 cells were stimulated only with forskolin (Figure 17).

Comparing the AC activity of AC V when stimulated with forskolin to the control, there was a 29% increase in the presence of $G\alpha_t$ and a 50% decrease in the presence of $G\beta\gamma$. Comparing the AC activity of AC II when stimulated with isoproterenol, there was a 54% decrease in AC activity in the presence of $G\alpha_t$ and a 53% increase in AC activity in the presence of $G\beta\gamma$. Here it can be clearly seen that $G\beta\gamma$ has an inhibitory effect on AC V when stimulated with forskolin. This was further confirmed also by the effect of $G\alpha_t$ on AC V whose effect was stimulatory and indirectly an inhibition of endogenous $G\beta\gamma$ on AC V when stimulated with forskolin. AC II COS-1 cells behaved similar to AC II HEK 293 cells (Figure 12 and Figure 14). In the presence of $G\beta\gamma$ and upon stimulation with isoproterenol, the AC II activity was stimulatory. In the presence of $G\alpha_t$, the cAMP level decreased to approximately 50%. This was due to the capturing of endogenous $G\beta\gamma$, which was then no longer available for stimulation of AC II. The results of the effect of $G\beta\gamma$ on AC II COS-1 cells were similar to the results of the effect of $G\beta\gamma$ on AC II HEK 293 cells. It can be seen that there was 50% inhibition by $G\beta\gamma$ on forskolin-stimulated AC V COS-1 cells; whereas, $G\alpha_t$ showed an increase of about 30% in the level of cAMP (Figure 17). This effect of $G\beta\gamma$ on AC V COS-1 cells was not seen on AC V HEK 293 cells. Thus, it can be suspected that it was not possible to see the inhibitory effect of $G\beta\gamma$ on AC V *in vivo* in HEK 293 cells due to an excess amount of $G\beta$ in HEK 293 cells. Consequently, the experiments were carried out in stably expressing AC II in COS-1 cells and stably expressing AC V in COS-1 cells.

5.2.3 Generation of COS-1 Cells Stably Expressing AC II and AC V

In order to investigate the regulation of AC by $G\beta\gamma$, COS-1 cells stably expressing AC II (Feinstein *et al*., 1991) or AC V (Feinstein *et al*., 1991) were established. Monoclonal cell lines were obtained by selection with 0.8mg/ml G-418 and were maintained using 0.4mg/ml G-418. The expression of AC II and AC V in COS-1 cells was compared to wild type COS-1 cells by Western blot analysis as well as by AC activity assays. Subsequent to isolation of AC transfectants, a series of preliminary experiments was carried out to characterize the enzyme activity in different clonal cell lines.

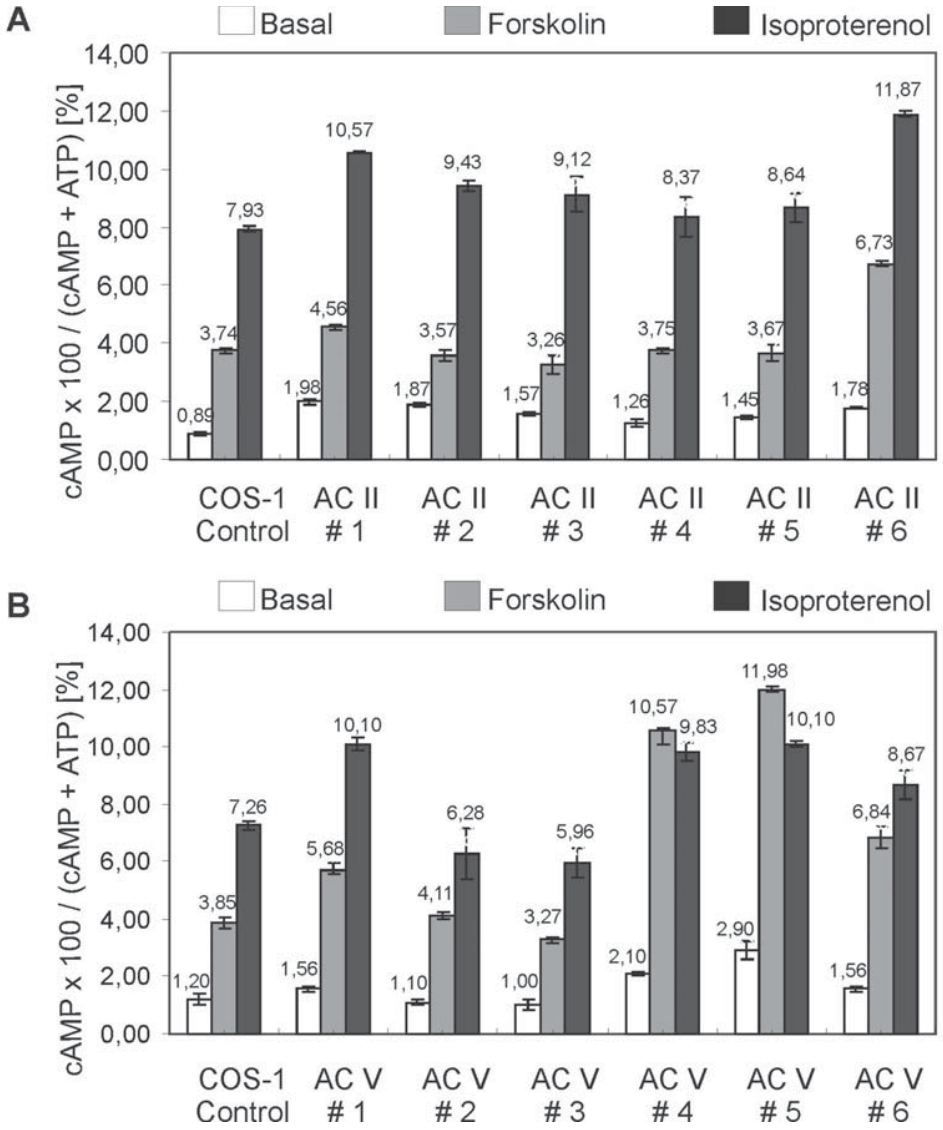


Figure 18: AC activity in COS-1 cells stably expressing AC II (A) and AC V (B)

A) COS-1 cells were transfected with AC II and selected with G-418 (0.8mg/ml). B) COS-1 cells were transfected with AC V and selected with G418 (0.8mg/ml). AC II COS-1 cells and AC V COS-1 cells were maintained with 0.4mg/ml G418 after seeding a cell per well. To analyze AC activity, cells were labeled with [$^{[3H]}$]-adenine for 60 min and stimulated with forskolin (10µl; 10min) and isoproterenol (1µl; 10min). cAMP formation was measured as described in section 4. Indicated are mean values ± S.D. of three independent experiments.

As shown in Figure 18, the forskolin-stimulated and isoproterenol-stimulated AC activity of COS-1 cells stably expressing AC II or AC V was compared to the control vector-transfected COS-1 cells. For AC II COS-1 cells (specifically AC II #6), the basal, forskolin- and isoproterenol-stimulated AC activity was two fold, two fold and one and half fold respectively higher than the control COS-1 cells. Similarly, for AC V COS-1 cells (specifically AC V #5), the basal, forskolin- or isoproterenol-stimulated AC activity

was two and half fold, three fold and one and half fold higher than in control COS-1 cells. This increase in AC activity clearly indicated that AC II and AC V have been stably expressed in COS-1 cells. Figure 19 shows the Western blot analysis of clonal cell lines with higher AC activity specifically AC II #1, AC II #6, AC V #4, AC V #5 expressed stably in COS-1 cells from Figure 18.

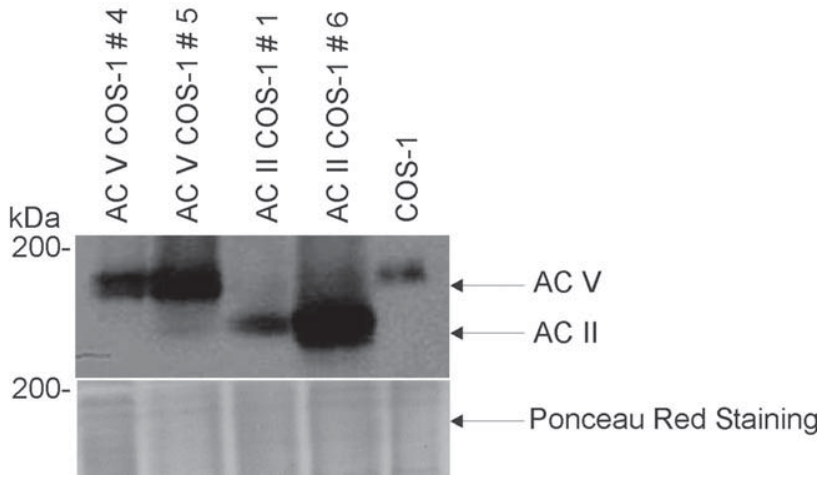


Figure 19: Detection of AC II and AC V in COS-1 cells by immunoblot assays.

Membranes from COS-1 (Lane 5, wild type, 25µg), AC II COS-1 cells (AC II #1 lane 3 AC II #6 lane 4, 25µg each) and AC V COS-1 cells (AC V #4 lane 1 AC V #5 lane 2, 25µg each) were lyzed. All lysates were separated by SDS-PAGE, blotted onto PVDF membrane and visualized by antibody BBC-2-AP and chemiluminescence as described in section 4. Vertical numbers indicate molecular mass (kDa) of iodinated standard proteins (upper panel). Equal loading was confirmed by staining the membranes with Ponceau Red (bottom panel).

COS-1 cell lines expressing AC II or AC V were analyzed for the level of expression of AC II or AC V in these cells. In Figure 19, the strong signals show the establishment of COS-1 cells stably expressing AC II or AC V, thus confirming the presence of AC II and AC V being expressed in COS-1 cells.

The results from Figure 18 and 20 confirmed the expression of AC II and AC V in COS-1 cells. For further studies AC II # 6 and AC V #5 were used for the detailed experimental analysis.

5.2.4 Effect of Gβγ on COS-1 Cells Stably Expressing AC II and AC V

In order to see the effect of Gβγ on AC II or AC V, COS-1 cells, AC II COS-1 cells and AC V COS-1 cells were transfected with Gβγ. Each type of cells was further stimulated with forskolin or isoproterenol. The AC activity of the forskolin- and isoproterenol-stimulated cells was compared to the control. Figure 20 presents the AC activity for each

of the three cell types under the basal condition (control) (in the presence and absence of Gβγ) and under the two experimental conditions i.e: forskolin or isoproterenol.

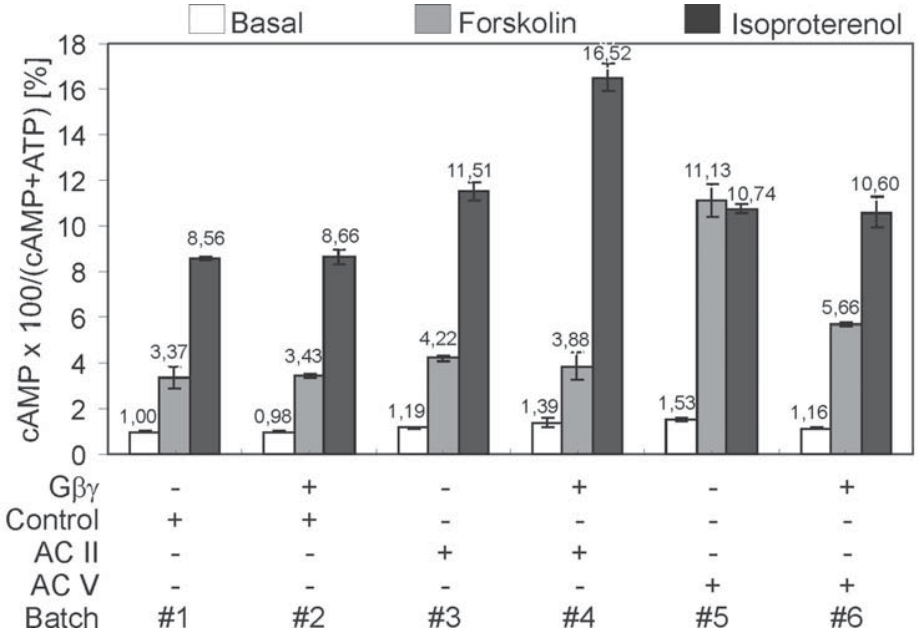


Figure 20: Effect of Gβγ in COS-1 cells stably expressing AC II and AC V.

COS-1 cells stably expressing AC II or AC V were transfected together with Gβγ. 48 h after transfection, cotransfected AC II COS-1 cells and AC V COS-1 cells were labeled with [^{3}H]-adenine for 60 minutes. These cells were stimulated with forskolin (10µl; 10min) and isoproterenol (1µM; 10min). cAMP formation was measured as described in section 4. Indicated are mean values ± S.D. of three independent experiments.

Comparing Batch 1 and Batch 2, there was no change in AC activity for wild type cells with overexpression of Gβγ (Figure 20). Observing AC activity in AC II COS-1 cells (Batch 3 and Batch 4), there is a 15% or 31% increase for the basal or isoproterenol-stimulated activity respectively. As for the forskolin-stimulated AC II, there was a decrease in AC activity by 8%. One could say there was no change in basal AC activity as well as forskolin-stimulated AC activity. What is definitely clear here was, similar to AC II HEK 293 cells; Gβγ had a stimulatory effect on AC II COS-1 cells. The effect of Gβγ on AC II is only seen when stimulated with isoproterenol, due to the release of stimulated $G\alpha_s$.

Comparing Batch 5 and Batch 6, the AC activity in AC V COS-1 cells showed a decrease of 31% or 50% for basal or forskolin-stimulated activity in the presence of Gβγ, respectively. There was no change in AC activity in AC V COS-1 cells when stimulated with isoproterenol. These results corresponded with the results seen in Figure 17.

An interesting observation was that this inhibitory effect of Gβγ on AC V in COS-1 cells

was seen also on the basal activity. Moreover, there was no change in cAMP level upon stimulation with isoproterenol. It is known that forskolin stimulates AC V directly, whereas isoproterenol stimulates AC V *via* $G\alpha_s$. The inhibitory effect of $G\beta\gamma$ on AC V was seen only in the presence of forskolin and not when stimulated with isoproterenol. The contrary was seen with AC II COS-1 cells. It was found that $G\beta\gamma$ has a stimulatory effect on AC II only when stimulated with isoproterenol but no stimulatory effect was seen when stimulated with forskolin or in its basal activity, for both HEK and COS-1 cells.

To summarize the effect of $G\beta\gamma$ on AC V HEK cells or AC V COS-1 cells, $G\beta\gamma$ had no stimulatory or inhibitory effect on AC V when stimulated with isoproterenol. But when stimulated with forskolin or left in its basal state, there was a strong inhibitory effect of $G\beta\gamma$ on AC V in case of COS-1 cells and stimulatory effect in the presence of $G\alpha_t$ (indirect inhibition) on AC V in the case of HEK 293 cells. It is interesting to note the differences in the effect of $G\beta\gamma$ on AC II and AC V. AC II can be stimulated by $G\beta\gamma$ only in the presence of $G\alpha_s$; whereas $G\beta\gamma$ has no effect on AC V in the presence of isoproterenol. The reverse is seen when stimulated with forskolin. $G\beta\gamma$ has no effect on AC II in the presence of forskolin; whereas AC V can be stimulated by $G\beta\gamma$ in the presence of forskolin. This indicates that $G\alpha_s$ could play a role in the inhibitory effect of $G\beta\gamma$ on AC V.

5.3 $G\alpha_s$-Regulation of $G\beta\gamma$-Inhibited AC V Activity

5.3.1 Establishment of a System to Down-Regulate $G\alpha_s$ in COS-1 Cells

Two experimental approaches were pursued in order to determine whether or not $G\alpha_s$ plays a role for the inhibitory effects of $G\beta\gamma$ on COS-1 cells expressing AC V: 1) the amount of $G\alpha_s$ in a cell was down-regulated (including any active $G\alpha_s$) or 2) free endogenous $G\alpha_s$ was captured.

5.3.1.1 Down-Regulation of $G\alpha_s$ in COS-1 Cells with Anti-$G\alpha_s$-Oligonucleotides

Two ways of down-regulating $G\alpha_s$ in COS-1 cells were used: the antisense technique and cholera toxin. Employing the antisense technique, the conditions for target-gene knock-down needed to be empirically determined, because they depend on the cell type and the protein whose expression is to be blocked. A fluoroisothiocyanate (FITC)-coupled oligonucleotide for anti-$G\alpha_s$ was used to find the optimum uptake for down regulation.

Due to FITC oligonucleotides' fluorescent properties, it is possible to observe whether the specific oligonucleotide in this case anti-$G\alpha_s$ has been taken up by the cell. The parameters considered are described in section 4. As shown in Figure 21 time for incubation was varied, in order to estimate the optimum time required for maximum uptake of the FITC-oligonucleotides into COS-1 cells.

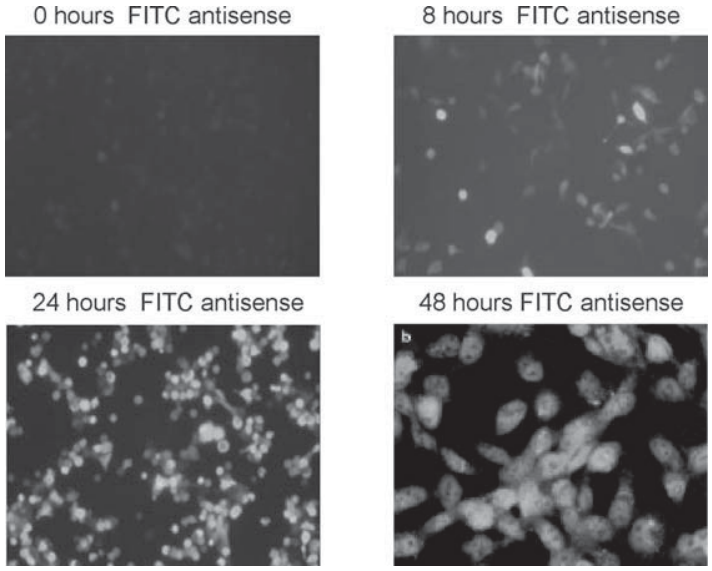


Figure 21: Time-dependent intracellular uptake of oligonucleotides in COS-1 cells.

COS-1 cells were grown in 24-well plates at a density of 4 x 10^5 per well. The cells were incubated with 2µM FITC at various time intervals as described in section 4. Fluorescence uptake was examined by conventional fluorescence microscopy at different time points. The upper panel shows fluorescence images obtained after incubation for 0 and 8 hours (from left to right). The lower panel shows fluorescence images obtained after incubation for 24 and 48 hours (from left to right).

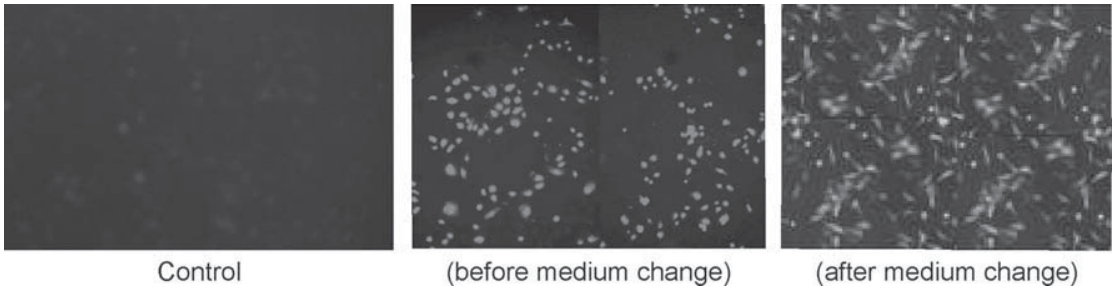


Figure 22: Incubation of FITC oligonucleotides in COS-1 cells for 24 h before/after change of medium

Cells were seeded in three wells at a density of 4 x 10^5 per well. Transfection of COS-1 cells was carried out in all wells. The total transfection process took 48 hours. The image to the most left is the control where no FITC oligonucleotides were added to transfected COS-1 cells. The middle image shows COS-1 cells after addition of 2µM FITC to transfected COS-1 cells for the first 24 hours of transfection. The most right image shows COS-1 cells after addition of 2µM FITC to transfected COS-1 cells for the second 24 hours of transfection.

The maximum uptake was measured by intensity of fluorescence. It was clearly visible

that the maximum uptake of FITC oligonucleotides by COS-1 cells was after 24. After 48 hours, the intensity of fluorescence had decreased.

In the above experiment, the optimum time for the addition of FITC-oligonucleotides to the cells was determined by the intensity of the fluorescence of the FITC-oligonucleotides. FITC-oligonucleotides were added to the transfected cells in the first 24 hours or in the second 24 hours of transfection. As shown in Figure 22, the intensity of the fluorescence emitted by COS-1 cells was stronger when FITC oligonucleotides were added for the second 24 hours of transfection. Hence it was decided to add anti-$G\alpha_s$ to the transfected cells after the medium was changed.

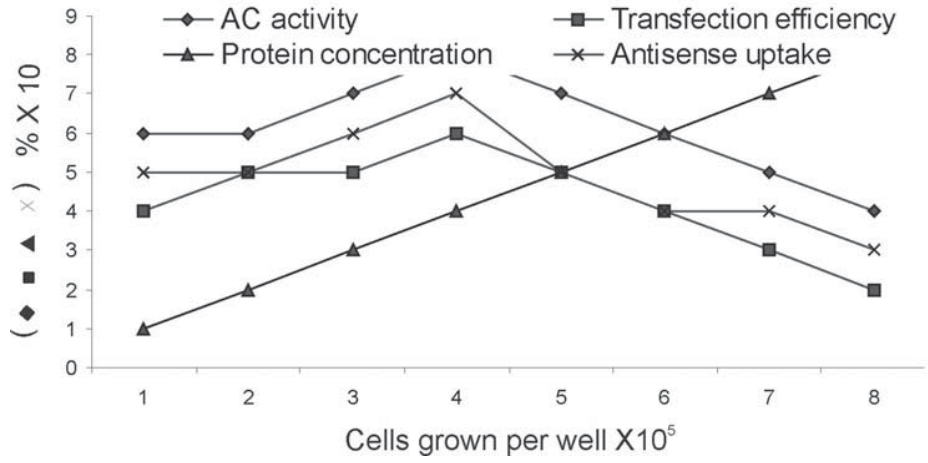


Figure 23: Relationship between seeding density of COS-1 cells per well and AC activity, immunoblot analysis, transfection efficiency and antisense uptake

COS-1 cells were seeded per well in two 24-well plates. All cells were transfected with AC V. 24 hours later 2μM anti-$G\alpha_s$ was added for 24 hours to 32 wells of the 48 wells and 2μM FITC to 16 of the 48 wells. These 16 wells were test for AC activity *versus* increase in cells per well and is represented by ♦. The AC activity is measure in % Conversion of ATP to cAMP. AC activity indicated the AC V activity of transfected AC V *versus* increase in cells per well and is represented in the diagram by ■. Transfection efficiency is measured by number of blue colored cells to the total of number of cells grown per well in % X 10. The second Batch of 16 wells was tested for transfected AC V as well as down regulation of $G\alpha_s$ by Western blot analysis and is represented by ▲. Protein content is measured in μg protein per well X10. The last Batch of 16 wells were tested for antisense uptake and is represented by ×. Antisense uptake is measured by number of cells emitting fluorescence over the total no of cells grown per well in % X10.

After finding the optimum conditions for the uptake of antisense as well as transfection, these conditions were applied to observe the down regulation of $G\alpha_s$ *via* adenylyl cyclase assays as well as Western blot analysis. As shown in Figure 23, cells were seeded in increasing density per well and tested for its AC activity as well as immunoblots assays under different conditions.

Observing the AC activity of AC V transfected in COS-1 cells *versus* the increase in

COS-1 cells per well, it was seen that the AC activity increased as cells increased proportionally with the cellnumber and reached a maximum at 4 x 10^5 per well. The AC activity decreased when cells were grown at 5 x 10^5 per well. It is important to note that cells seeded at 5 x 10^5 per well were hyperconfluent and grew in double layers. Furthermore, floating dead cells floating were observed, which indirectly affected AC activity. Observing the transfection efficiency which was tested by measuring AC activity of AC V transfected in COS-1 cells, an additional X-Gal staining was carried out. The ratio of transfected (blue) cells to total cells also showed that the optimum number of cells grown per well was 4 x 10^5. Similarly, antisense uptake efficiency was also measured by the ratio of fluorescence emitted by the cells to the total cell number per well. The maximum flourescence emission was seen when cells were grown at 4 x 10^5 cells per well. By Western blot analysis, we observed that the abundance of endogenous $G\alpha_s$ increased proportionally with cell number. As for the transfected AC V in COS-1 cells, the intensity of AC V signal was independant of the number of cells grown per well. The intensity of the signal in Western blot analysis was not quantitative. Therefore it could not be concluded if $G\alpha_s$ was down regulated or not since increase in cells per well showed a stronger signal. Hence, it was decided to grown cells at 4 x 10^5 per well, and to investigate the regulation of $G\beta\gamma$ on AC V after down regulating $G\alpha_s$ for 24 hours by AC activity assays.

The fluorescent oligonucleotides were replaced with the required anti-$G\alpha_s$ oligonucleotides and its control. This was done in order to down-regulate the $G\alpha_s$ in AC V COS-1 cells co-transfected with $G\beta\gamma$ or $G\alpha_t$. The cells were tested by stimulating them only with forskolin. The AC activity was tested in order to see the effect of $G\beta\gamma$ on AC V in the absence of $G\alpha_s$.

As shown in Figure 24, these wild type COS-1 cells were used as control. Comparing Batch 1 to Batch 2, 3 and 4, it was clear that AC V has been successfully transfected in COS-1 cells because a ten fold increased AC activity in Batch 2 and five fold increased AC activity in Batch 3 and 4 was detected. $G\alpha_t$ and $G\beta\gamma$ were coexpressed in Batch 2, 3 and 4. Coexpression of $G\beta\gamma$ triggered 50% inhibition of AC V in Batch 2 and approx 30% inhibition of AC V in Batch 3 and 4 (observing white bar *versus* black bar with respect to its specific Batch). Similarly, coexpression of $G\alpha_t$ triggered the stimulation of AC V by 27% for Batch 2 and 30% stimulation of AC activity for Batch 3 and 4 (grey bar *versus* white bar).

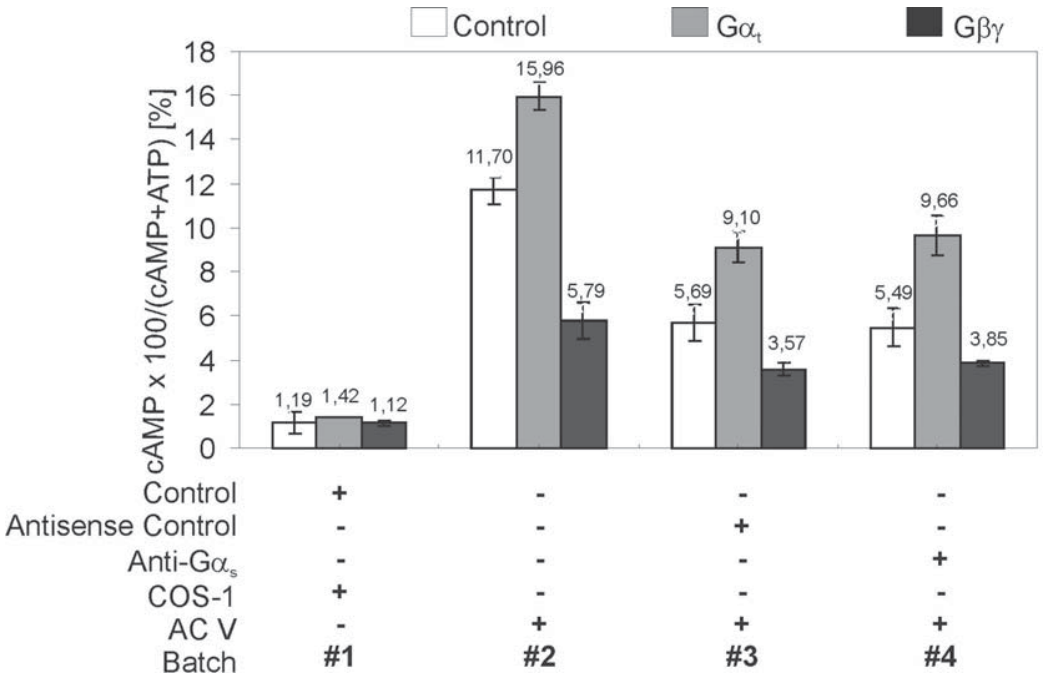


Figure 24: Regulation of Gβγ on AC V COS-1 cells coexpressing $G\alpha_t$ or Gβγ in the presence of antisense control and anti-$G\alpha_s$ oligonucleotides.

COS-1 and AC V COS-1 cells were coexpressed with Gβγ or $G\alpha_t$. In this case the white bars represent COS-1 and AC V COS-1 cells transfected with mock, the grey bars represent COS-1/AC V COS-1 cells transfected with $G\alpha_t$ and the black bars represent COS-1/AC V COS-1 cells transfected with Gβγ. The three bars represent a Batch under the four conditions: COS-1 cells (Batch 1), AC V COS-1 cells (Batch 2), antisense control (Batch 3) and anti-$G\alpha_s$ (Batch 4). 24 hours after transfection 2µM antisense control (Batch 3) and 2µM anti-$G\alpha_s$ oligonucleotides (Batch 4) were added. 48 h after transfection, all cells were labeled with [^{3}H]-adenine for 60 minutes. These cells were stimulated with forskolin (10µl; 10min). cAMP formation was measured as described in section 4. Indicated are mean values ± S.D. of three independent experiments.

Comparing Batch 3 and 4 to Batch 2, it was also clear that the AC activity decreased by 50% (comparing the white bars). This could be due to the incubation period of antisense in COS-1 cells. Comparing Batch 4 to Batch 3, a change in AC activity was expected to be seen, if $G\alpha_s$ was down regulated. Since there was no difference observed, it indicated that anti-$G\alpha_s$ did not down regulate $G\alpha_s$. Thus it could not be concluded whether $G\alpha_s$ plays a role or not, therefore it first had to be determined whether $G\alpha_s$ was down-regulated. To verify the down-regulation of $G\alpha_s$, the following experiment was performed.

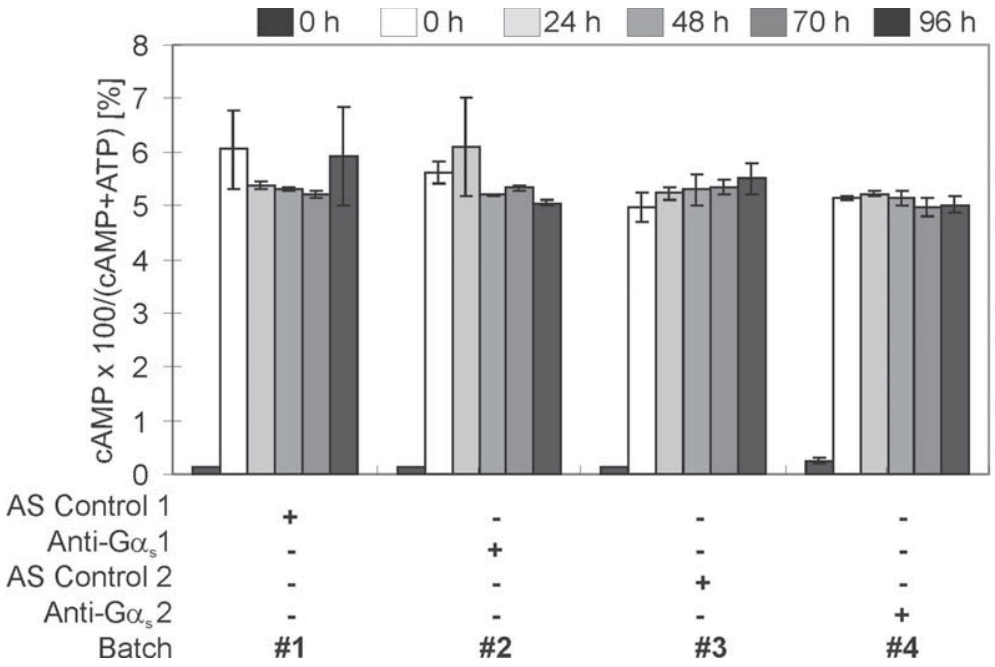


Figure 25: Effect of anti-$G\alpha_s$ oligonucleotide in AC V COS-1 cells over a period of time.

Antisense-$G\alpha_s$ 1 and antisense-$G\alpha_s$ 2 were the two different types of anti-$G\alpha_s$ oligonucleotides used. Each of this antisense-$G\alpha_s$ has its respective antisense control. AC V COS-1 cells were incubated with the respective antisense and its control over a period of four days. On the day of test, all cells were labeled with [^{3}H]-adenine for 60 minutes. These cells were stimulated with isoproterenol (1µl; 10min) except for the first black bar which represents AC V COS-1 cells without stimulation of isoproterenol. cAMP formation was measured as described in section 4. Indicated are mean values ± S.D. of three independent experiments.

Isoproterenol stimulates adenylyl cyclase *via* $G\alpha_s$, therefore it was decided to stimulate AC V COS-1 cells with isoproterenol after pretreatment with anti-$G\alpha_s$. As shown in Figure 25, the stimulation with isoproterenol and preincubation with antisense triggered a 35% decrease in AC activity as compared to cells not treated with antisense (Figure 20, Batch 5 observing only isoproterenol stimulation). Comparing the first black bar (Figure 25) at 0 hour to the white bar at 0 hour clearly shows the activation of AC V *via* $G\alpha_s$. Comparing Batch 1 or 3 to Batch 2 or 4 respectively, there was no decrease in AC activity seen over the period of time. We expected to observe in that Batch 2 and 4 show a gradual decrease in AC activity. Irrespective of the amount of time the cells were pretreated with anti-$G\alpha_s$, the AC activity remained constant. Since this decrease in cAMP level was not observed, it may be concluded that there was no down-regulation of $G\alpha_s$ in these cells. A Western blot analysis was carried out to investigate the efficiency of $G\alpha_s$ down regulation.

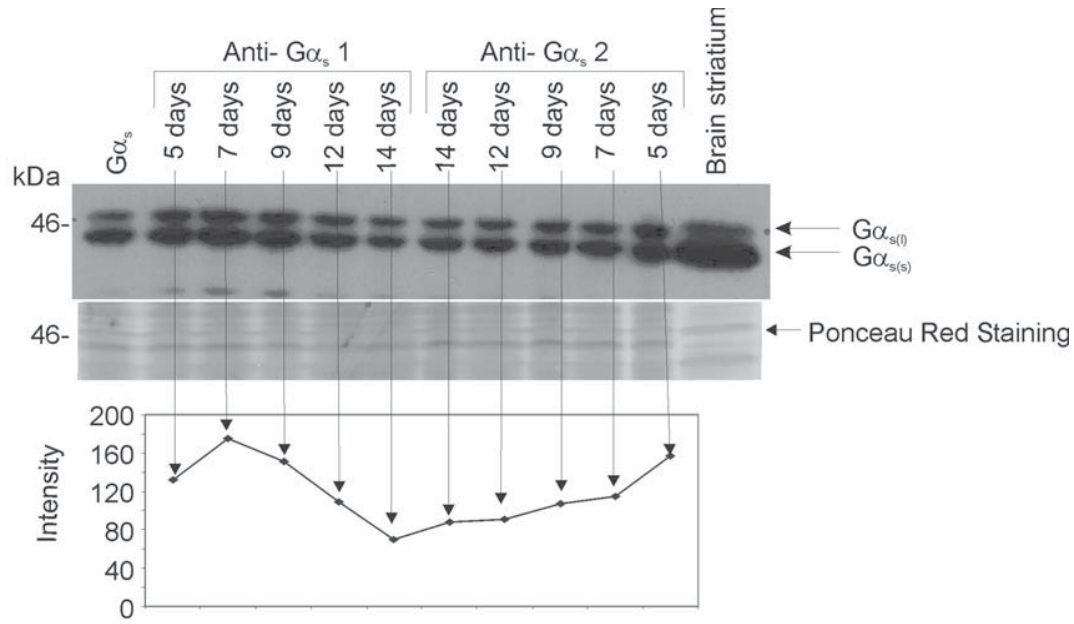


Figure 26: Effect of AS Gα$_{s1}$ and AS Gα$_{s2}$ on COS-1 cells over a period of 14 days

AC V COS-1 cells were incubated at the time points 14, 12, 9, 7, 5 days with two different anti-Gα$_s$ oligonucleotides. Gα$_s$ endogenously expressed in COS-1 cells were used as control. On the 14th day, COS-1 cells (wild type, 25µg) as well as AC V COS-1 cells from each time point (25µg per lane) were lyzed. All lysates were separated by SDS-PAGE, blotted onto a PVDF membrane and Gα$_s$ was visualized with a Gα$_s$-specific antibody followed by anti-rabbit secondary antibodies as described in section 4. The doublet signal represents the long and short form of Gα$_s$, respectively. Vertical numbers indicate molecular mass (kDa) of iodinated standard proteins (upper panel). Equal loading was confirmed by staining the membranes with Ponceau Red (middle panel). Band intensities on the blot were analyzed by densitometric analysis using Aida software (lower panel).

As shown in Figure 26, immunoblot analysis was carried out on AC V COS-1 cells pretreated with anti-Gα$_s$. The cells were pretreated for a period of 0 hours to 14 days. Here the intensity of the band representing Gα$_s$ was expected to decrease over a period of time. As shown in Figure 26, Gα$_s$ decreased over a period of 14 days but not completely. It is necessary to down regulate Gα$_s$ 100%, in order to investigate the role Gα$_s$ plays in the Gβγ regulation on AC V. The band intensities were evaluated densitometrically using "Aida" software. In Figure 26 it clearly showed a decrease in band intensity by 30% with anti-Gα$_s$1 over a period of 14 days and a decrease of 12% in band intensity with anti-Gα$_s$2 over a period of 14 days. As this knockdown efficiency is insufficient for our experimental purposes, the following experiments with cholera toxin were carried out.

5.3.1.2 Down-Regulation of $G\alpha_s$ in COS-1 Cells with Cholera Toxin

Cholera toxin catalyses the ADP-ribosylation of the α chain of $G\alpha_s$, thereby stabilizing the protein in its active form. The expression of this active form of $G\alpha_s$ results in increased adenylyl cyclase activity. According to Chang *et al*, it was seen that pretreatment with cholera toxin over a period of 48 hours leads to degradation of $G\alpha_s$ (Chang *et al*., 1989). So it was decided to treat COS-1 cells with cholera toxin for a period of 48 hours. A Western blot analysis was done to analyze the decrease in $G\alpha_s$ signal over a period of 48 hours.

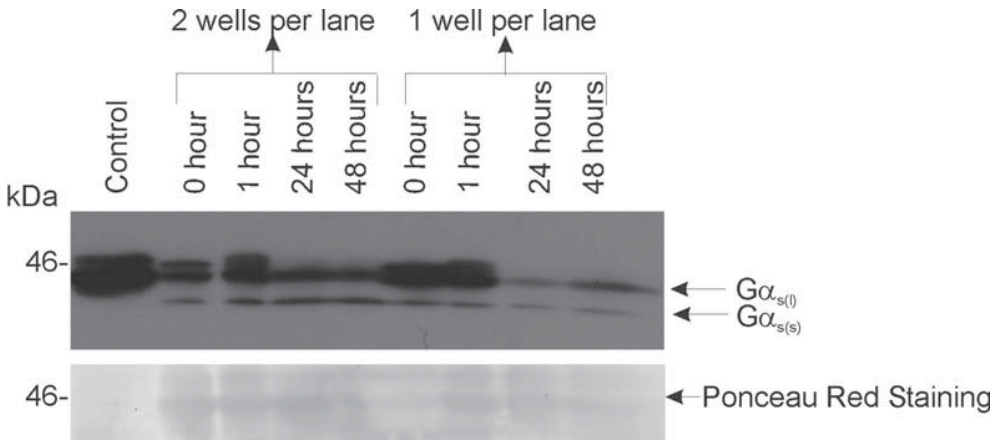


Figure 27: Down regulation of $G\alpha_s$ in COS-1 cells over a period of 48 hours with cholera toxin

COS-1 cells were incubated at the time points 48, 24, 1 and 0 hours with cholera toxin. After 48 hours, all these cells from each time point (25µg per lane) were lyzed. All lysates were separated by SDS-PAGE, blotted onto a PVDF membrane and $G\alpha_s$ was visualized with a $G\alpha_s$-specific antibody followed by anti-rabbit secondary antibodies as described in section 4. The two signals over each other in each case represent the long and short form of $G\alpha_s$. Vertical numbers indicate molecular mass (kDa) of iodinated standard proteins (upper panel). The loading was confirmed by staining the membranes with Ponceau Red (bottom panel).

As shown in Figure 27 COS-1 cells were pretreated with Cholera toxin over a period of 48 hours. It can be clearly seen that $G\alpha_s$ was down regulated by cholera toxin after 24 hours and was present after 48 hours, but to a much lower extent than pretreatment with anti-$G\alpha_s$, seen in Figure 26. Even though $G\alpha_s$ was present to a smaller amount, it was decided to carry out an AC activity assay to analyze the effect of $G\alpha_s$ on AC activity over the period of time. Hence the following experiment was run. Therefore, in order to evaluate the putative decrease in AC activity, the cells were stimulated with isoproterenol. If $G\alpha_s$ was down-regulated, there would be a decrease in cAMP level over this period of time.

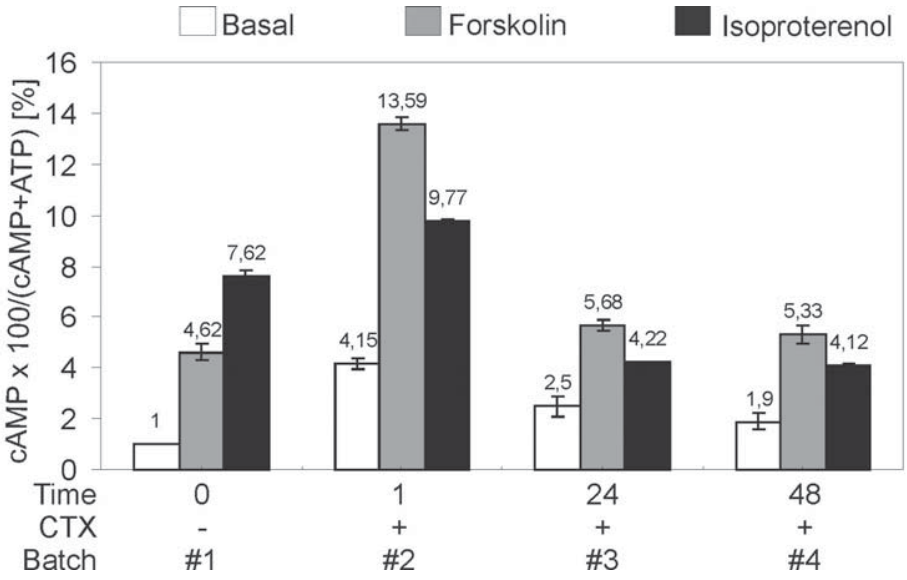


Figure 28: Effect of CTX-mediated down-regualtion of $G\alpha_s$ on AC activity

COS-1 wild-type cells were grown in 2 x 24-well plates and pretreated with cholera toxin (1µg/ml for 0 h, 1 h, 24 h, or 48 h). On the day of the experiment, cells were labeled with [^{3}H]-adenine for 60 minutes. These cells were stimulated with forskolin (10µM; 10min) and isoproterenol (1µl; 10min). cAMP formation was measured as described in section 4. Indicated are mean values ± S.D. of three independent experiments.

In Figure 28, comparing Batch 1 to Batch 2, the increase in AC activity was four fold, three fold and one and one third for the basal, forskolin- and isoproterenol-stimulated COS-1 cells respectively after an hour of treatment with cholera toxin. It is interesting to note that after 24 hours of pretreatment with cholera toxin, there was still a strong increase of two and half fold and 30% for the basal or forskolin-stimulated AC activity, respectively, and a decrease of 20% in isoproterenol-stimulated AC activity (observing Batch 3 *versus* Batch 1). Whereas after 48 hours, there was a 90% or 14% increase in basal or forskolin-stimulated AC activity, respectively, (Batch 4 to Batch 1) and a 16% decrease in isoproterenol-stimulated AC activity (Batch 4 to Batch 1). Observing the overall AC activity for isoproterenol-stimulated COS-1 cells, it was clearly seen that 46% of the $G\alpha_s$ mediated activity had been down regulated. But for the basal and forskolin-stimulated AC activity, the endogenous $G\alpha_s$ present in COS-1 cells was activated and remained in its activated state even 48 hours after pretreatment with cholera toxin. For a complete degradation of $G\alpha_s$ after pretreatment with cholera toxin and stimulation of isoproterenol, the AC activity (black bar, Batch 4) should have decreased to or been equal to the AC activity seen for the basal activity of COS-1 cells at 0 hours (white bar, Batch1). Therefore it was concluded not to use this process for further experiments since $G\alpha_s$ -although partly down-regulated- would always be in its activated state. As also confirmed

by experiments shown in Figure 27, $G\alpha_s$ did decrease over this period of time but was still present in small quantities. The small quantity of $G\alpha_s$ present was the CTX-activated $G\alpha_s$ that was responsible for the high AC activity seen in Figure 27.

5.4 Adenylyl Cyclase Activity in S49 and S49 cyc$^-$ cells

Since $G\alpha_s$ could not be down-regulated completely, the cell line was changed. S49 and S49cyc$^-$ human lymphoma cells were used. The S49cyc$^-$ cells are devoid of $G\alpha_s$ (Akiyama *et al*., 1983) but express AC V and AC VI isoforms (Premont *et al*., 1992).

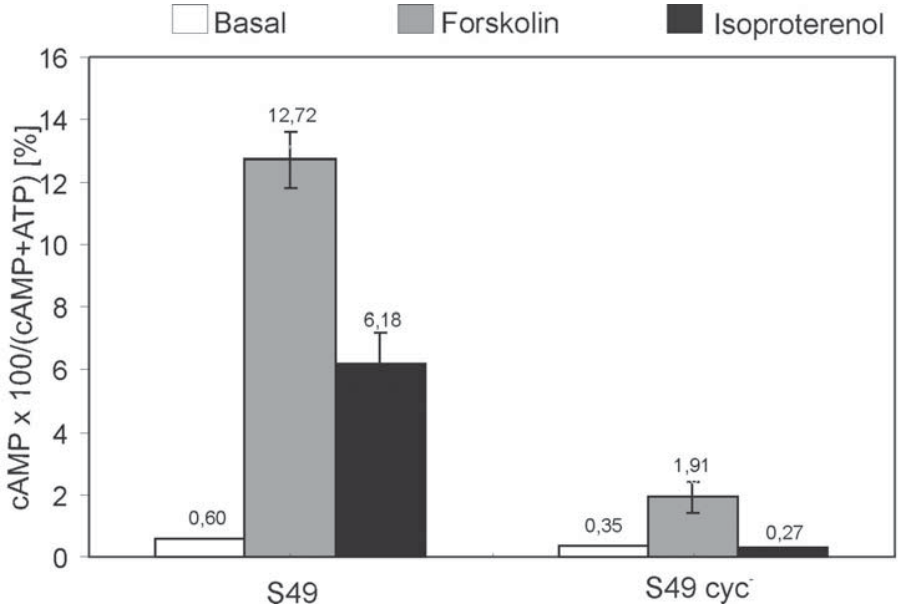


Figure 29: AC activity in forskolin- and isoproterenol-stimulated S49 and S49cyc$^-$ cells

S49 cells and S49cyc$^-$ cells were labeled with [^{3}H]-adenine for 60 min and stimulated with forskolin (10µl; 10min) and isoproterenol (1µM; 10 mins). cAMP formation was measured as described in section 4. Indicated are mean values ± S.D. of three independent experiments.

AC V and AC VI in S49 cells and S49cyc$^-$ cells were tested for their AC activity, and the results are shown in Figure 29. The AC activity in S49 cells showed a 0.6%, 12.7% and 6.0% increase in basal, forskolin and isoproterenol- stimulated activity, respectively. The AC activity in S49cyc$^-$ cells showed a 0.3%, 1.9%, 0.3% increase in the basal, forskolin- and isoproterenol-stimulated activity respectively. It can be clearly seen that $G\alpha_s$ was absent in S49cyc$^-$ cells, as there was no detectable stimulation when cells were activated with isoproterenol. On the other hand, forskolin stimulated adenylyl cyclase in this experimental setup.

AC V and AC VI are the isoforms present in S49 cells according to previous PCR studies

(Premont *et al*., 1992; Tang *et al*., 1992). The ratio of AC V to AC VI present in these cells could not be quantified by immunoblot analysis, due to the close resemblance of AC V to AC VI and the lack of isoforms-specific antibodies. As shown in Figure 30, Western blot analysis demonstrated the presence of AC V/AC VI in S49 and S49cyc$^-$ cells, and confirmed that S49cyc$^-$ are devoid of $G\alpha_s$, whereas $G\alpha_s$ was readily detecable in S49 wild type cells.

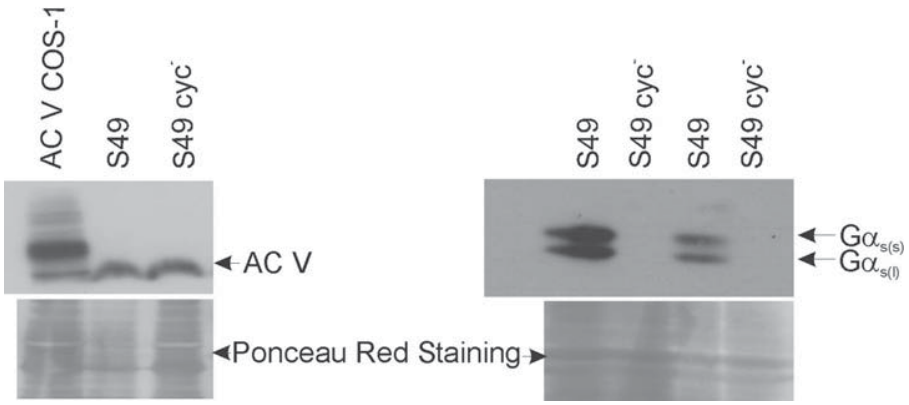


Figure 30: Detection of AC V/AC VI in S49 and S49cyc$^-$ cells and presence and absence of $G\alpha_s$ in S49 and S49cyc$^-$ cells respectively.

S49 cells and S49cyc$^-$ cells were (25µg per lane) were lyzed. All lysates were separated by SDS-PAGE, blotted onto PVDF membrane. AC V/AC VI were visualized by antibody BBC-2-AP (left panel) and $G\alpha_s$ was visualized by antibody $G\alpha_s$ followed by antirabbit (right panel) and chemiluminescence as described in section 4. The two signals over each other in right panel represent the long and short form of $G\alpha_s$. Vertical numbers indicate molecular mass (kDa) of iodinated standard proteins (upper panel). Equal loading was confirmed by staining the membranes with Ponceau S Red (bottom panel).

In order to express $G\beta\gamma$ or $G\alpha_s$ in these cells, different transfection methods and electroporation methods had to be employed. Neither of these processes proved successful for expressing $G\beta\gamma$ or $G\alpha_s$. Therefore, *in vitro* adenylyl cyclase assays were performed.

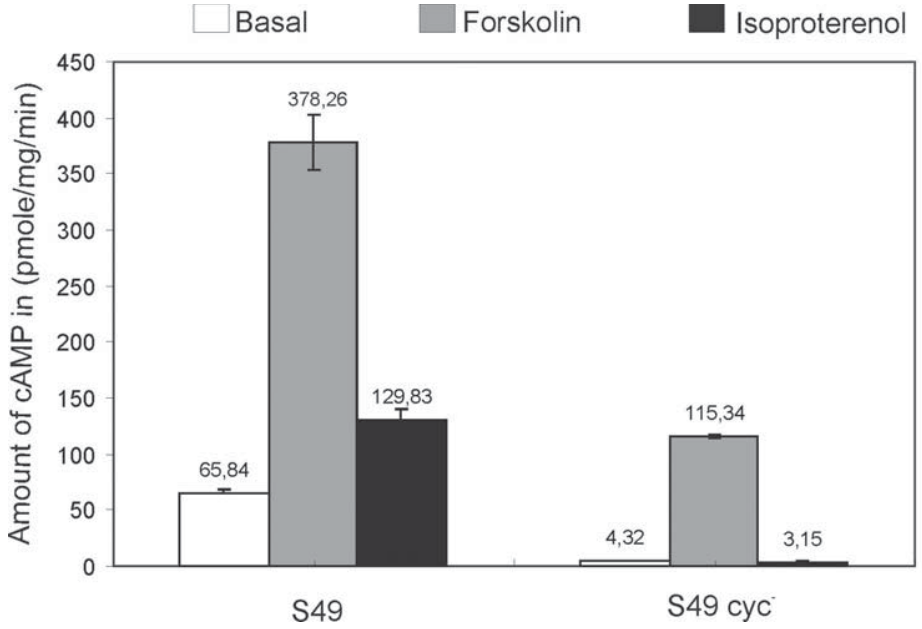


Figure 31: Adenylyl cyclase activity in S49 and S49cyc$^-$ cells in vitro

Membranes of S49 cells and S49cyc$^-$ cells were prepared as described in section 4. These membranes were labeled with [^{32}P]-ATP for 20 minutes and stimulated with forskolin (100µM; 10min) and isoproterenol (100µM; 10 mins). cAMP formation was measured as described in section 4. Indicated are mean values ± S.D. of three independent experiments.

The cell membranes of S49 cells and S49cyc$^-$ cells were stimulated with forskolin and isoproterenol. Here the AC activity was determined by measuring the amount of cAMP in pmole/mg/min. In Figure 31, S49 cells showed for basal, forskolin-stimulated, and isoproterenol-stimulated a ten, 288, and 63 pmol/mg/min AC activity respectively. The basal, forskolin-stimulated and isoproterenol-stimulated AC activity of S49cyc$^-$ membranes were two, 54, and two pmole/mg/min, respectively. Similar to the *in vivo* experiment (Figure 29), it was observed that S49cyc$^-$ membranes showed no AC activity compared to the S49 wild type cells when stimulated with isoproterenol.

The S49 membranes showed an AC activity that was six times higher than the S49cyc$^-$ membranes, when stimulated with forskolin. One can conclude from the *in vivo* and *in vitro* adenylyl cyclase assays that S49cyc$^-$ cells are devoid of $G\alpha_s$ (Figure 29 and 32). This was further verified by immunoblots which showed the presence of $G\alpha_s$ in S49 wild-type cells and the absence of $G\alpha_s$ in S49cyc$^-$ cells (Figure 30). Hence, the $G\beta\gamma$ regulation on AC V in S49 and S49cyc$^-$ membranes could be investigated.

5.4.1 Effect of Gβγ on Endogenous AC V/VI in S49 and S49cyc$^-$ Cells

To investigate the effect of $G\beta\gamma$ on AC V, the following experiments were carried out *in*

vitro. S49 cell membranes and S49cyc$^-$ cell membranes were preincubated with Gβγ. The AC activity was measured after stimulating the membranes with forskolin and isoproterenol. The S49 cell membranes and S49cyc$^-$ cell membranes without Gβγ were used as controls.

Comparing the AC activity for S49 cells to AC activity for S49 cells in the presence of Gβγ, there was a 31% and 28% decrease for the basal and forskolin-stimulated cells, and no detectable change was seen when cell membranes were stimulated with isoproterenol (Batch 2 *versus* Batch 1). The Gβγ inhibition of 30% and 50% was also seen with AC V COS-1 cells in the basal activity and forskolin-stimulated AC activity, respectively (Figures 18 and 21).

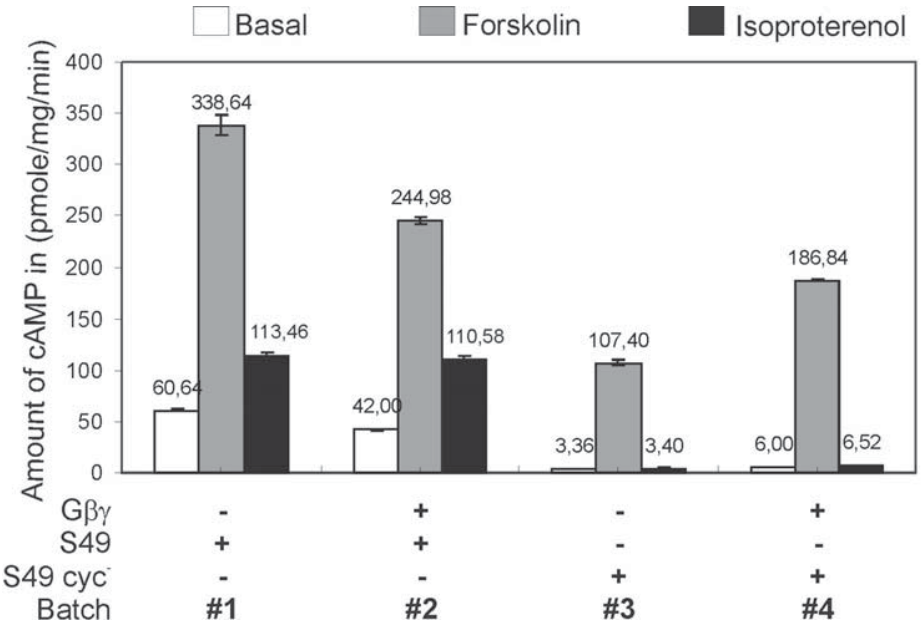


Figure 32: Effect of Gβγ on S49 cells and S49cyc$^-$ cells in the presence and absence of Gβγ

Membranes of S49 cells and S49cyc$^-$ cells were prepared as described in section 4. 100nM Gβγ were added to 10µg of membrane of either S49 or S49cyc$^-$ cells. These membranes were labeled with [^{32}P]-ATP for 20 minutes and stimulated with forskolin (100µM; 10min) and isoproterenol (100µM; 10 mins). cAMP formation was measured as described in section 4. Indicated are mean values ± S.D. of three independent experiments.

Similarly, comparing the AC activity of S49cyc$^-$ cells to the AC activity of S49cyc$^-$ cells in the presence of Gβγ, there was 44%, 43% and 48% increase in the basal, forskolin- and isoproterenol- stimulated cells (Batch 4 *versus* Batch 3). This experiment was carried out to examine the effect of Gβγ in the total absence of $G\alpha_s$ on AC V. There are two observations. What is clearly seen is that when $G\alpha_s$ is present but not stimulated (Figure 32 Batch 2) Gβγ has an inhibitory effect on AC V. In contrast, when $G\alpha_s$ is present and

activated there is no effect of Gβγ on AC V (Figure 32, Batch 2). Interestingly, in $G\alpha_s$-deficient cells, Gβγ has a stimulatory effect on AC V for basal, forskolin- and isoproterenol-stimulated cells (Figure 32 Batch 4). It is important to note that in all cases – *i.e.* transiently and stably expressing AC V in either HEK 293 cells or COS-1 cells and endogenously expressing AC V and AC VI in S49 membranes – AC V was not inhibited when stimulated with isoproterenol, and this suggested that $G\alpha_s$ in its active state hinders the inhibition of AC V by Gβγ. The presence of non-activated $G\alpha_s$ is important for the inhibition of AC V, as no inhibition is seen in the absence of $G\alpha_s$.

In order to investigate the role $G\alpha_s$ plays when Gβγ inhibits AC V and AC VI, $G\alpha_s$ in different states of (activated, not activated, and denatured) was added to S49cyc$^-$ membranes. These membranes were investigated for their basal-, forskolin-, and isoproterenol-dependant AC activity.

5.4.2 Effect of $G\alpha_s$ on Gβγ-Mediated Inhibition of AC V/VI

In Figure 33, it can be seen that the isoproterenol-stimulated S49cyc$^-$ membranes were similar to the basal values (Batch 1), thus confirming that there was no $G\alpha_s$ present (also represents Figure 32, Batch 3). Comparing Batch 1 to Batch 2, a 42%, 36% and 36% increased with respect to basal, forskolin-stimulated and isoproterenol-stimulated AC activity respectively. In Batch 3, the basal AC activity was equal to the isoproterenol-stimulated AC activity. This was due to the fact that the $G\alpha_s$ that was added to the S49cyc$^-$ cells was already in its activated state. More specifically, this was the effect seen also when S49 cells are stimulated with isoproterenol. As for the forskolin-stimulated AC activity, the synergistic effect of $G\alpha_s$ and forskolin on AC V and AC VI makes the AC activity higher than the isoproterenol-stimulated AC activity. In Batch 4, there was no change in cAMP level in all three conditions and is similar to Batch 3.

In Batch 5, non activated $G\alpha_s$ was added to S49cyc$^-$ cell membranes, thus mimicking the behavior of S49 wild type cells. On stimulation of cell membranes in batch 5, a 55, 300 and 109 pmole/mg/min AC activity for basal, forskolin- and isoproterenol-stimulated activity was observed. Upon addition of Gβγ to Batch 6, the basal and forskolin-stimulated activity decreased by 32% for both, whereas the isoproterenol-stimulated cells showed no difference. The effect of Gβγ on S49cyc$^-$ cells with non-activated $G\alpha_s$ corresponded to the effect of Gβγ on S49 (Figure 32).

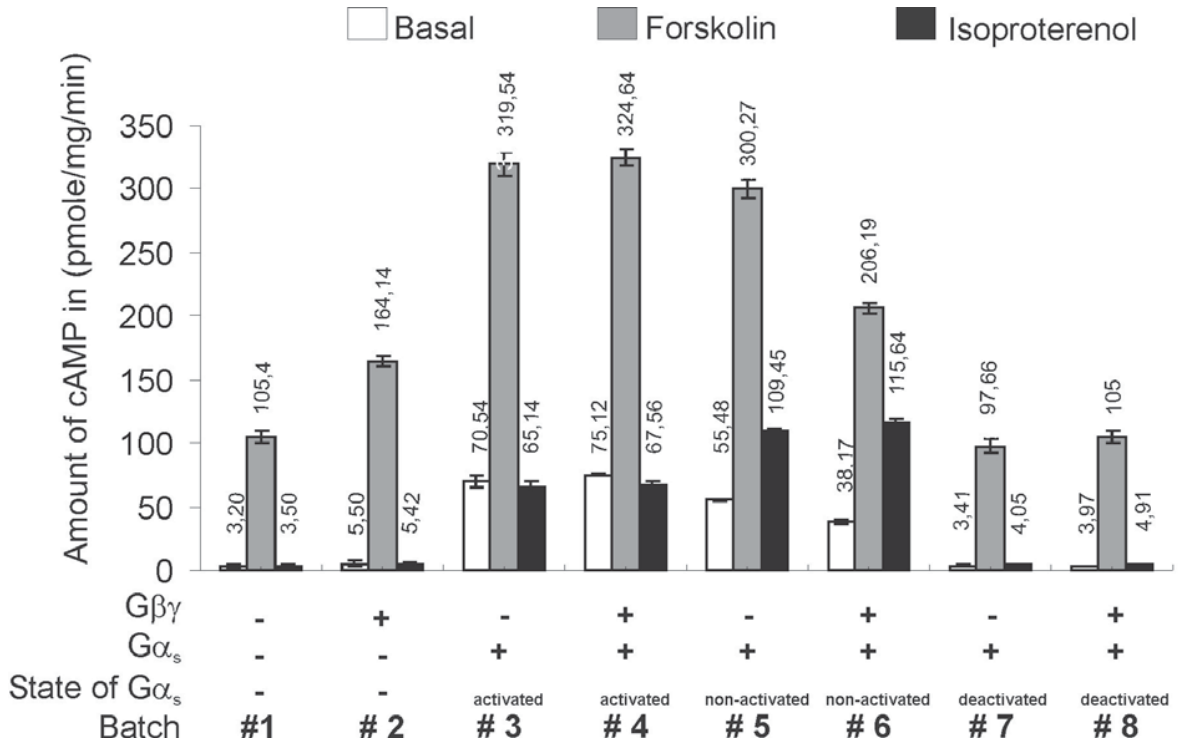


Figure 33: Effect of different states of $G\alpha_s$ on AC V/AC VI

Membranes of S49cyc$^-$ cells were prepared as described in section 4. 100µg/ml activated $G\alpha_s$, 100µg/ml non-activated $G\alpha_s$, 100µg/ml inactive $G\alpha_s$, and 100nM $G\beta\gamma$ were added to S49cyc$^-$ membranes as follows: All Batches have 10µg of S49cyc$^-$ cell membranes. To Batch 1 no form of $G\alpha_s$ was added. Batch 2 is the same as Batch 1 with the addition of 100nM $G\beta\gamma$. To Batch 3 activated-$G\alpha_s$ was added. Batch 4 is the same as Batch 3 with the addition of 100nM $G\beta\gamma$. To Batch 5 non-activated-$G\alpha_s$ was added. Batch 6 is the same as Batch 5 with the addition of 100nM $G\beta\gamma$. To Batch 7 deactivated-$G\alpha_s$ was added. Batch 8 is the same as Batch 7 with the addition of 100nM $G\beta\gamma$. These Batches were labeled with [^{32}P]-ATP for 20 minutes and stimulated with forskolin (100µM; 10min) and isoproterenol (100µM; 10 mins). cAMP formation was measured as described in section 4. Indicated are mean values ± S.D. of three independent experiments.

In Batches 7 and 8, denatured $G\alpha_s$ was used. As denatured $G\alpha_s$ cannot bind to the AC, no difference is expected to be seen with respect to its AC activity upon stimulation with forskolin or isoproterenol. Batches 7 and 8 are also expected to show equal results to Batches 1 and 2, respectively.

In Figure 33, it is confirmed that $G\alpha_s$ plays a role in the inhibitory effect of $G\beta\gamma$ on AC V and AC VI. More specifically, this inhibition takes place only when $G\alpha_s$ is in its non-active state.

5.5 *Gβγ-Regulation of AC Activity in COS-1 Cells Expressing AC VI*

5.5.1 *Generation of COS-1 Cells Stably Expressing AC VI*

It is known that AC V and AC VI are present in S49 and S49cyc$^-$ cells. Therefore COS-1 cells stably expressing AC VI were generated. COS-1 cells stably expressing with AC VI (Bayewitch *et al*., 1998) were established. Monoclonal cell lines were obtained by selection with 0.8mg/ml G-418 and were maintained using 0.4mg/ml G 418. The expression AC VI in COS-1 cells was compared to wild type COS-1 cells by Western blot analysis as well as by AC activity assays. Subsequent to isolation of AC transfectants, a series of preliminary experiments were carried out to characterize the enzyme activity in different clonal cell lines.

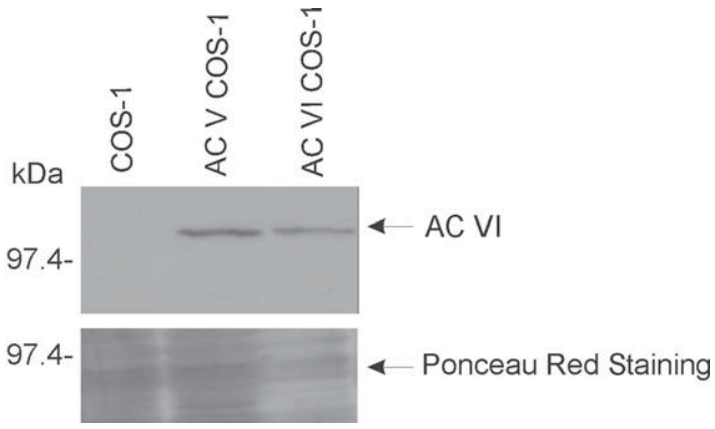


Figure 34: Detection of COS-1 cells stably expressing AC VI by Western blot analysis

Membranes from COS-1 cells (wild type 25µg), AC V COS-1 cells (25µg) and AC VI COS-1 cells (25µg) were lyzed. All lysates were separated by SDS-PAGE, blotted onto PVDF membrane and visualized by antibody BBC-2-AP and chemiluminescence as described in section 4. Vertical numbers indicate molecular mass (kDa) of iodinated standard proteins(upper panel). Equal loading was confirmed by staining the membranes with Ponceau Red (bottom panel).

The stable expression of AC V and AC VI in COS-1 cells was confirmed by immunoblotting (Figure 34). These were tested for AC activity and were further tested to see if Gβγ specific has an inhibitory effect on AC VI.

5.5.2 *Effect of Gβγ on COS-1 Cells Stably Expressing AC VI*

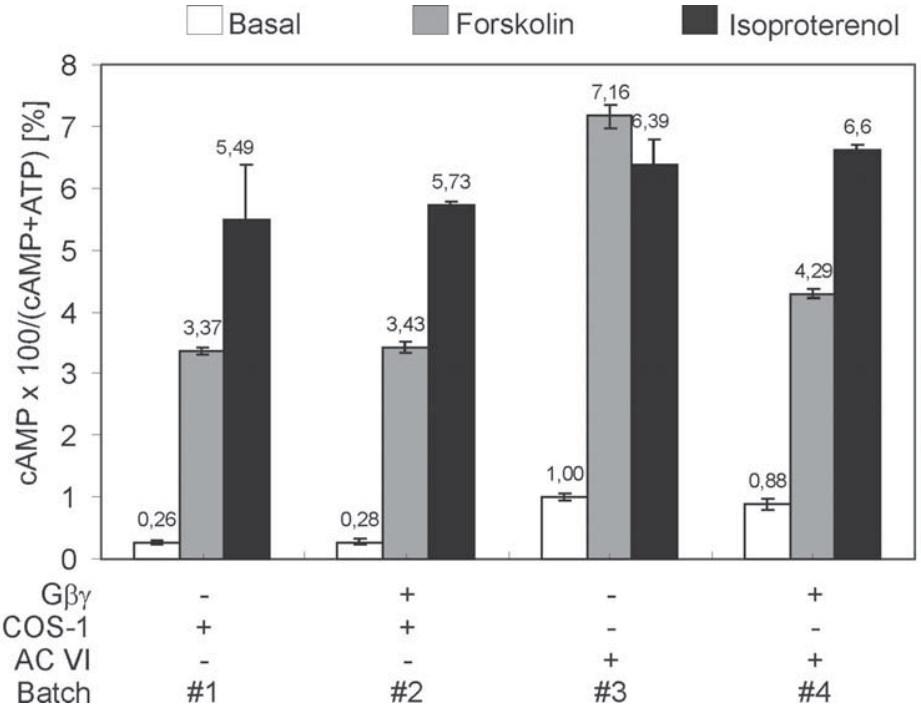


Figure 35: Effect of Gβγ on COS-1 cells stably expressing AC VI

COS-1 cells and AC VI COS-1 were transfected with Gβγ (Batch 2 and 4). 48 h after transfection, these cells were labeled with [^{3}H]-adenine for 60 minutes. These cells were stimulated with forskolin (10µl; 10min) and isoproterenol (1µM; 10min). cAMP formation was measured as described in section 4. Indicated are mean values ± S.D. of three independent experiments.

Comparing the AC activity of wild type COS-1 cells to AC VI COS-1 cells in Figure 35, the AC activity was increased by a factor of five in the basal state, by a factor of two in the forskolin-stimulated state, and by a factor of 1,2 in the isoproterenol-stimulated state. This clearly indicated the presence of AC VI being transfected into COS-1 cells. Thereafter these cells were tested to see the effect of Gβγ on AC VI COS-1 cells.

The addition of Gβγ to AC VI COS-1 cells inhibited the forskolin-stimulated activity and basal activity by 33% and 12% respectively; whereas, the isoproterenol-stimulated activity showed no change in the cAMP level. This demonstrates that the AC VI COS-1 cells, like AC V COS-1 cells, are regulated by Gβγ *in vivo*.

This research showed that Gβγ has an inhibitory effect on AC V and AC VI *in vivo* and *in*

vitro. Gβγ inhibition was demonstrated in the membranes of S49 cells and S49cyc$^-$ cells, in COS-1 cells expressing AC V or AC VI, and indirectly in HEK 293 cells.

Moreover, inhibition by Gβγ in S49 wild-type cells was seen not only in the basal state but also when stimulated with forskolin, *i.e.* when non-stimulated $G\alpha_s$ was present. In the presence of isoproterenol, this inhibitory effect of Gβγ on AC V and AC VI in S49 cells was not seen, *i.e.* when stimulated $G\alpha_s$ was present; on the contrary, an increase of cAMP level was observed. This increase in cAMP level is due to the Gβγ scavenging $G\alpha_i$, where $G\alpha_i$ has an inhibitory effect on AC V and VI (Gilman *et al*., 1991).

The Gβγ inhibition on AC V and AC VI was observed in COS-1 cells stably expressing AC V and AC VI. More specifically, this inhibition was observed only when these COS-1 cells were in their basal state or when stimulated with forskolin. This inhibition did not take place in the absence of $G\alpha_s$. Thus, it can be concluded that Gβγ can inhibit AC V and AC VI only when endogenous $G\alpha_s$ is in its non-activated state.

The stimulation of AC II by Gβγ took place *via* direct interaction with Gβγ in the presence of activated $G\alpha_s$; whereas, the inhibition of AC V and AC VI by Gβγ takes place only *via* unactivated $G\alpha_s$.

6 DISCUSSION

6.1 Regulation of Adenylyl Cyclases by G-Proteins

Adenylyl cyclase catalyzes the conversion of ATP to 3',5'-cyclic AMP (cAMP) and pyrophosphate. The resulting cAMP acts as a second messenger by interacting with and regulating other proteins such as protein kinase A (PKA) and cyclic nucleotide-gated ion channels. Over the last two decades, it became evident that adenylyl cyclase (AC) activity is regulated by multiple effectors, which include not only the Gα-subunits of G_s and G_i proteins but also PKC, PKA and Gβγ. The most important regulators of adenylyl cyclases (ACs) are Gα-subunits. AC can be activated or inhibited by Gα proteins, which are coupled to heptahelical trans-membrane receptors and can be activated by various extracellular stimuli. There are multiple classes of Gα-subunits that regulate AC (see Table 1 and Figure 8), either in a stimulatory or inhibitory manner. The Gβγ-subunit regulates AC in a subtype-specific manner. Gβγ inhibits of AC I, AC III, and AC VIII, stimulates AC II, AC IV, and AC VII, only when $G\alpha_s$ is co-activated. In fact, Gβγ is among the most potent of all negative regulators of AC I, AC III, and AC VIII and can markedly inhibit the effect of forskolin, $G\alpha_s$, and Ca^{2+}/CaM on AC activities (Gao *et al*., 1991; Tang *et al*., 1992; Taussig *et al*., 1992).

Bayewitch and colleagues have shown *in vitro* that Gβγ can inhibit AC V and AC VI (Bayewitch *et al*., 1998). Till to date there have been no studies showing the Gβγ regulatory effect on AC V in intact cells. Therefore, this study investigates the effect of Gβγ on AC V in various intact cell lines. As mentioned earlier, Gβγ has a stimulatory as well as an inhibitory effect on other adenylyl cyclases. More specifically, the inhibition of AC I by Gβγ takes place in the presence of Ca^{2+}/CaM, whereas the stimulation of AC II regulated Gβγ is synergistic in the presence of stimulated $G\alpha_s$ (Tang *et al*., 1992). Since the effect of Gβγ on AC II is stimulatory, AC II was used as positive control. Forskolin and isoproterenol were used to stimulate the cellular AC activity. Forskolin has a direct stimulatory effect on all isoforms of adenylyl cyclase except for AC IX. Isoproterenol is an agonist for the β-adrenergic receptors. β-adrenergic receptors are bound to GDP-bound-$G\alpha_s\beta\gamma$ heterotrimers. Stimulation with isoproterenol triggers a conformational change that causes an exchange of GDP-bound-$G\alpha_s$ to GTP-bound-$G\alpha_s$ which subsequently dissociates into GTP-bound-$G\alpha_s$ and Gβγ. GTP-$G\alpha_s$ has a stimulatory effect

on all isoforms of adenylyl cyclase.

6.1.1 Stimulatory Effect of Gβγ on AC II

Previous reconstitution (Tang *et al*., 1991) and transfection (Feder *et al*., 1986) studies have established that the stimulation of AC II by Gβγ requires the presence of activated $G\alpha_s$ and have been performed using proteins purified from Sf9 cells. With the knowledge that AC II is stimulated by Gβγ in the presence of activated $G\alpha_s$, it was decided to trigger the release of Gβγ in HEK 293 cells expressing AC II and to study its effect *in vivo* in HEK 293 cells. Figure 36 shows a simplified schematic representation of signalling transduction cascades relevant to this research.

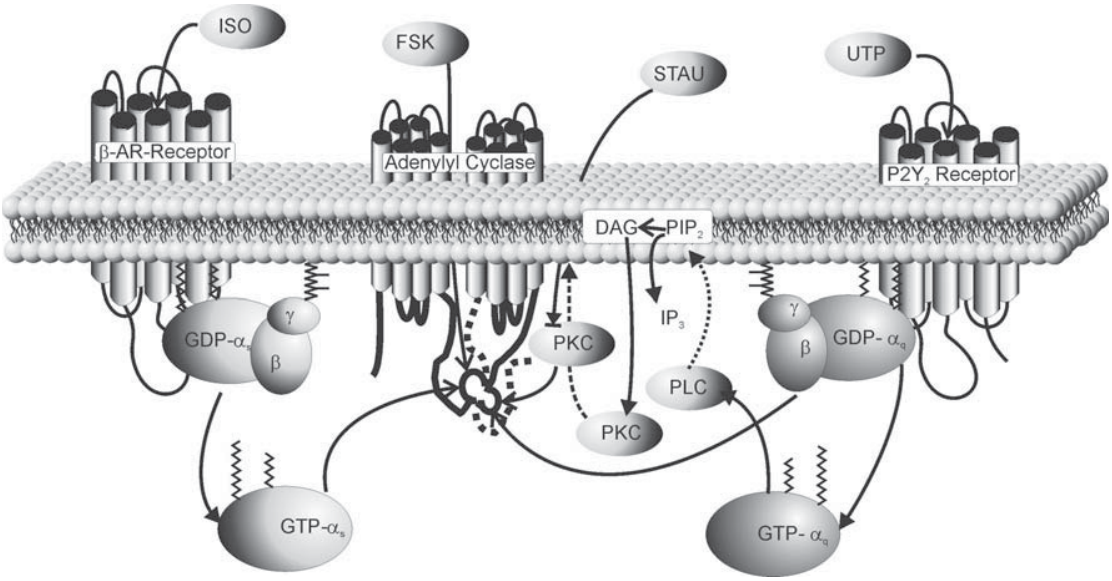


Figure 36: Simplified schematic signal transduction cascade

$G\alpha_s\beta\gamma$ is bound to the β-ARs. Isoproterenol is used to trigger the release of $G\alpha_s$ and Gβγ. Forskolin is hydrophobic and therefore passes through the membrane and binds to the catalytic core of adenylyl cyclase directly. $G\alpha_q\beta\gamma$ is bound to the $P2Y_2$ receptor. UTP, an agonist for $P2Y_2$ receptors, is used to trigger the release of $G\alpha_q$ and Gβγ. PLC isoforms are stimulated by $G\alpha_q$ as well as Gβγ. On stimulation of PLC, PLC translocates to the membrane. Its substrate PIP_2 is hydrolyzed to DAG and IP_3. The hydrophilic IP_3 moves to the endoplasmic reticulum, where IP_3 stimulates the release of Ca^{2+} from storage. The lipophilic DAG remains in the membrane and activates PKC which in turn activates adenylyl cyclase isoforms in an isoforms specific manner. Staurosporin is used to inhibit the PKC activity. The idea is to observe the effect of endogenous Gβγ (released from β-AR and $P2Y_2$ receptors) on AC II and AC V. FSK: Forskolin, ISO: isoproterenol, β-AR: β-adrenergic receptor, UTP: uridine triphosphate, Stau: staurosporin, PLC: phospholipase C, PKC: protein kinase C, PIP_2: phosphatidylinositol bisphosphate, IP_3: inositol 1,4,5-trisphosphate, DAG: diacylglycerol, GDP: guanosine diphosphate, GTP: guanosine triphosphate.

The following three different approaches were preformed to observe the effect of Gβγ on AC II. I) Endogenous Gβγ was released by stimulating $P2Y_2$ receptors that are

endogenous present in HEK 293 cells. II) HEK 293 cells expressing AC II were transfected with Gβγ and III) HEK 293 cells expressing AC II were transfected with $G\alpha_t$ to observe the reverse effect of Gβγ on AC II. The outcome of these approaches is discussed below. I) Endogenous Gβγ was released by stimulating endogenus $P2Y_2$ receptors in HEK 293 cells with UTP and further stimulated with isoproterenol. The adenylyl cyclase activity of AC II increased by one third (Figure 10). It is also known that the G_q-dependent pathway for the activation of PKC can be circumvented by Gβγ-sensitive phospholipase-β isoforms (Camps *et al*., 1992; Smrcka *et al*., 1993), but activation of PLC by Gβγ is much less efficient than that by G_q (Tsu *et al*., 1997). Besides the release of $G\alpha_q$, release of Gβγ also triggers colocalization of PLC isoforms with AC II and stimulates the formation of cAMP (Kuang *et al*., 1996). It was interesting to note that the basal PKC activity was already elevated before stimulation with UTP. The decrease in AC II activity when treated with staurosporin only confirms the elevated basal PKC activity (compare Batch 1 to Batch 3 in Figure 10). A possible explanation could be the stimulation of Gβγ (released from $G\alpha_s$) which directly activates PLC, PLC in turn activates the PKC which stimulates AC II. It has been reported that the PKC effect on AC activity is PKC-isoform dependent (Lustig *et al*., 1993). This is supported by a study which demonstrated that modulation of stimulated β-adrenergic receptor activity also depends on the complement of PKC isoforms present in a given cell type (Guimond *et al*., 2005). Therefore, one can conclude that the elevated basal PKC activity is likely due to the Gβγ released on stimulation of β-adrenergic receptors. This approach confirms the release of endogenous Gβγ in HEK 293 cells from β-adrenergic receptors as well as $P2Y_2$ receptors (compare Batch 2 to Batch 1 in Figure 10).

II) The transfection of Gβγ in HEK 293 cells or COS-1 cells stably or transiently expressing AC II clearly demonstrates Gβγ stimulation on AC II *in vivo* and in the presence of stimulated $G\alpha_s$ (Figures 13 and 15). Till to date this experiment was always carried out *in vitro*. Moreover, this stimulation is not seen when cells were stimulated with forskolin. In addition, it is also known that the binding site of $G\alpha_s$ on AC II that invokes AC II stimulation lies in the C1b region and AC II has two binding sites for Gβγ. The first Gβγ binding site that regulates the AC II stimulation lies in the C1b domain of AC II. This region was confined to the amino acid sequence "PFAHL" (Diel *et al*., 2006). The second Gβγ binding site is the amino acid sequence "QEHA" located in the C2 region of AC II (Chen *et al*., 1995). On stimulating AC II with forskolin, we did not observe an increase in cAMP level (compare Batch 1 to Batch 3 in Figures 13 and 15). This is supported by Tang

and collegues who also showed that stimulation of AC II by Gβγ takes place only in the presence of $G\alpha_s$ (Tang *et al*., 1991).

III) The transfection of $G\alpha_t$ in HEK 293 cells stably expressing AC II demonstrated no change in AC II activity when stimulated with isoproterenol and forskolin (comparing Batch 2 to Batch 1 in Figure 12). One would expect to see a decrease in AC II activity triggered by the $G\alpha_t$ which scavenges endogenous Gβγ then not available for stimulation. Interestingly, the decrease in AC II was seen in HEK 293 cells transiently expressing AC II. The fact that releasing endogenous Gβγ from β-adrenergic receptors in HEK 293 cell stably expressing AC II did not trigger a decrease in AC II activity implies that there could be excess amount of Gβγ in HEK 293 cells (compare Figure 12 to Figure 14). The elimination of the stimulatory effect of endogenous Gβγ on AC II in HEK 293 cells transiently expressing AC II is likely due to the capturing of endogenous Gβγ, which was then no longer available for the stimulatory effect of $G\alpha_s$ and Gβγ on AC II (compare Batch 2 to Batch 1 in Figure 14). In this case, stimulation of AC II occurs only when Gβγ and stimulated GTP-bound-$G\alpha_s$ are present. It is also known that $G\alpha_s$ and AC II bind to Gβγ at the same site (Li *et al*., 1998).

6.1.2 Inhibitory Effect of Gβγ on AC V

Previous *in vitro* studies have shown that Gβγ can have an inhibitory effect on AC V (Bayewitch *et al*., 1998). These experiments were carried out in COS-1 cell membranes. Confirming the release of Gβγ and its stimulation on AC II *in vivo*, the same procedure was carried out with HEK 293 cells expressing AC V. Similarly the following three approaches were used to study the effect of Gβγ on AC V *in vivo*. I) Endogenous Gβγ was released by stimulating $P2Y_2$ receptors that are endogenous present in HEK 293 cells expressing AC V. II) HEK 293 cells expressing AC V were transfected with Gβγ and III) HEK 293 cells expressing AC V were transfected with $G\alpha_t$. The outcome of these approaches is discussed below.

I) In comparision to observing the stimulation of Gβγ on AC II (Figure 10), the release of endogenous Gβγ in HEK 293 cells expressing AC V showed no AC activity (Figure 11). In addition, the PKC basal activity that was observed with AC II (compare Batch 2 to Batch 1 in Figure 10), was not observed with AC V either (Figure 11). A possible explanation for the absence of PKC activity with respect to AC V could be the following. It is known that PKCγ has a stimulatory effect on AC II but not on AC V (Kawabe *et al*.,

1996). Furthermore, PKCγ is also present in HEK 293 cells (Kawabe *et al*., 1994). This is consistent with my findings where PKC activity is seen with AC II (Figure 10). With respect to AC V, both PKCα and PKCζ are known to exert stimulatory effects on AC V (Chakrabarti *et al*., 2003) but there is no evidence that PKCα and PKCζ are present in HEK 293 cells. This is probably the explanation why AC II and not AC V was stimulated. Another possibility is that the specific subtype of Gβγ dimers expressed in HEK 293 cells do not have an inhibitory effect on AC V. Baywitch showed that $G\beta_5\gamma_2$ has no inhibitory effect on AC V (Bayewitch *et al*., 1998). Specifically what type of endogenous Gβγ is present in HEK 293 cells is not known.

II) When HEK 293 cells stably and transiently expressing AC V were transfected with $G\beta_1\gamma_2$, no inhibitory effect of Gβγ on AC activity was observed (Figures 14 and 16). In contrast, we found an inhibitory effect of Gβγ on AC V activity in COS-1 cells (Figure 17). Gβγ inhibition on AC V was also shown by Bayewitch *et al*. *in vitro*. It is interesting to note that this inhibition was only seen in its basal state and upon treatment with forskolin, but not when stimulated with isoproterenol.

III) Although no inhibition of AC V activity was seen in HEK 293 transfected with Gβγ, an inhibition of AC V activity by Gβγ was seen in COS-1 cells. The coexpression of $G\alpha_t$ triggered a stimulation of AC V activity in the presence of forskolin and isoproterenol (Figures 14 and 16). The increase in AC activity when stimulated with forskolin in the presence of $G\alpha_t$ can be explained by the fact that $G\alpha_t$ scavenged free Gβγ, thus eliminating the inhibitory effect of Gβγ on AC V. This also implies that the Gβγ present in HEK 293 cells could be $G\beta_1\gamma_2$. The stimulatory effect of AC V upon incubation with isoproterenol could be due to the $G\alpha_s$ released from G_s heterotrimers bound to β-adrenergic receptors. $G\alpha_t$ captures endogenous Gβγ as well as Gβγ released from G_s heterotrimers; hence this stimulation can be $G\alpha_s$-related stimulation.

The question arises as to why we do not see the expected inhibition when Gβγ is released. There are two possible answers. One, it could be that the subtype of Gβγ present in HEK 293 cells does not inhibit AC V activity. It has been reported that $G\beta_5\gamma_2$ has no effect on AC V or AC VI *in vitro* in COS-1 cell membranes (Bayewitch *et al*., 1998). Jones and collegues have also shown that the effector activation varies with respect to Gβγ specificity (Jones *et al*., 2004). A second possibility could be the high amount of Gβγ present in HEK 293 cells. The fact that $G\alpha_t$ scavenges endogenous Gβγ, which results in the elimination of the inhibitory-effect of Gβγ (compare Batch 2 to Batch 1 in Figures 14 and 16) suggests that endogenous Gβγ is present. If Gβγ is present in excess in HEK 293 cells

suggests that AC V is already at its maximally inhibited state. One could therefore conclude that $G\alpha_t$ eliminates the (inhibitory) effect of Gβγ on AC V.

It is also apparent that the AC activity in the transient cell lines showed distinct AC II or AC V activity compared to stable cell lines. When comparing Table 11, 12, 13 and 14, the cAMP level (endogenous Gβγ scavenged by $G\alpha_t$) was always greater in the transiently transfected cell lines as compared to the stable cell lines.

Table 11: AC activity of AC II stably expressed in HEK 293 cells cotransfected with $G\alpha_t$ and Gβγ

	Basal	Forskolin-stimulated	Isoproterenol-stimulated
Control → $G\alpha_t$	-	15%↓	7%↓
Control → Gβγ	-	-	50%↑

Table 12: AC activity of AC II transiently expressed in HEK 293 cells cotransfected with $G\alpha_t$ and Gβγ

	Basal	Forskolin-stimulated	Isoproterenol-stimulated
Control → $G\alpha_t$	10%↓	3%↓	40%↓
Control → Gβγ	12%↓	6%↓	16%↑

Table 13: AC activity of AC V stably expressed in HEK 293 cells cotransfected with $G\alpha_t$ and Gβγ

	Basal	Forskolin-stimulated	Isoproterenol-stimulated
Control → $G\alpha_t$	23%↑	35%↑	26%↑
Control → Gβγ	-	-	-

Table 14: AC activity of AC V transiently expressed in HEK 293 cells cotransfected with $G\alpha_t$ and Gβγ

	Basal	Forskolin-stimulated	Isoproterenol-stimulated
Control → $G\alpha_t$	34%↑	45%↑	53%↑
Control → Gβγ	-	-	-

This led to the hypothesis that cell lines stably expressing AC have a higher amount of free Gβγ when compared to the cell lines transiently expressing AC. The higher amount of endogenous Gβγ present in stably expressed AC HEK 293 cell lines could be a result of the stable expression of the adenylyl cyclase isoform in HEK 293 cells, which probably down regulates or up regulates Gβγ over a period of time (Holmer *et al*., 1996; Zhou *et al*., 2007) respectively. However, the amount of endogenous Gβγ was still high in cell lines transiently expressing AC to observe Gβγ inhibition on AC V, thus suggesting that Gβγ could have reached its maximum inhibition on AC V cell lines in stably and transiently expressing AC. In order to investigate this possibility, different concentrations of Gβγ were added to HEK 293 cells stably or transiently expressing AC V. The inhibition

remained unchanged with increasing concentrations of Gβγ. Nevertheless, it could be demonstrated that Gβγ exerted an indirect inhibitory effect on AC V when transfected with $G\alpha_t$.

Interestingly, this *in vivo* Gβγ inhibition on AC V in COS-1 cells transiently and stably expressing AC V was seen only when stimulated with forskolin (Figure 17). Similarly, indirect Gβγ inhibition on AC V was also seen in HEK 293 cells stably and transiently expressing AC V were cotransfected with $G\alpha_t$. Hence it can be concluded that the presence of excess Gβγ is what made it impossible to see the inhibition in HEK 293 cells directly, since Gβγ inhibition was already at its maximum. The question still remains though why one cannot measure this inhibition when AC V is stimulated with isoproterenol. Does stimulated $G\alpha_s$ play a role here, as this inhibitory effect is also seen in basal activity? This led to further research on the role of $G\alpha_s$ in the Gβγ-inhibited AC V.

6.2 *The Role of $G\alpha_s$ for the Gβγ-Mediated Inhibition of AC V and AC VI*

From the previous results it has been shown that Gβγ inhibits AC V only when stimulated with forskolin. Till now the effect of Gβγ on AC V has been investigated when $G\alpha_s$ is stimulated (using isoproterenol) and $G\alpha_s$ is not stimulated (using forskolin since it stimulates AC directly). Three different processes were used to investigate the effect of Gβγ on AC V. Since the inhibition was seen in COS-1 cells it was decided to use the first two techniques to down regulate $G\alpha_s$ in COS-1 cells. They are as follows: I) Using antisense technique to down-regulate $G\alpha_s$, II) Using cholera toxin to degrade $G\alpha_s$ and III) In parallel, S49cyc$^-$ cells were tested since these cells are devoid of $G\alpha_s$.

Using antisense technique to down-regulate $G\alpha_s$, proved to be unsuccessful (Figures 25 and 27). Cholera toxin down-regulated $G\alpha_s$ and $G\alpha_s$ that was present in COS-1 cells were in its GTP-bound state (Figures 28 and 29). It was necessary to completely eliminate $G\alpha_s$ from the cellular system. As shown by Chang, $G\alpha_s$ did get degraded to 80% upon CTX-treatment (Chang *et al*., 1989). The 20% of $G\alpha_s$ present was in its activated state. The Gβγ effect on AC V was not seen in the presence of activated $G\alpha_s$ and it was important to observe the Gβγ effect on AC V in cells totally void of $G\alpha_s$. Since the down regulation of $G\alpha_s$ was not possible in COS-1 cells, S49 and S49cyc$^-$ cells were used to investigate the inhibitory-Gβγ effect on AC V. S49cyc$^-$ cells are devoid of $G\alpha_s$.

S49 and S49cyc$^-$ cells are known to express endogenous AC V and AC VI (Chang *et al*., 1989; Premont *et al*., 1992). Upon adding Gβγ to S49 wild type cell membranes, it was

clearly seen that there was a decrease in cAMP level (a Gβγ inhibition) not only in the presence of forskolin but also basal state of AC activity (Figure 32). These results are consistent with the experiments carried out in COS-1 cells expressing AC V (Figures 18 and 21). Moreover, it was interesting to note that this inhibition was not observed when these cell membranes were stimulated with isoproterenol. Similar results were observed also when COS-1 cells expressing AC VI and transfected with Gβγ were activated by stimulated $G\alpha_s$ *via* isoproterenol – Gβγ inhibition of AC V was not observed (Figure 35). On the contrary, when a similar test was carried out in S49cyc$^-$ cells, a slight elevation in AC activity was seen upon addition of Gβγ to $G\alpha_s$-deficient S49cyc$^-$ cell membranes when stimulated with forskolin. Tang and collegues reported that $G\alpha_i$ are present in S49 and S49cyc$^-$ cell membranes (Tang *et al*., 1991) and it is known that $G\alpha_i$ has an inhibitory effect on AC V in S49cyc$^-$ cell membranes (Dessauer *et al*., 1998). Upon addition of Gβγ to S49cyc$^-$ cell membranes, Gβγ scavenges $G\alpha_i$, hence eliminating the inhibitory effect of $G\alpha_i$ on AC V (Bokoch *et al*., 1983).

From the experiments carried out on S49 cells and S49cyc$^-$ cells, it can be concluded that Gβγ has an inhibitory effect on AC V when stimulated with forskolin. This inhibitory effect of Gβγ on AC V was seen in S49 cells, COS-1 cells expressing AC V, and indirectly in HEK 293 cells expressing AC V, only when they are stimulated with forskolin. When these cells were stimulated with isoproterenol, no inhibition was observed. These results confirmed that $G\alpha_s$ plays a role, therefore S49cyc$^-$ cells were incubated with $G\alpha_s$ in its basal (non-stimulated) state and with AlF^{4-}-activated $G\alpha_s$ representing (stimulated $G\alpha_s$). It was clear that the Gβγ inhibition on AC V was taking place only when $G\alpha_s$ was in its non-activated state (forskolin-stimulated) and not when it was in its activated state (isoproterenol-stimulated). Gβγ had an inhibitory effect on AC V only when $G\alpha_s$ was in its non-active state. In the total absence of $G\alpha_s$, Gβγ had no inhibitory effect on AC V or AC VI but a stimulatory effect. This stimulatory effect is due to the Gβγ scavenging $G\alpha_i$, where $G\alpha_i$ inhibits AC V.

Since AC VI is endogenously present in S49 cells and S49cyc$^-$ cells (Krupinski *et al*., 1992), it was important to express AC VI in COS-1 cells and to further test if Gβγ has the same regulatory effect on AC VI like on AC V. Regulation of AC VI by Gβγ showed a decrease in the formation of cAMP for basal adenylyl cyclase activity as well as the forskolin-stimulated activity but no Gβγ inhibitory effect on AC VI was observed, when stimulated with isoproterenol. So the regulation of AC V and VI in COS-1 cells and S49 wild-type cell membranes by Gβγ was inhibitory only in the presence of non-activated

$G\alpha_s$.

In a recent paper Gao´s group demonstrated that Gβγ has a stimulatory effect on AC V and AC VI when stimulated with $G\alpha_s$ and forskolin. All his experiment were carried out *in vitro* using proteins in Sf9 insect cells (Gao *et al*., 2007). This paper also mentions that this stimulatory effect was not seen in canine AC V or AC VI. He triggered the release of endogenous Gβγ from G_i- or G_s-coupled receptors to investigate the Gβγ effect on AC VI. According to Gao, the stimulation of Gβγ on AC VI was not seen in intact COS-7 cells due to low concentration of endogenous Gβγ in COS-7 cells. His paper suggests that the inhibition seen in intact cells is due to an indirect effect from overexpression of Gβγ which modulates many other effectors such as PLCβ, PI3K and ion-channels which in turn increases intracellular Ca^{2+} concentration and thereby inhibit the Ca^{2+}-sensitive AC V and AC VI, resulting in inhibitory effects that over rule the stimulation of the Gβγ effect.

More specifically, Gβγ stimulates AC V and AC VI only in S49cyc$^-$ cells. This is also been shown by Katada *et al*. This stimulation is due to the sequestering of $G\alpha_i$ by Gβγ. Comparing the stimulation of Gβγ on AC V with forskolin as seen by Gao´s group is not seen at all in HEK 293 cells, COS-1 cells and S49 cells except in S49cyc$^-$ cells. On the contrary, inhibition of AC V and AC VI by Gβγ is observed and with isoproterenol no effect of Gβγ on AC V and AC VI. A likely explanation is that it could be that $G\alpha_s$ is absent in Sf9 cells. Sf9 cells were used by Gao´s group when they reported that there was a Gβγ-stimulation seen on AC V activity when forskolin-stimulated. The laboratories of Guenet and Manning have independently shown that endogenous $G\alpha_s$ is not present in Sf9 cells, whereas $G\alpha_i$ and its subtypes are expressed. This has been proven by immunoblot assays as well as ADP-ribosylation (Barr *et al*., 1997; Heitz *et al*., 1995). The stimulation of Gβγ on AC V seen in Sf9 cells when stimulated with forskolin and isoproterenol (Gao´s group) was seen only in S49cyc$^-$ cells (my work). This is due to sequestering of $G\alpha_i$ by Gβγ.

The isoforms AC V and AC VI used in this research were isolated from rabbit heart tissue and all experiments were carried out in COS-1 cells, HEK 293 cells, S49 cells and S49cyc$^-$ cells. Stimulation of Gβγ on AC V and AC VI was seen in only S49cyc$^-$ cells when stimulated with forskolin and isoproterenol. This stimulation is due to the capturing of $G\alpha_i$ by Gβγ, an indirect elimination of the effect of $G\alpha_i$ on AC V and AC VI. Premont *et al*. also mentioned that he did not see any effect of Gβγ on AC V in isoproterenol-stimulated HEK 293 cells which is consistent with my findings (Premont *et al*., 1992). This is due to the excess amount of endogenous Gβγ in HEK 293 cells. Premont *et al*. had not tested the

effect of Gβγ on AC V or AC VI by stimulating AC V with forskolin only. No inhibitory effect of Gβγ on AC V was seen in HEK 293 cells when stimulated with forskolin, on the contrary the Gβγ-inhibition could be seen only indirectly when stimulated with forskolin and in the presence of $G\alpha_t$.

7 CONCLUSIONS

Pfeuffer *et al*. postulated in their research work that basal adenylyl cyclase isolated from rabbit myocardial membranes differs from adenylyl cyclase from bovine brain cortex (Pfeuffer *et al*., unpublished). Although isolated from non-activated membranes, the purified enzyme displayed a GDP-bound $G\alpha_s$ complex with adenylyl cyclase devoid of the $G\beta\gamma$-dimer.

In this study the following could be shown for the first time:

- $G\beta\gamma$ stimulates AC II in intact HEK 293 cells and in COS-1 cells
- $G\beta\gamma$ indirectly inhibits AC V in intact HEK 293 cells
- $G\beta\gamma$ inhibits AC V and AC VI in intact COS-1 cells and in S49 cells
- $G\beta\gamma$ inhibits AC V/VI in S49cyc$^-$ cell membranes
- $G\beta\gamma$ inhibiton on AC V and AC VI takes place only in the presence of GDP-bound-$G\alpha_s$.

The above five points mentioned imply two novel facts:

- GDP-bound-$G\alpha_s$ exists in a complex with AC V/VI without being associated with $G\beta\gamma$
- GDP-bound-$G\alpha_s$ has regulatory effects on AC V/VI.

It is important to note that till to date it was always known that $G\alpha_s$ regulates its various effectors only in its GTP-bound state. Later it was discovered that $G\beta\gamma$ also has a regulatory effect and acts as a negative regulator of $G\alpha$ signaling by binding to $G\alpha$ and by reducing the rate of spontaneous GDP release. So far, nothing is known about the regulatory effects of non-activated $G\alpha_s$. We have reported for the first time that "non-activated" $G\alpha_s$ which we prefer to refer to as "GDP-bound-$G\alpha_s$" can exist as a complex with AC V/VI. Secondly, this existing complex has a regulatory effect on its effector AC V/VI. It contradicts all current knowledge that GDP-bound-$G\alpha_s$ has a regulatory effect. The basic paradigm describing G-protein signaling through activated $G\alpha$-subunits can be enlarged to include a number of novel regulatory mechanisms. Does GDP-bound $G\alpha_s$ coexist with other effectors in a complex or is AC V/VI the only effector to which GDP-bound-$G\alpha_s$ binds to?

What is the reason that triggers GDP-bound-$G\alpha_s$ to form a complex with AC V and AC VI? Does $G\beta\gamma$ scavenge the GDP-bound $G\alpha_s$ and thereby eliminate the GDP-bound-$G\alpha_s$-stimulation of AC V and AC VI? Or does $G\beta\gamma$ trigger a direct inhibitory effect on

AC V/VI GDP-bound $G\alpha_s$ complex only?

Do GTP-bound $G\alpha_s$ and GDP-bound $G\alpha_s$ bind at the same binding site to AC V and AC VI isoforms? It could be that the GDP-bound $G\alpha_s$ binds specifically on the C2a domain of AC V and does not induce a change in the relative orientation of the C1 and C2 domains that, in turn, primes the active sites for catalysis. If it is the same site, it could be that GTP-bound-$G\alpha_s$ triggers the alignment of the C1 and C2 domains of AC V for catalysis.

Till to date the binding site of $G\alpha_s$ on AC V suggested a presence of high and low affinity sites on AC V, which interact with $G\alpha_s$. More specifically, a 10-aa region within the C2a domain of AC V interacts with a 112-aa in C1b region of AC V and this intermolecular interaction modulates the stimulation of enzyme activity by $G\alpha_s$ (Scholich *et al*., 1997). It has been reported that this 10-aa region in the C2a domain of ACII is located in the β2-strand that is positioned outside of the catalytic cleft (Yoo *et al*., 2004). This location of the 10-aa region encompassed by L^{1042}–T^{1051} in AC V would allow interactions with the 112-aa in the C1b domain of AC V. From the crystal structure of the C2a domain of AC II, Zhang *et al*. speculated that this region is a focal point for regulating the stability and alignment of the active molecule (Zhang *et al*., 1997).

In my experimental model, there are three types of interactions between the three forms of AC V/VI and $G\alpha_s$. The three forms are I) AC V/VI, II) AC V/VI-GDP-bound-$G\alpha_s$ complex and III) AC V/VI-GTP-bound-$G\alpha_s$ complex.

Figure 37 shows the activity of the three various states in which AC V exists. From Figure 37 it is clear that the adenylyl cyclase activity of AC V/VI-GTP-bound-$G\alpha_s$ complex (Figure 37 see Batch 1 black bar) has a higher level of AC activity compared to the AC V/VI-GDP-bound-$G\alpha_s$ complex and AC V/VI-GDP-bound-$G\alpha_s$ complex (Figure 37 Batch 1 white bar) has a higher level of AC activity than AC V (Figure 37 Batch 3 white bar).

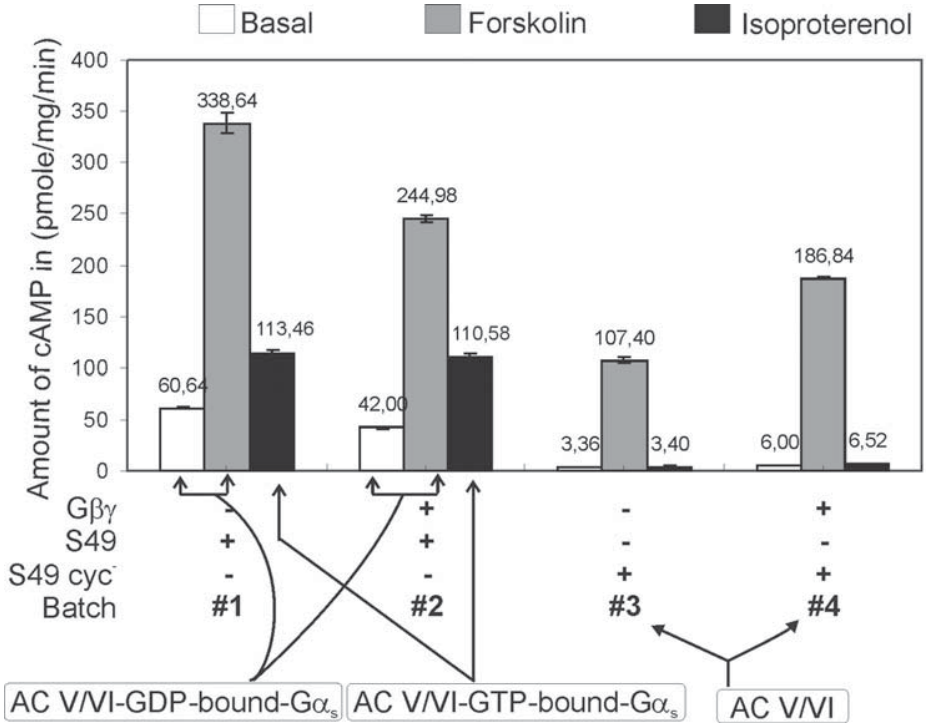


Figure 37: Adenylyl cyclase activity of AC V/VI, AC V/VI-GDP-bound-$G\alpha_s$ and AC V/VI-GTP-bound-$G\alpha_s$

The basal adenylyl cyclase activity for AC V/VI and AC V/VI-GDP-bound-$G\alpha_s$ is 3,36 and 60,64pmole/mg/min respectively. AC V/VI and AC V/VI-GDP-bound-$G\alpha_s$ changes to AC V/VI-GTP-bound-$G\alpha_s$ when stimulated with isoproterenol. The adenylyl cyclase activity of AC V/VI-GTP-bound-$G\alpha_s$ is 113,46 pmole/mg/min.

According to my model, AC V exists in two forms, which are AC V and AC V/VI-GDP-bound-$G\alpha_s$. On stimulation with isoproterenol, both forms of AC V/VI change to their GTP-bound-$G\alpha_s$ state. The AC V state is converted to AC V/VI-GTP-bound-$G\alpha_s$ *via* the β-AR whereas the AC V/VI-GDP-bound-$G\alpha_s$ has two possible ways to get converted to its GTP-bound-$G\alpha_s$ state (Figure 40).

The change in adenylyl cyclase activity when Gβγ is added AC V/VI (Figure 38), AC V/VI-GDP-bound-$G\alpha_s$ (Figure 39) and AC V/VI-GTP-bound-$G\alpha_s$ (Figure 40) are shown in the following simplified schematic diagram.

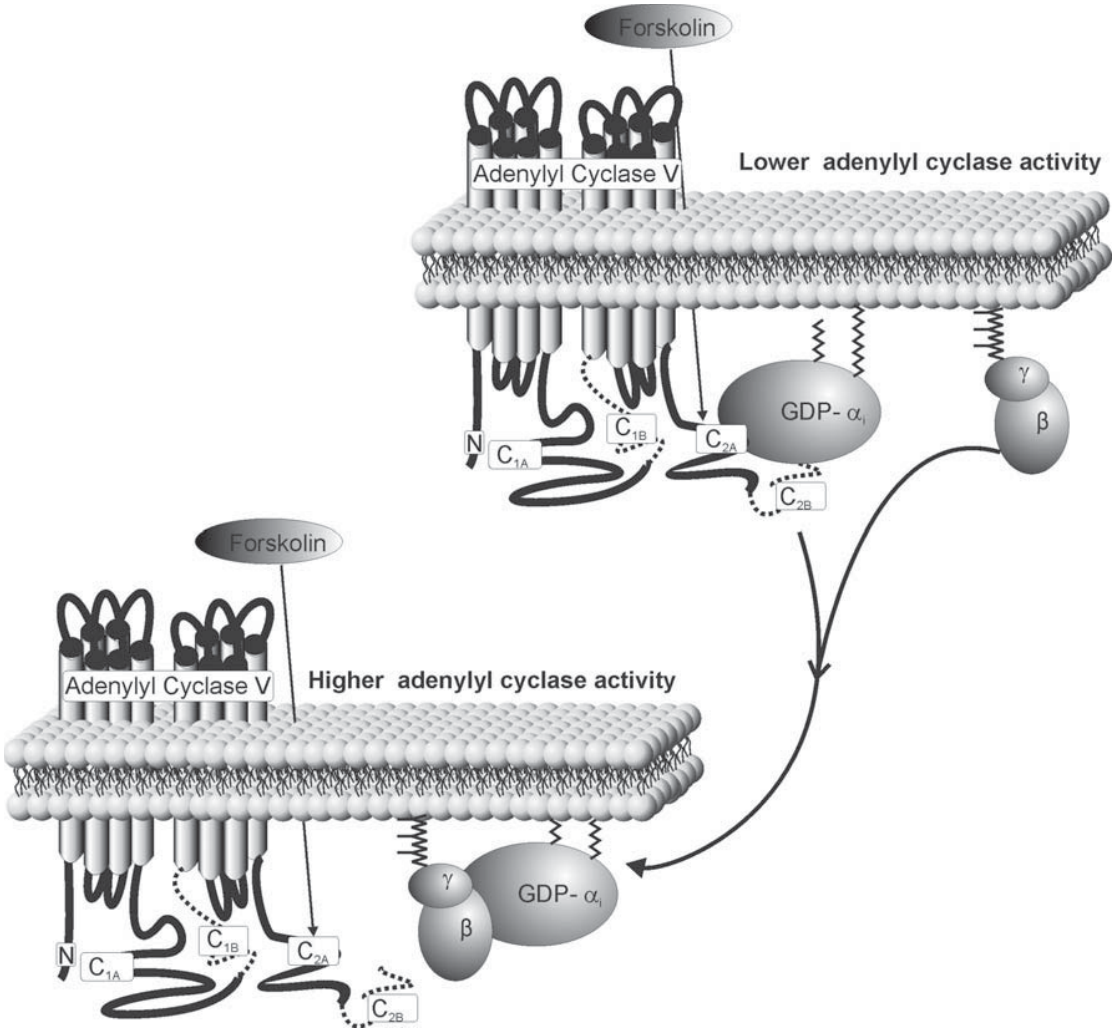


Figure 38: Effect of Gβγ on forskolin-stimulated AC V/VI

The change in adenylyl cyclase activiy is seen in Figure 37. Comparing the grey bars in Batch 3 and Batch 4. In this model AC V exist on its own. Addition of Gβγ shows increase in AC activity. But this activity is due to Gβγ scanvenging $G\alpha_i$. The stimulation of AC V/VI is seen in the basal, forskolin- as well as isoproterenol-stimulated activity.

As mentioned earlier, S49 cyc^- cells are devoid of $G\alpha_s$, whereas $G\alpha_i$-isoforms are expressed. S49 cyc^- cells represent the model depicited in Figure 38. When Gβγ is added to forskolin-stimulated AC V/VI, the AC V/VI activity increases (Figure 37 compare Batch 3 to Batch 4, white and grey bars). As mentioned earlier, $G\alpha_i$ has an inhibitory effect on AC V/VI which gets eliminated on addition of Gβγ as shown in Figure 38 and is consistent with the data in Figure 37.

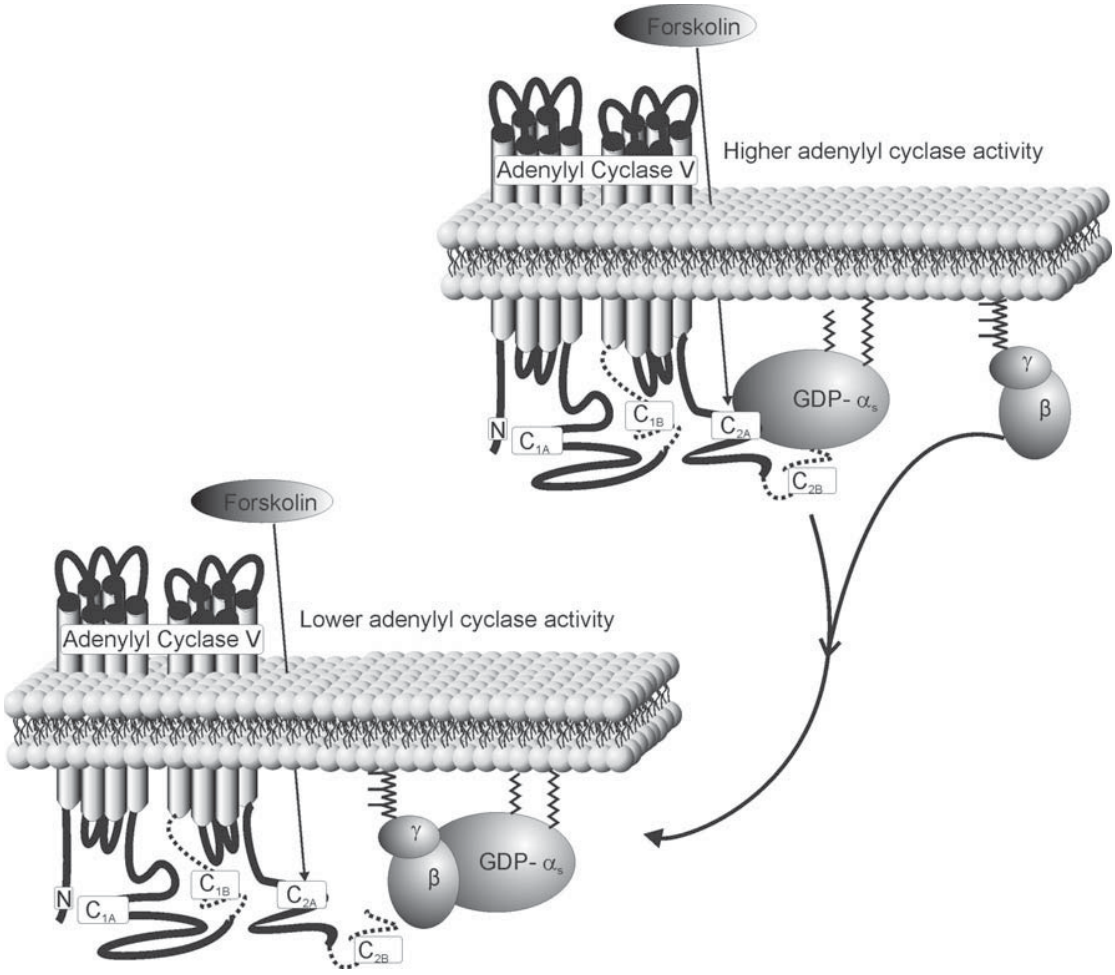


Figure 39: Effect of Gβγ on forskolin-stimulated AC V/VI-GDP-bound-$G\alpha_s$

The change in adenylyl cyclase activiy is seen in Figure 37 (comparing the white and grey bars in Batch 1 and Batch 2) This model suggests that AC V forms a complex with GDP-bound-$G\alpha_s$. Addition of Gβγ to AC V/VI-GDP-bound-$G\alpha_s$ triggers the formation of GDP-bound-$G_s\beta\gamma$ heterotrimer, thus reducing the AC acticvity of the complex to activity of AC V solely. The basal and forskolin stimulation activity shows the decrease in AC activity before GDP-bound-$G\alpha_s$ is scanvenged by Gβγ and after GDP-bound-$G\alpha_s$ is scanvenged by Gβγ

It is known that S49 cells express $G\alpha_s$. Addition of Gβγ to the AC V/VI-GDP-bound-$G\alpha_s$ complex results in in a decrease in AC V/VI activity. The decrease in AC V/VI activity is triggered by the binding of Gβγ to GDP-bound-α_s. Due to Gβγ having a stronger affinity to the GDP-bound-α_s from the AC V/VI-GDP-bound-$G\alpha_s$ complex, it triggers the formation of a G_s-trimer (GDP-bound-$G\alpha_s\beta\gamma$). As a result the AC activity seen here is AC V/VI. The higher activity of AC V/VI-GDP-bound-$G\alpha_s$ demonstrates that GDP-bound-$G\alpha_s$ has regulatory effect on AC V/VI (compare white/grey in Batch 1(AC V/VI-GDP-bound-$G\alpha_s$) to Batch 3 (AC V/VI).

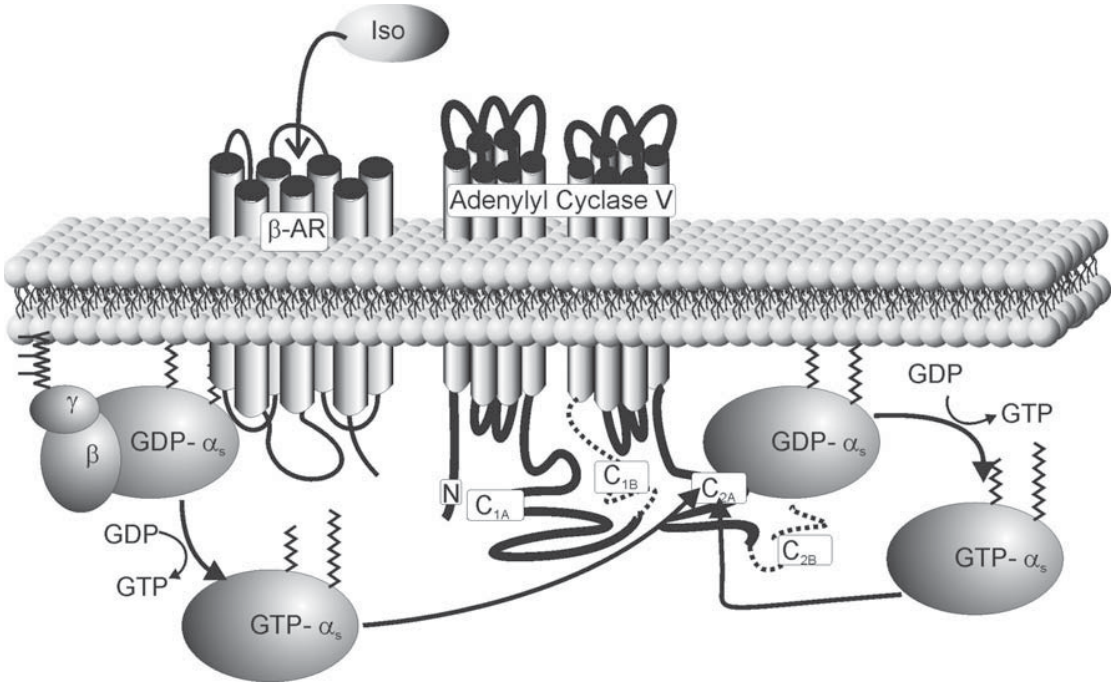


Figure 40: Effect of Gβγ on isoproterenol-stimulated AC V/VI and AC V/VI-GDP-bound-$G\alpha_s$

This model suggests that AC V forms a complex with GTP-bound-$G\alpha_s$. Addition of Gβγ to this model triggers the capturing of GDP-bound-$G\alpha_s$. But activation of β-AR by stimulation with isoproterenol triggers one or both of the two processes here. i) It exchanges the GDP to GTP on $G\alpha_s$ which is bound to the AC V and now this GTP-bound $G\alpha_s$ on the AC V triggers the higher activation of AC V. ii) The exchange of GDP to GTP on $G\alpha_s$ which is bound to the receptor. This GTP-bound $G\alpha_s$ (from the receptor) has a stronger affinity to AC V and replaces the GDP-bound-$G\alpha_s$ on the AC V and stimulates AC V.

Both AC V/VI as well as AC V/VI-GDP-bound $G\alpha_s$ have a stronger affinity to GTP-bound-$G\alpha_s$. In the case of AC V, upon stimulation with isoproterenol, $G\alpha_s$ bound to the receptor is in its GTP state, therefore binds to AC V (AC V-GTP-bound-α_s). In the case of AC V/VI-GDP-bound-$G\alpha_s$ complex, there are two possibilities of how AC V/VI-GDP-bound $G\alpha_s$ changes to AC V/VI-GTP-bound $G\alpha_s$. I) GDP exchange to GTP on $G\alpha_s$ takes place at the receptor and then dissociates and stimulates AC V/VI-GDP-bound-$G\alpha_s$ where GTP-bound-$G\alpha_s$ replaces GDP-bound-$G\alpha_s$ on AC V/VI-GDP-bound-$G\alpha_s$ complex. II) The exchange of GDP to GTP takes place on the GDP-bound-$G\alpha_s$ bound to AC V/VI-GDP-bound-$G\alpha_s$ complex. On addition of Gβγ to both systems, the capturing of bound GTP-$G\alpha_s$ does not take place, since the interaction of Gβγ to GTP-$G\alpha_s$ is weak. Therefore no change in adenylyl cyclase activity is seen (Figure 37, black bars in batch 1 and batch 2).

It can be concluded that this inhibition is due to dissociation of the AC V/VI-GDP-bound-$G\alpha_s$ complex triggered by the presence of Gβγ. Gβγ sequesters GDP-bound-$G\alpha_s$ from the AC V/VI-GDP-bound-$G\alpha_s$ complex and does not bind directly to AC V/VI from AC V/VI-

GDP-bound-$G\alpha_s$ complex.

The next step should be to investigate what triggers the formation of AC V/VI-GDP-bound-$G\alpha_s$ complex in myocardial tissues. As AC V/VI-GDP-bound-$G\alpha_s$ complex is not found when isolated from brain, is this inhibition of $G\beta\gamma$ seen on AC V isolated from brain?

8 SUMMARY

cAMP is a second messenger in the intracellular signalling pathways initiated by hormones, neurotransmitters, odorants, and chemokines. By activating PKA and cyclic nucleotide-gated ion-channels, this second messenger can change cellular attributes as diverse as the membrane potential and the rate of cell division. The key step in regulating intracellular cAMP levels is the modulation of adenylyl cyclase activity. Adenylyl cyclase, the enzyme that synthesizes cAMP, is subject to coincident regulation by both extracellular and intracellular stimuli.

It is known that the regulation of adenylyl cyclase isoforms is diverse and depends on the upstream regulatory G-proteins. The same G-protein may stimulate certain adenylyl cyclase isoforms, inhibit other isoforms and have no effect on the remaining isoforms. For example, Gβγ inhibits AC I, while it stimulates AC II, AC IV and AC VII in the presence of $G\alpha_s$. Gβγ has inhibitory effect on AC III and AC VIII. This research has shown that similar to AC II, Gβγ conditionally inhibits AC V and AC VI, whereas Gβγ conditionally stimulates AC II only in the presence of $G\alpha_s$. Specifically, $G\alpha_s$ forms a complex with AC V in its non-activated state i.e: GDP-bound state. Due to the complex formation of GDP-bound-$G\alpha_s$ with AC V, the adenylyl cyclase activity is increased. Adding Gβγ to this complex triggers the formation of the trimer *i.e.* $G\alpha_s\beta\gamma$, hence leading to a decrease in adenylyl cyclase activity. This implies that different "signalling units" of one and the same adenylyl cyclase may coexist in cells. Upon extraction of adenylyl cyclase from myocardial tissues, AC V was found in complex with GDP-bound $G\alpha_s$. In contrast, this AC complex was not found when ACs were extracted from brain tissues. The Gβγ inhibition is due to the dissociation of the AC V/VI-GDP-bound-$G\alpha_s$ complex triggered by the affinity of Gβγ to GDP-bound-$G\alpha_s$, and not due to direct binding of Gβγ to the AC V in the AC V/VI-GDP-bound-$G\alpha_s$ complex.

Adenylyl cyclase V is the dominant isoform in the adult heart, and it thereby plays a key role in determining the cardiac response to a variety of stimuli. For example, one of the initial effects of increased cAMP concentrations by stimulation with a β-adrenergic receptor agonist is an increase in Ca^{2+} influx. Ca^{2+} desensitizes the adenylyl cyclase V and VI, thus preventing their stimulation by β-adrenergic receptors. In this research, triggering the release of Gβγ induce an inhibition of AC V due to the dissociation of GDP-bound-$G\alpha_s$ from AC V/VI-GDP-bound-$G\alpha_s$ complex. Hence, such an inhibitory pathway could be important in heart tissue.

9 ZUSAMMENFASSUNG

Zyklisches Adenosinmonophosphat (cAMP) ist ein wichtiger sekundärer Botenstoff in einer Vielzahl von Signalkaskaden, die durch Hormone, Neurotransmitter, Odoratien oder Chemokine initiiert werden. Ein Schlüsselschritt in der Regulation der intrazellulären cAMP-Konzentration besteht in der Regulation der Adenylyl-Zyklase (AC)-Aktivität. AC, also das Enzym, das cAMP aus ATP generiert, kommt in verschiedenen Isoformen vor, deren jeweilige Regulation jedoch immer noch unzureichend verstanden ist. Bekannt ist, dass die verschiedenen AC-Isoformen von α- und βγ-Untereinheiten heterotrimerer G-Proteine unterschiedlich moduliert werden können. Ein und dasselbe G-Protein kann bestimmte AC-Isoformen stimulieren, während es andere AC-Isoformen inhibiert oder nicht in ihrer Aktivität beeinflusst. So wirkt Gβγ inhibitorisch auf AC I, -III und –VIII. In Gegenwart von $G\alpha_s$ kann Gβγ darüber hinaus AC II, -IV und -VII stimulieren. Zum Zeitpunkt der Anfertigung dieser Dissertation war nichts über die Regulation der AC V und VI in Zellen bekannt. AC V und -VI ist die im adulten Herzgewebe dominant exprimierte AC-Isoform. Interessanterweise haben frühe Ergebnisse aus unserer Arbeitsgruppe gezeigt, dass die aus dem Myokard von Kaninchen extrahierte, endogene AC V in einem Komplex mit $G\alpha_s$-GDP vorliegt, wobei die Bedeutung dieses Komplexes allerdings unklar blieb.

Die vorliegende Arbeit demonstriert erstmals, dass $G\alpha_s$ in seinem nicht-aktivierten, GDP-gebundenen Zustand einen Komplex mit AC V und -VI bildet, wodurch die AC-Aktivität erhöht wird. Interessanterweise kann Gβγ jedoch sowohl AC V als auch AC VI in Gegenwart von $G\alpha_s$ inhibieren. Unsere Daten zeigen, dass der inhibitorische Effekt von Gβγ nicht auf einer direkten Bindung von Gβγ an die AC V im AC V/$G\alpha_s$-GDP Komplex beruht, sondern durch die hohe Affinität von Gβγ zu GDP-gebundenem $G\alpha_s$ bewirkt wird. Hierdurch dissoziiert der AC V/$G\alpha_s$-GDP Komplex, und die Gβγ–stimulierte AC-Aktivität nimmt wieder ab. Diese Befunde werden auch durch zelluläre Untersuchungen unterstützt, in denen wir zeigen konnten, dass die Freisetzung von Gβγ in intakten Zellen zu einer Inhibition der AC V führt. Zusammengenommen implizieren diese Ergebnisse, dass in Abhängigkeit von extrazellulären Signalen in Zellen unterschiedlich aktive „Signal-Module“ derselben AC-Isoform existieren können. Weiterhin läßt die Existenz eines myokardialen AC V/$G\alpha_s$-GDP-Komplexes auf eine mögliche, physiologische Bedeutung dieses Mechanismus zur cAMP-Konzentrationsregulation in Herzgewebe schliessen.

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11 CURRICULUM VITAE

Personal Data

Celsa Antao-Paetsch
Born on 29.12.1967 in Manama, Bahrain
Indian nationality
Indefinite german residence
Married with two children

Schools / Universities

Sep.1971– Mar.1976	Indian High School, Manama, Bahrain
Jun.1976 – Mar.1982	St Mary's of Angels Convent High School / Goa, India (**Abschluss:** Abitur mit Auszeichnung)
Jun.1982 – Mar.1984	Shri Parvathibai Chowgules College of Science Goa/India (**Abschluss:** Post Abitur / Voraussetzung für weiteres Studium in Indischen Universitäten)
Jun.1984 – May. 1988	St. Xavier's College- University of Mumbai / Mumbai, India (Aufnahme des Bachelorsstudiums) **Emphasis:** Chemistry
May.1988	Degree: Bachelor of Science in Chemistry
Oct.1988 – Aug.1989	Goa Medical College / Goa University / Goa, India (Aufnahme des Diplomstudiums) **Emphasis:** Biochemistry (Frühzeitige Beendigung wegen Aufnahme des Diplomsstudium in SF, USA)
Aug.1989 – May.1992	University of San Francisco / California, U.S.A **Emphasis:** Photo organic chemistry **Thesis:** Study of Photochemical single electron transfer of Oxa (n.1.0) bicycloalkanes. Published in October 1993 in Tetrahedron Letters
May 1992	Degree: Master of Science in Photochemistry
Aug.1992 – Dec.1992	Clemson University/ South Carolina, USA (Aufnahme des Doktorsstudiums) **Emphasis:** Environmental Chemistry Frühzeitige Beendigung wegen Eheschließung / Umzug nach Deutschland
Jan.1993 – Oct.1994	German language for foreigners at University of München (**Abschluss:** Prüfung zum Nachweis deutscher Sprachkenntnisse)
Oct.1994 – Jun.1999	Two children (now 14 und 12) and Motherhood break
Jun.1999 – present	University of Düsseldorf / Düsseldorf, Germany (Aufnahme des Doktorsstudiums) **Emphasis:** Biochemistry **Doctoral thesis:** Regulation of Gβγ on adenylyl cyclase isoforms Type V and Type VI

Work Experience

Aug. 1989 – May. 1992 Research/Teaching Assistant for Microbiology und Chemistry
University of San Francisco / San Francisco, U.S.A

Spectroscopy: Fourier transformer - infrared spectroscopy, fourier transformer nuclear magnetic resonance, ultra violet / visible spectroscopy, atomic absorption/ atomic emission.

Chromatography: Column chromatography, thin layer chromatography, gas liquid chromatography, high pressure liquid chromatography

Electrochemistry: Potentiometric titration, cyclic voltametry and differential pulse voltametry

Teaching experience: Preparing as well as teaching lab classes and grading of undergraduate chemistry students (freshmen and sophomores)

Jun 1999 – Jun 2004 Research/Teaching Assistant for Biochemistry
University of Düsseldorf / Düsseldorf, Germany

Molecular biological methods: Production and transformation of *E.coli*, amplification und purification of plasmids, analysis of nucleic acids by gel electrophoresis, transfection of various cell lines, application of antisense oligonucleotides in adherent cells

Protein biochemical methods: Polyacrylamide gel electrophoresis for the separation of proteins, electrotransfer of proteins onto the PVDF membranes, immunodetection of proteins on PVDF membranes, generation of [^{32}P]-cAMP, [^{3}H]-Adenine assays of enzymes in vivo as well as in vitro, cultivation of various cells lines, generation of cell lines stably expressing the required protein

Teaching experience: Preparing and supervising lab classes for medical and dental students (2. und 4. Semester)

Jun. 2004 – Jun 2006 Consultant - Smart Minds Consulting / Pune, India
Jun.2006 – present Senior Consultant - Smart Minds Consulting / Pune, India

Turn key services for U.S and European companies interested in setting up their operations in India: Organization and supervision of tasks like premarket entry research, clarification of comprehensive legal issues, complete local registration processes, negotiations of lease and property contracts as well as consulate administration required in Germany. Furthermore I was responsible for the local operations which includes company formation, setting up bank accounts, organizing chartered accountants, manufacturing setup, office search, interior designing for the offices as well as factories, executive and staff recruitment, interim management for U.S and European companies in India thereby interfacing directly with the top management of our various customers

Computers Knowledge Excel, Power point, Windows 2007 & Vista, Corel Draw

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13 DECLARATION

Die hier vorgelegte Dissertation habe ich eigenständig und ohne unerlaubte Hilfe angefertigt. Die Dissertation wurde in der vorgelegten oder in ähnlicher Form noch bei keiner anderen Institution eingereicht. Ich habe bisher keine erfolglosen Promotionsversuche unternommen.

Düsseldorf, den 01.09.2008

(Celsa Antao-Paetsch)