Realia et naturalia

DISSERTATIONES
CHIMICAE
UNIVERSITATIS
TARTUENSIS
144

OLGA MAZINA

Development and application of the biosensor assay for measurements of cyclic adenosine monophosphate in studies of G protein-coupled receptor signaling

TARTU ÜLIKOOL · UNIVERSITAS TARTUENSIS · 1632

DISSERTATIONES CHIMICAE UNIVERSITATIS TARTUENSIS
144

DISSERTATIONES CHIMICAE UNIVERSITATIS TARTUENSIS
144

OLGA MAZINA

Development and application of the biosensor assay for measurements of cyclic adenosine monophosphate in studies of G protein-coupled receptor signaling

UNIVERSITY OF TARTU
PRESS
1632

Institute of Chemistry, Faculty of Science and Technology, University of Tartu, Estonia.

Dissertation is accepted for the commencement of the Degree *of Doctor philosophiae* in Chemistry on June 18, 2015 by the Doctoral Committee of the Institute of Chemistry, University of Tartu.

Supervisors: Professor Ago Rinken
Institute of Chemistry, University of Tartu, Estonia

Dr. Sergei Kopanchuk
Institute of Chemistry, University of Tartu, Estonia

Opponent: Professor Alexander P. Savitsky
Russian Academy of Sciences,
A.N. Bach Institute of Biochemistry

Commencement: August 24, 2015 at 10:00, Ravila 14a, room 1021

This work was funded by the Estonian Science Foundation (7569, 8011, 8314) by the Estonian Ministry of Education and Science (SF0180032s12, IUT 20-17) and by the European Union through the European Regional Development Fund (Centre Excellence "Mesosystems: Theory and Applications", TK114 and Competence Centre on Health Technology, EU30020).

This work has been supported by Graduate School "Functional materials and technologies" receiving funding from the European Social Fund under project 1.2.0401.09-0079 in University of Tartu, Estonia.

ISSN 1406-0299
ISBN 978-9949-32-877-2 (print)
ISBN 978-9949-32-878-9 (pdf)

University of Tartu Press
www.tyk.ee

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

The current thesis is based on the following original publications, referred to in the text by corresponding Roman numerals:

I Mazina, O., Reinart-Okugbeni, R., Kopanchuk, S., & Rinken, A. (2012). BacMam system for FRET-based cAMP sensor expression in studies of melanocortin MC1 receptor activation. *Journal of Biomolecular Screening*, *17*(8), 1096–1101.

II Mazina, O., Tõntson, L., Veiksina, S., Kopanchuk, S., & Rinken, A. (2013). Application of Baculovirus Technology for Studies of G Protein-Coupled Receptor Signaling. In *Nanomaterials Imaging Techniques, Surface Studies, and Applications* (pp. 339–348). Springer New York.

III Mazina, O., Allikalt, A., Heinloo, A., Reinart-Okugbeni, R., Kopanchuk, S., & Rinken, A. (2015). cAMP Assay for GPCR Ligand Characterization: Application of BacMam Expression System. In *G Protein-Coupled Receptor Screening Assays* (pp. 65–77). Springer New York.

IV Mazina, O., Luik, T., Kopanchuk, S., Salumets, A., & Rinken, A. (2015). Characterization of the Biological Activities of Human Luteinizing Hormone and Chorionic Gonadotropin by a Förster Resonance Energy Transfer-based Biosensor Assay. Accepted for publication in *Analytical Letters.*
DOI:10.1080/00032719.2015.1052140

Author's contribution:

I The author was the principal investigator responsible for the paper – planning and performing of experiments as well as writing the manuscript.

II The author performed the cAMP detection experiments and wrote the corresponding part of the manuscript.

III The author was the principal investigator responsible for planning and writing the manuscript.

IV The author was the principal investigator responsible for the paper – planning and performing of most of the experiments as well as writing the manuscript.

ABBREVIATIONS

AC	adenylate cyclase
AcMNPV	*Autographa californica* multiple nucleopolyhedrovirus
AMP	adenosine-5'-monophosphate
ATP	adenosine-5'-triphosphate
BacMam	a recombinant baculovirus for delivering genes of interest in to mammalian cells
BP	base pair
BRET	bioluminescence resonance energy transfer
B16F10	mouse melanoma cell line B16F10
cAMP	3'-5'-cyclic adenosine monophosphate
CFP	cyan fluorescent protein
cGMP	3'-5'-cyclic guanosine monophosphate
COS-7	African green monkey kidney fibroblast-like cell line COS-7
CREB	cAMP-response-element-binding protein
DMEM	Dulbecco's Modified Eagle's Medium
DNA	deoxyribonucleic acid
DPBS	Dulbecco's Phosphate-Buffered Saline
EC_{50}	The molar concentration of an agonist that produces 50% of the maximal possible effect of that agonist.
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
Epac	exchange protein directly activated by cAMP
FLIM	fluorescence lifetime imaging
FP	fluorescence polarization
FRET	fluorescence (Förster) resonance energy transfer
FSH	follicle-stimulating hormone
GEF	guanine nucleotide exchange factor
GDP	guanosine-5'-diphosphate
GTP	guanosine-5'-triphosphate
GPCR	G protein-coupled receptor
hCG	human chorionic gonadotropin
HEK293	human embryonic kidney cell line HEK293
hLH	human luteinizing hormone
IBMX	3-isobutyl-1-methylxanthine
IC_{50}	The molar concentration of an antagonist that reduces the response to an agonist by 50%.
IP_3	inositol-1,4,5-triphosphate
JEG-3	human choriocarcinoma cell line JEG-3
MCR	melanocortin receptor
MEM	Minimum Essential Medium
MSH	melanocyte-stimulating hormone
PDE	phosphodiesterase

PKA	protein kinase A
PLC	phospholipase C
RET	resonance energy transfer
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
Sf9	*Spodoptera frugiperda* cell line Sf9
YFP	yellow fluorescent protein

INTRODUCTION

Pharmacologically active compounds or drugs are inseparable from today's society. The world's population growing and ageing and the total number of patients suffering from some sort of disease is constantly rising. Illnesses are caused or result in dysregulation of cellular metabolism and signaling. G protein-coupled receptors (GPCRs) form a large family of proteins that regulate biosignaling between the cells of the human organism. Modulation of GPCRs by administration of specific compounds is one of the possibilities to cure or alleviate many diseases and more than one third of all prescription drugs are targeting GPCRs.

However, new synthetic compounds need to be carefully designed, selected, tested and compared with other compounds of known pharmacological profile. Such characterization needs to be performed both *in vitro* and on living cells before entering expensive animal testing.

There are close to 800 different GPCRs in the human genome. One of the common pathways for GPCR signal transduction across the cell membrane is modulation of adenyalte cyclase resulting in the production of cyclic adenosine monophosphate (cAMP). cAMP is a cellular secondary messenger molecule that in turn can activate many downstream signaling proteins. Monitoring changes in cAMP concentration is one of the direct approaches for detection of GPCR activation. Genetically encoded biosensor proteins have proven as suitable tools for this task. Such biosensors are applicable in living cells for cAMP detection in real time.

For characterization and functional screening of GPCR ligands cAMP biosensor proteins must be present in the cells expressing the receptor of interest. Reliable expression of the biosensor protein enables setting up a robust assay for cAMP detection for different GPCR in various cellular systems. Moreover, monitoring of cAMP levels in living cells is important for deeper studies of GPCR signaling mechanisms. To achieve reliable biosensor protein expression for the cellular receptor activation assays we implemented a baculovirus based BacMam expression system.

After successful generation and optimization of the BacMam based assay system, we proceeded with the validation of the assay system using different GPCRs expressed in different cell lines by characterizing their activation with a set of known ligands. We were able to distinguish between full and partial agonists (activators) and were able to monitor both, the increase and the decrease in cAMP concentrations in response to receptor activation. The developed biosensor assay was thereafter adapted for detection and quantification of gonadotropins from various preparations.

1. G PROTEIN-COUPLED RECEPTORS

With approximately 800 members, GPCRs form the largest family of cell surface receptors. Genes encoding GPCRs account for approximately 2% of the human genome (Fredirksson et al., 2003). These proteins are characterized by a seven-transmembrane α-helical structure that transduces extracellular signals into intracellular effector pathways via heterotrimeric G-protein activation (Pierse et al., 2002; Rosenbaum et al., 2009; Heng et al., 2013). GPCRs are known to modulate a wide variety of different cellular responses. They respond to endogenous neurotransmitters and hormones as well as to exogenous ligands and stimuli, eg. therapeutic drugs, photons, tastants, and odorants. More generally, GPCRs play a critical role in cell growth, differentiation, migration, and death.

Disturbances in GPCR signaling can result in metabolic, immunological, and neurodegenerative disorders, but also in cancer and infectious diseases. About 40% of today's prescription drugs mediate their effects by modulating GPCR signaling pathways (Heng et al., 2013; Garland 2013). Representing a large number of therapeutic targets currently in use or in development, the family of G protein-coupled receptors is of outmost importance for drug screening and discovery.

GPCRs are commonly divided into five families based on their sequence and structural similarity: rhodopsin (family A), secretin (family B), glutamate (family C), adhesion and Frizzled/Taste2, with the rhodopsin family being the largest and most diverse of these families (Fredriksson et al., 2003; Rosenbaum et al., 2009). The ligands for the GPCRs are of wide structural variation; ions, organic odorants, amines, peptides, proteins, lipids, nucleotides, and even photons are able to mediate their message through these proteins. Based on their effect on the receptor function, ligands can be classified as full, partial and inverse agonists and antagonists. Agonists exert a positive signaling response, while inverse agonists reduce the level of basal or constitutive activity below that of the unliganded receptor. Antagonists compete and inhibit the binding of agonists but do not produce a cellular response (Rosenbaum et al., 2003).

After agonist binding, a conformational rearrangement of the receptor and the subsequent activation of the heterotrimeric G proteins is initiated (Rosenbaum et al., 2003). These are gunanine nucleotide binding proteins (hence the name, G proteins) composed of α-, β-, and γ-subunits. The conformational change of the agonist-bound GPCRs catalyzes the exchange of guanidine diphosphate (GDP) for guanidine triphosphate (GTP) on the α-subunit (Gα) of the G proteins, thereby dissociating Gα the dimeric β-, and γ-subunits (Gβγ). The functions of G proteins are regulated cyclically by association of GTP with Gα, hydrolysis of GTP to GDP and P_i, and dissociation of GDP. Binding of GTP is closely linked with "activation" of the G protein and consequent regulation of the activity of the appropriate effector. Hydrolysis of GTP initiates deactivation (Gilman, 1987).

In 1994 the Nobel Prize in physiology or medicine was awarded to Alfred G. Gilman and Martin Rodbell for their discovery of G-proteins and the role of these proteins in signal transduction in cells. In 2012 the Nobel Prize in chemistry was awarded to Robert J. Lefkowitz and Brian K. Kobilka for their contribution to the studies of GPCRs. This illustrates the fundamental importance of studies on GPCR signaling. Although many aspects of cellular signal transmission have been thoroughly investigated and characterized, much information remains elusive. The need for sensitive methods allowing investigation of the GPCR mediated signaling processes is driving today's scientists to develop novel analytical approaches or to implement well known techniques in an innovative way resulting in new data and information.

GPCRs vary in structure and function but also in the structure and size of their natural ligands. The widest expression of GPCRs in the brain accounts for receptors binding the low molecular weight neurotransmitters (Brady et al., 2011). The biogenic amines such as dopamine, epinephrine, norepinephrine, histamine, serotonin and glutamate regulate vital physiological and cognate functions via their specific GPCRs. As an example, dopamine receptor targeted compounds are significant tools for the management of several neuropsychiatric disorders including schizophrenia, bipolar disorder, depression and Parkinson's disease (Carlsson, 2001; Beaulieu and Gainetdinov, 2011; Beaulieu et al., 2015). There are five receptors for dopamine differing in their expression in the organism and the ability to modulate downstream signaling. The most widespread of the dopamine receptors is the D1 subtype. Followed by the D2 and to a lesser extent by the D3 receptors that are mainly expressed in limbic areas of the brain. Compared to the first three subtypes of dopamine receptors, D4 and D5 subtypes exhibit only modest expression patterns in the brain (Beaulieu and Gainetdinov, 2011).

Also widely expressed in the brain and the periphery are peptide receptors. Endogenous peptides like melanocortins, opioid peptides, neuropeptide Y, galanin and vasopressin regulate pigmentation, food intake, sexual arousal, sleep cycle, blood pressure and water retention. Consistent with the variety of physiological roles of peptide receptors, the range of conditions in which their regulation is distorted includes cardiovascular, endocrine and metabolic disorders. The melanocortin system is composed of five melanocortin receptors (MCRs) modulated by the agonists adrenocorticotropic hormone and α, β and γ melanocyte-stimulating hormone (MSH), and two naturally occurring antagonists, agouti and agouti-related protein. MCRs are one of the smallest GPCRs composed of about 300 amino acid residues (Wikberg et al., 2000; Rodrigues et al., 2014). Most widely expressed is the MC1 receptor subtype. It is found in the skin and in the brain, in immune cells, adipocytes, pituitary, corpus luteum and in placenta. It is primarily responsible for the regulation of skin and hair pigmentation, but is also involved in anti-inflammatory actions and regulation of pain signaling (Rodrigues et al., 2014). The ligands for the MCR can be endogenous peptides (α, β and γ-MSH), synthetic peptides (NDP-α-MSH, MS-05) or cyclic synthetic peptides (SHU-9119, HS-024) and low molecular weight

non-peptide ligands (THIQ) (Sawyer et al., 1980; Hruby et al., 1995; Kask et al., 1998; Szardenings et al., 2000; Mutulis et al., 2004).

A group of GPCRs binding larger peptides and proteins are the hormone receptors. Their ligands (up to 40 kDa) can be comprised of several subunits that can vary in the level of glycosylation depending on the tissue and the metabolism cycles. Hormone receptors are mostly responsible for regulating hormone production and secretion, growth and endocrine functions. Examples of the latter are gonadotropins: follicle-stimulating hormone (FSH), luteinizing hormone (hLH), chorionic gonadotropin (hCG). hLH and hCG act via a shared GPCR (LH receptor) that regulates diverse reproductive mechanisms that are essential for ovulation, early pregnancy and placental function in females as well as spermatogenesis and testosterone production in males (Choi and Smitz, 2014). LH receptor is a glycoprotein consisting of 675 amino acid residues. The mature LH receptor has the molecular weight of 85–95 kDa depending on the level of glycosylation (Ascoli et al., 2002). Gonadotropins FSH, LH and hCG are heterodimers that consist of non-covalently linked α- and β-subunits. Their α-subunits of these hormones are identical, but the β-subunits differ to the extent that FSH shows no comparable binding affinity or biological activity on the LH receptor (Ascoli et al., 2002).

1.1. SECONDARY SIGNALING PATHWAYS OF GPCR ACTIVATION

Based on the amino acid sequence homologies of their Gα subunit, heterotrimeric G proteins are classified into four families: $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$, and $G\alpha_{12/13}$ (Gilman, 1987). Upon activation of GPCRs, each Gα subunit stimulates and integrates a distinct signaling pathway. $G\alpha_s$ subunits stimulate the effector enzyme adenylate cyclase (AC) to catalyze the production of cAMP from ATP. This initiates different cAMP-dependent cellular pathways leading to the activation of downstream signaling proteins. In contrast, $G\alpha_i$ subunits inhibit the production of cAMP. $G\alpha_{q/11}$ subunits activate phospholipase C (PLC), an enzyme that cleaves phosphatidylinositol 4,5-biphosphate into second messengers inositol-1,4,5-triphosphate (IP_3) and diacylglycerol. IP_3 activates the release of Ca^{2+} stores from endoplasmatic reticulum and diacylglycerol activates protein kinase C, promoting further signaling cascades. $G\alpha_{12/13}$ subunits act through RhoGEF (guanine nucleotide exchange factors) to activate Rho GTPase family members and regulate actin cytoskeleton remodeling and cell migration. In addition, free Gβγ dimers also activate effector molecules such as ion channels and PLC, thus inducing independent signaling pathways (Rosenbaum et al., 2003; Lin, 2013).

Despite extensive studies, a comprehensive characterization of the G protein-coupling profile of GPCRs is complicated: for example, some GPCRs can couple only to a single type of G protein, whereas many GPCRs couple to a

broader range of G protein families depending on the tissue type and the developmental stage of the organism (Miyano et al., 2014).

Aside from the activation of the G proteins, GPCR signaling is also modulated by G-protein coupled receptor kinases and β-arrestins (Gurevich et al., 2012; Reiter and Lefkowitz, 2006). The modulation of GPCR signaling by these proteins can be divided into three distinct actions: (a) silencing: the functional uncoupling of the receptor from its cognate G protein by a mechanism known as "homologous desensitization"; (b) trafficking: receptor internalization, "resensitization" and/or degradation; and (c) signaling: the activation or inhibition of intracellular signaling pathways independently of heterotrimeric G proteins (Reiter and Lefkowitz, 2006). It has been demonstrated, that agonists can display functional selectivity where activated receptors are biased to either G protein- or arrestin-mediated downstream signaling pathways (Seifert, 2013).

1.2. cAMP SIGNALING PATHWAY

cAMP was first described by Rall et al. (1957), who showed that sympathomimetic amines and glucagon were able to induce the synthesis of a heat-stable factor, cAMP, formed by particulate fractions of liver homogenates in the presence of ATP and Mg^{2+} (Beavo and Brunton, 2002; Godinho et al., 2015). In the same year Earl Sutherland described this molecule as an intracellular "second" messenger that is activated in response to epinephrine (the "first" messenger), that cannot pass through the cell membrane (Sutherland, 1972). Sutherland was awarded the Nobel Prize in Physiology or Medicine in 1971 for his discoveries concerning the mechanisms of the action of hormones (Beavo and Brunton, 2002).

cAMP generation and degradation is regulated by the adenylate cyclase (AC) and phosphodiesterase (PDE) families of enzymes, respectively (Tasken and Aandahl, 2004; Bender and Beavo, 2006). These enzymes are differentially expressed and regulated in different tissues. Both, AC and PDE enzymes are responsible for modulation of physiological levels of the second messenger.

Adenylate cyclases are ATP-pyrophosphate lyases that convert ATP to cAMP and pyrophosphate. There are nine mammalian transmembrane AC isoforms (AC1-AC9), and one soluble isoform that has distinct catalytic and regulatory properties resembling the cyanobacterial enzymes. All isoforms of transmembrane ACs are stimulated by the GTP-bound $G\alpha_s$. GTP-bound $G\alpha_i$ protein inhibits AC1, AC5, and AC6 resulting in reduced intracellular cAMP content (Sadana and Dessauer, 2009).

Cyclic nucleotide PDEs are enzymes that regulate the cellular levels of the second messengers, cAMP and cGMP, by catalyzing the hydrolysis of the 3' cyclic phosphate bonds resulting in non-signaling AMP and GMP. There are 11 different PDE families, with each family typically having several different isoforms and splice variants. PDEs differ in their structure, kinetic properties, modes of regulation, intracellular localization and cellular expression (Bender

and Beavo, 2006). PDEs also differ in their substrate specificity: PDE4, PDE7, and PDE8 are specific for cAMP, PDE5–PDE6, and PDE9 are specific for cGMP and PDE1–PDE3 and PDE10–PDE11 hydrolyze both cAMP and cGMP (Bender and Beavo, 2006; Godinho et al., 2015).

cAMP is mostly produced in close proximity to the plasma membrane. However, its degradation by specific PDEs underpins compartmentalized cAMP signaling in cells and the activation threshold is thus regulated for downstream effectors in spatially defined intracellular complexes (Houslay, 2010). The so-called cAMP microdomains were first visualized in rat cardiomyocytes (Zaccolo and Pozzan, 2002)

The first identified downstream target of cAMP was protein kinase A (PKA) (Walsh et al., 1968). It was shown that PKA is a heterotetramer composed of two regulatory and two catalytic subunits (Corbin and Krebs, 1969). Upon cooperative binding of four cAMP molecules to the regulatory subunits of PKA heterotetramer, the catalytic subunits dissociate from the regulatory dimer and are then able to phosphorylate downstream targets. PKA activity results in regulation of target enzymes and transcription factors (Tasken and Aandahl, 2004; Godinho et al., 2015). PKA has served as a prototype for understanding protein kinases and the role of phosphorylation as means of modifying the activity of proteins (Kandel, 2012). For discoveries concerning the reversible protein phosphorylation as a biological regulatory mechanism Edmond H. Fischer and Edwin G. Krebs were awarded the Nobel Prize in Physiology or Medicine in 1992 (Beavo and Brunton, 2002).

Intracellular cAMP is also able to directly modulate ion channels (Fesenko et al., 1985; Bradley et al., 2005), and specific guanine nucleotide exchange factors (GEFs). These cAMP responsive GEFs were named exchange proteins directly activated by cAMP (Epac) (De Rooij et al., 1998). Epac proteins were discovered as mediators of cAMP-induced signaling pathways, which were not regulated by either PKA or cyclic-nucleotide-gated channels, the only previously known cAMP-target proteins (De Rooij et al., 1998, Gloerich and Bos, 2010). There are two isoforms of cAMP specific GEFs, Epac1 and Epac2, differing in the amount of the binding domains for cAMP, one and two, respectively (De Rooij et al., 1998 and 2000). The N-terminal cyclic nucleotide binding domain of Epac2 binds cAMP with relatively low affinity (~ 90 μM) compared to the cAMP binding domain (~ 1.2 μM) proximal to the catalytic domain, which is present in both Epac1 and Epac2 (De Rooij et al., 2000). The second cAMP binding domain in Epac2 has been speculated to only serve a modulatory role (De Rooij et al., 2000; Gloerich and Bos, 2010). Epacs are present in most tissues, albeit with different expression levels. Epac1 is highly abundant in blood vessels, kidney, adipose tissue, central nervous system, ovary, and uterus, whereas Epac2 is mostly expressed in the central nervous system, adrenal gland, and pancreas (Gloerich and Bos, 2010).

In the late 1960s, long-term effects and transcriptional regulation of cAMP effectors started to emerge as areas of investigation. Systems in which elevated cAMP and the activation of PKA seemed to be coupled to the induction of

specific proteins were reported (Wicks et al., 1969). The first transcription factor found to be regulated by PKA was the cAMP-response-element-binding protein (CREB). It was also the very first transcription factor whose activity was shown to be regulated by phosphorylation (Mayr and Montminy, 2001). The free catalytic subunits of PKA can diffuse into the nucleus and induce cellular gene expression by phosphorylating CREB at serine residue 133 (Gonzalez and Montminy, 1989). CREB mediates the activation of cAMP-responsive genes by binding as a dimer to a conserved cAMP-responsive element. Transcription of cellular genes usually peaks after 30 minutes of stimulation with cAMP, coinciding with the time required for the catalytic subunit levels of PKA to become maximal in the nucleus (Mayr and Montminy, 2001).

As discussed above, ligands of different GPCRs represent a variety of molecules of very different structure and size (low molecular weight molecules, e.g. dopamine, oligopeptides, e.g. melanocortins, large hormone proteins, e.g. gonadotropins). The cellular responses may be very different for these ligands and their receptors, but if coupled to the $G\alpha_s$ system, elevation of cellular cAMP level is the common first step in the signaling cascades. One of the central tasks of this study was setting up a system for characterization of GPCR signaling upon receptor activation of ligands with various structures. GPCRs investigated in this study (dopamine, melanocortin and gonadotropin receptors) are all coupled either to the $G\alpha_s$ or to $G\alpha_i$ proteins, stimulating or inhibiting cellular cAMP synthesis respectively. For real-time detection of receptor activation in living cells, an assay for measurement of cAMP levels was implemented.

2. METHODS FOR cAMP DETECTION

The need of assaying cAMP became an issue almost immediately after discovering of the new second messenger molecule in 1957. Sutherland's original assay, which was the only one available for more than ten years, was based on the ability of cAMP to activate glycogen breakdown *in vitro*. The assay depended on the purification of several enzymes and varied with the characteristics of the dog-liver supernatant fraction that contained the necessary protein kinases for cAMP binding (Cook et al., 1957; Beavo and Brunton, 2002). Measuring of the cellular response to the extracellular agents provided a new level of scientific analysis and compound characterization. Research focused on development of new, faster, reliable and more sensitive assays carried on for half a century and is still very timely today.

2.1. RADIOACTIVELY LABLED cAMP ACCUMULATION ASSAYS

A considerable progress in cAMP detection was achieved more than a decade after the first measurements of cAMP, when a protein-binding assay (Gilman, 1970) made the cellular *in vitro* studies more accessible. Gilman's procedure was easy and cheap, and could detect 0.1 pmoles of cAMP, which is sensitive enough for most purposes, because basal levels of cAMP are ~ 5 pmoles/mg of cell protein (Beavo and Brunton, 2002). The assay used a commercially available ligand, [^{3}H]cAMP and only one protein, the cAMP-sensitive protein kinase, needed to be prepared. Assay conditions were such that a binding constant approaching 1 μM was obtained (Gilman, 1970). This assay was widely used and improved during several decades (Nordstedt et al., 1990; Vonk et al., 2008).

One of the most direct approaches to monitoring cAMP generation from ATP in living cells is to follow this conversion by pre-labeling cellular adenine nucleotide pool with [^{3}H]adenine. After stimulating the cells with agonist, column chromatography is used to separate [^{3}H]cAMP from all other tritium-labelled adenine derivatives (Huang et al., 1971; Donaldson et al., 1988; Hill et al., 2010). This method provides a direct read-out of [^{3}H]cAMP accumulation, but is relatively time-consuming. Also, concern that is often raised with a radioactive readout of the accumulation of any intracellular second messenger such as cAMP, is that it is a measure of turnover rather than absolute levels (Hill et al., 2010).

2.2. IMMUNOASSAYS FOR cAMP DETECTION

Immunoassays for cAMP accumulation measurements follow a general principle, with changes in intracellular cAMP being detected by the competition between cellular cAMP and a labelled form of cAMP for binding to an anti-cAMP antibody (Brooker et al., 1978; Williams, 2004). Same principle was used in the protein-binding assay (Gilman, 1970; Nordstedt et al., 1990), where instead of a specific antibody, PKA binding and subsequent sequestration of the complex was used for cAMP quantification.

The radioimmunoassay developed by Harper and Brooker in 1975 brought the sensitivity for cyclic nucleotide measurements into the femtomole range. They improved the assay by acetylating the cyclic nucleotides at the 2' position with acetic anhydride. This was especially important for measuring cGMP, which often exists at the level of one tenth of the tissue content of cAMP (Beavo and Brunton, 2002).

Homogeneous radiometric assays, such as scintillation proximity assays (Biosciences Amersham, 1996) and Flashplate® technology (NEN Life Science, 1998) enable the direct detection of [^{125}I]cAMP captured by the antibody in close proximity to a solid scintillate surface. These methods are fast and have high reproducibility. There are clear advantages over more traditional radiometric enzyme-linked immunosorbent assays (ELISAs) in terms of convenience, because the stimulation and detection can be carried out in the same well (Hill et al., 2010). Yet, the radiometric technologies started to be superseded in the early 2000's with emerging of safer and cheaper methods of non-radiometric read-outs, which were more readily miniaturized (Williams, 2004). Most pharmaceutical company laboratories prefer to employ fluorescent or luminescent assays due to safety, cost and throughput considerations (Hill et al., 2010).

One of the first fluorescent technologies to emerge was the fluorescence polarization (FP) technology. cAMP detection is based on a decrease in the extent of molecular rotation of a fluorescently labelled cAMP that occurs following binding to the larger anti-cAMP antibody (Williams 2004). The assay detects cytoplasmic cAMP content exposed by cell lysis (Prystay et al., 2001). FP assays are relatively insensitive to low levels of cAMP, with detection limits in the range of 100 fmol (Hill et al., 2010).

During the 2000's more fluorescent and luminescent technologies emerged to overcome the issues of speed, sensitivity and compound interference that posed as shortage in FP assays. Some of these assays rely on chemiluminescent proximity (ALPHAScreen®, Perkin Elmer), electro-chemiluminescence detection (Meso Scale Discovery) and time resolved fluorescence resonance energy transfer (FRET) (HTRF®, Cis Bio & LANCE®, DELFIA®, Perkin Elmer). In assays employing HTRF technology, the anti-cAMP antibodies are labelled with europium cryptate and the cAMP is labelled with a modified allophyocyanin. When these two fluorescent molecules are in close proximity, FRET occurs and long lifetime fluorescence is emitted at two different wavelengths (Williams 2004). All these technologies rely on high quality antibodies;

vary in terms of their costs, reader compatibility and impact of compound interference (Williams, 2004; McLoughlin et al., 2007; Hill et al., 2010).

2.3. REPORTER-GENE ASSAYS

In reporter-gene assays receptor-mediated changes in intracellular cAMP concentrations are detected via changes in the expression level of a particular gene (the reporter), the transcription of which is regulated by the transcription factor cAMP response-element binding protein (CREB) binding to upstream cAMP response elements (Naylor 1999, Williams, 2004). Based upon the splicing of transcriptional control elements to a variety of reporter genes (with easily measurable phenotypes), the assay "reports" the effects of a cascade of signaling events on gene expression inside cells (Naylor, 1999). Products of various genes have been used as readout of reporter-gene assays (e.g. β-galactosidase, green fluorescent protein, luciferase and β-lactamase). Depending on the chosen reporter-gene, the readout can be a change in color (upon substrate chromophore cleavage), change in fluorescence intensity or the light produced as a byproduct of the chemical reaction. Due to additional signal amplification (the readout is resulting at the end of the cellular signaling cascade) and the fact that these assays generally require longer incubation times to allow for the gene transcription event, reporter-gene assays can provide a significant advantage over accumulation assays for the detection of weak agonists. However, attention should be paid when evaluating ligands with partial agonist activity. In most reporter gene systems these molecules are likely to manifest as full agonists because of the signal amplification (Baker et al., 2003, Hill et al., 2010).

2.4. BIOSENSORS FOR cAMP DETECTION

Most of the methods for cAMP detection discussed above rely on measuring the accumulated pool of the second messenger. Moreover, most of the methods are limited to end-point measurements in cell lysates and therefore generally contain little temporal and no spatial information. The need for real-time monitoring of the signaling events was evident. In the beginning of 2000's several laboratories set out to develop methods for monitoring GPCR activation and signaling in intact cells and ultimately also in intact organs or organisms (Lohse et al., 2007). In 2008, Osamu Shimomura, Martin Chalfie, and Roger Tsien received the Nobel Prize in Chemistry for their discovery of the green fluorescent. The reason for such success lies on the fact that soon after introducing of the potential of the genetically encoded fluorescent proteins (Heim and Tsien, 1996; Matz et al., 1999) many laboratories have utilized these to develop fluorescent biosensors. These developments have permitted the visualization of cellular processes turning fluorescent proteins into key tools of biology and biochemistry (Newman et al., 2011; Okumoto et al., 2012).

Humankind has known the use of biosensors for hundreds of years. For example, the canary in a coalmine is a sensor for detection of toxic gases as methane or carbon monoxide. However, such assay is not quantitative and the sensor may not survive the assay. Today, biosensors are engineered constructs that couple the detection of a biochemical event to an optical signal (Clister et al., 2015). Besides quantitative measurements of concentration and distribution of biomolecules (e.g. cellular second messengers), there are also other potential applications of biosensors. Discovery and modeling of metabolic pathways for systems biology as well as implementation of biosensor technology to complement other quantitative techniques of analytical chemistry (Okumoto et al., 2012). It is however always important to keep in mind that the introduction of a biosensor will at least to some extent affect the cellular environment and possibly modify cellular behavior.

The biosensor system generally consists of a sensory domain that recognizes the analyte of interest and the response domain that is responsible for yielding a detectable signal. For a sensor to reliably report the dynamics of the analyte, the affinity and dynamic range of the sensor must match the level of analyte and the kinetics of association and dissociation need to be fast enough to capture transient accumulations (Okumoto et al., 2012).

2.4.1. RESONANCE ENERGY TRANSFER BASED BIOSENSORS

Non-radiative fluorescence and bioluminescence resonance energy transfer processes, FRET and BRET, are based on energy transfer between two closely spaced probes (Boute et al., 2002; Lohse et al., 2007). The prototypical resonance energy transfer (RET) biosensor consists of a recognition element fused with a pair of fluorescent or bioluminescent proteins capable of RET. A conformational change in the recognition element can be exploited to induce change in the distance and orientation between the donor and acceptor molecule resulting in the change in RET efficiency (Lohse et al., 2007; Okumoto et al., 2012).

FRET from one dye to another is a quantum mechanical process occurring if these two chromophores are in close proximity (less than 10 nm apart). A German chemist Theodor Förster (Förster, 1948) first reported this phenomenon. The efficiency of energy transfer is given as $E = (1 + (R / R_0)^6)^{-1}$, where R is the inter-dye distance, and R_0 is the Förster radius at which E= 0.5 (Roy, Hohng and Ha, 2008, Lakowicz, 2007). R_0 (in Å) = $0.211(\kappa^2 n^{-4} Q_D J(\lambda))^{1/6}$. As shown, R_0 depends on the orientation factor (κ) accounting for the dipole-dipole orientation of donor and acceptor; the refractive index of the medium (n); the quantum yield of the donor (Q_D) and the spectral overlap integral (J) between the emission of the donor and excitation of the acceptor fluorophores (Lakowicz, 2007). Relative FRET efficiency can be determined from the ratio of acceptor to donor fluorescence when only the donor fluorophore is excited. Alternatively, the acceptor molecules can be photobleached, and the relative

increase in donor emission can be used as a measure of FRET efficiency (Willoughby and Cooper, 2008). A limitation of FRET is the requirement for external illumination to initiate the fluorescence transfer. BRET is a similar quantum mechanical process to FRET, but occurring if bioluminescent luciferase (usually from *Renilla reniformis*) produces initial photon emission compatible with the acceptor fluorophore, which re-emits light at another wavelength (Boute et al., 2002).

Several cAMP biosensors based on BRET (Prinz et al., 2006; Jiang et al., 2007) have been developed, still most of the widely used sensors rely on FRET. Among the FRET-based cAMP sensors the first generation sensors depended on PKA (Adams et al., 1991; Zhang et al., 2001; Zaccolo and Pozzan, 2002) and on cAMP regulated ion channels (Fagan et al., 2001; Rich et al., 2001). Yet probes based on Epac protein (DiPilato et al., 2004; Nikolaev et al., 2004; Ponsioen et al., 2004) proved to be most advantageous due to their monomolecular structure and uniform cellular expression. Epac-based probes have been much improved since the development of their prototypes (Nikolaev et al., 2006; van der Krogt et al., 2008; Klarenbeek et al., 2011) resulting in an array of cAMP sensors with improved sensitivity, signal-to-noise ratio, dynamic range and spatiotemporal resolution (Willoughby and Cooper, 2008). Very common fluorophores with overlapping spectra used in cAMP biosensors are the GFP variants cyan (CFP) and yellow (YFP) fluorescent proteins (Nikolaev and Lohse, 2006; van der Krogt et al., 2008; Willoughby and Cooper, 2008). In the first cAMP biosensor however, synthetic dyes were used a FRET pair. The biosensor consisted of catalytic (C) and regulatory (R) subunits of PKA, chemically labeled with fluorescein (Fl) and rhodamine (Rh), respectively. The sensor was termed FlCRhR reflecting its composition (Adams et al., 1991).

Fluorescent proteins as well as genetically encoded sensors consist of polypeptide chains and are inherently sensitive to changes in ionic strength and pH (Okumoto et al., 2012). Thorough studies have been conducted and several FRET pairs compared to account for these issues as well as for protein photostability, brightness and proper maturation as well as low phototoxicity (van der Krogt et al., 2008; Rusanov et al., 2010; Lam et al., 2012; Klarenbeek et al., 2011 and 2015).

The recognition elements of cAMP sensors are composed of polypeptides that are native to the cells. If unaltered these may interfere with cellular functions (Okumoto et al., 2012). To avoid this issue, mutations that make the recognition elements catalytically inactive have been introduced in several cAMP biosensors (Ponsioen et al., 2004; van der Krogt et al., 2008; Klarenbeek et al., 2011). The other approach has been the fusion of only the short peptide chain responsible for the binding of cAMP between the FRET pair, therefore eliminating the need to use full-length proteins (Nikolaev et al., 2004).

2.4.2. Epac2-camps BIOSENSOR

In this thesis, two biosensors were used for measurements of cAMP levels in living cells. The first generation Epac2-camps construct consists of enhanced cyan fluorescent protein (eCFP) and enhanced yellow fluorescent protein (eYFP) fused directly to the high-affinity cAMP-binding domain of Epac2 protein (K_d ~ 1.2 μM; De Rooij et al., 2000). Excitation of the eCFP at a wavelength of 436 nm leads to eCFP emission at ~ 480 nm as well as to eYFP emission at ~ 525 nm (Nikolaev et al., 2004). The fluorimetrically measured EC_{50} for Epac2-camps was 0.92 ± 0.07 μM, which is well suited for measurements of physiologically relevant cAMP concentrations (from sub-micromolar in the resting state up to 10–100 μM upon adenylate cyclase activation; Klarenbeek et al., 2011). At the time, this novel sensor had several advantages compared to the former PKA-based biosensors and cAMP accumulation assays. Epac2-camps contains only one binding site for cAMP thus lacking binding cooperativity (in PKA-based biosensors four cAMP molecules must bind to the sensor to induce dissociation and the subsequent change in FRET). Epac2-camps sensor is uniformly distributed throughout the cytosol and has no catalytic properties or interactions with other cellular proteins (Nikolaev et al., 2004). The specificity of the decrease in FRET in response to cAMP was demonstrated *in vitro* by using Epac2-camps purified from *Spodoptera frugiperda* (Sf9) cells. It was shown that other nucleotides were recognized only weakly (AMP, > 10 mM; ATP, 2.5 ± 0.4 mM; cGMP, 10.6 ± 0.4 μM) (Nikolaev et al., 2004). A direct comparison of Epac2-camps with the tetrameric PKA system (Zaccolo and Pozzan, 2002) in Chinese hamster ovary cells uncovered that Epac2-camps reacted to adenylate cyclase stimulation much more rapidly in accordance with the faster activation kinetics of the new indicator measured *in vitro* (Nikolaev et al., 2004). The response of the Epac-based sensor is limited by the kinetics of cAMP production, whereas the tetrameric PKA sensor is also limited by activation kinetics of the sensor itself (binding of four cAMP molecules and the subsequent subunit dissociation).

Due to the suitable affinity for cAMP, convenient FRET pair for detection of fluorescence intensity with our equipment, no kinetic limitations originating from the biosensor itself and the demonstrated applicability of the biosensor in many different cell lines, we selected Epac-camps as the biosensor for detection of cAMP upon GPCR stimulation. The cDNA construct of the biosensor was kindly provided by Professor Marin J. Lohse from University of Würzburg.

2.4.3. $^{T}Epac^{VV}$ BIOSENSOR

As time went on and many laboratories were successfully using the developed cAMP biosensors, scientists continued to improve and develop the biosensors. Starting from the biosensor variant based on the partially truncated Epac1 protein (Ponsioen et al., 2004), several "second generation" cAMP biosensors were developed by systematic variation of the donor and acceptor fluorescent

proteins (van der Krogt et al., 2008). By tilting of the dipole of the acceptor proteins FRET efficiency was improved (van der Krogt et al., 2008). This was achieved by relocating the amino- and carboxyl termini of the acceptor fluorophore to alternative locations on the surface of the fluorescent protein barrel, so-called circular permutation. During the studies by van der Krogt et al., the dynamic range of the biosensor was improved, if fluorescent donors were presented with duplicate (tandem) acceptors. The effective absorption of the larger acceptor construct is increased and the two fluorophores in a tandem acceptor are likely to be oriented differently, thereby easing on the requirement of donor-acceptor dipole alignment (van der Krogt et al., 2008). It was found that incorporation of Venus in the cAMP biosensor cured pH- and UV-sensitivity of the probe. Using the tandem acceptor consisting of Venus and circularly permutated (cp^{173}Venus, presenting the acceptor protein at a favorable angle) yielded the widest FRET span of 36% upon full AC stimulation in individual cells (van der Krogt et al., 2008).

Replacing enhanced CFP protein with mTurquoise, a very bright CFP variant (Goedhart et al., 2010) resulted in a "third generation" cAMP sensor, mTurquoiseΔ-Epac(CD,ΔDEP)-^{cp173}-Venus-Venus, called $^{T}Epac^{VV}$ (Klarenbeek et al., 2011). In ratiometric fluorescence intensity based assays the dynamic range of the new sensor significantly outperforms previous versions: maximal FRET change upto 50% in individual cells. Moreover, due to the single-exponentially decaying donor fluorophore the sensor is well suited for both time-domain and frequency-domain fluorescence lifetime imaging (FLIM) (Klarenbeek et al., 2011). Due to clear outperformance of $^{T}Epac^{VV}$ over the "first generation" Epac2-camps biosensor we switched to the "third generation" construct in the studies of GPCR systems. The cDNA construct was kindly provided by Dr. Kees Jalink from The Netherlands Cancer Institute.

2.4.4. METHODS FOR FRET DETECTION

The degree of FRET can be measured by various approaches, of which the most popular is represented by simple fluorescence ratiometry (Sprenger and Nikolaev, 2013). This method relies on the sensitized emission of the acceptor fluorophore when excited by a donor via FRET. In this case, emission intensities of the donor and the acceptor molecules upon donor excitation are detected, and the FRET signal is calculated on a donor/acceptor or acceptor/donor ratio. Quantitative FRET determinations derived from intensity measurements require an accurate reference for the acceptor-free donor signals and are difficult to achieve in practice (Jares-Erijman and Jovin, 2003; Lohse et al., 2012). Yet problems like spectral bleed-through and cellular background fluorescence can be overcome if the relative change of FRET is calculated from the donor/acceptor ratio. Taken that the contributions of spectral bleed-through and background fluorescence are the same before and after cell treatment, dividing the intensities results in data normalization during the calculation of FRET

change. This rough change in FRET signal can then be plotted against the various concentrations of the compound eliciting the measured effect.

Another popular method for FRET detection is acceptor photobleaching. This approach requires a microscope equipped with a laser for “turning off” of the acceptor in the FRET pair. After bleaching of the acceptor with bright light, emission of donor increases and this increase corresponds to FRET efficiency (Lohse et al., 2012).

The determination of fluorescence lifetime, either in the time or in the frequency domain, is one of the most direct measures of FRET (Jares-Erijman and Jovin, 2003). Upon FRET the lifetime of the donor fluorescence decays more compared to the acceptor lifetime. The change in FRET efficiency can be detected by the change in donor lifetime only (Jares-Erijman and Jovin, 2003). FLIM is relatively insensitive to variations in fluorophore concentration and optical path length. In general, the method of detection can dictate the complexity of the probe/object. By using FLIM combined with total internal reflection microscopy one can concentrate on the membrane region allowing single molecule detection with high data sampling (Roy, Hohng and Ha, 2008). With FLIM, much higher spatio-temporal resolution is achievable compared to experiments relying on detection of fluorescence intensities. Since FLIM detection if focused on donor lifetime only, there is no need for additional controls (van der Krogt et al., 2008; Becker 2012).

3. RECOMBINANT GENE EXPRESSION

To use genetically encoded biosensors in experiments on living cells, the DNA construct of the biosensor must be delivered into the cells of interest. There are many methods for expression of recombinant DNA in eukaryotic cells. Most popular is the use of transient transfection methods like calcium phosphate precipitation (Graham and Van der Eb, 1973) or complex formation with cationic lipophilic reagents like polyethylene imine (Boussif et al., 1995) or diethylethanolamine-dextran (Schenborn and Goiffon, 2000). If the sensors are used in single-cell microscopy experiments, these methods are very suitable. Inhomogeneous expression levels do not pose problems, because the cells of suitable biosensor expression can be selected by applying a threshold value. Since only a small number of cells are required there are no considerable cost issues regarding purified DNA and the transfection reagents.

However, for applications where large number of cells with a comparable transgene expression levels are desired, transient transfection methods are not optimal due to the cumulative high cost of material and variance of the transfection efficiency.

For the long-time transgene expression, generation of a cell line stably expressing the protein of interest can be achieved by co-transfection of a marker-gene (for antibiotic resistance). Upon addition of the selection antibiotics, the cell population where the transgene has been integrated into the genome can be identified and cultured. This method however is not optimal for all transgenic constructs. In case of cAMP biosensor constructs, the prolonged presence of non-native protein binding the second messenger molecules could interfere with normal cellular processes.

In nature, most efficient and highly evolved mechanisms for infecting cells are the viruses. There are many different methods developed to employ their high gene delivery efficiency. Commonly, virus-based gene delivery is accomplished by using replication-deficient RNA or DNA viruses containing the gene of interest, but with the disease-causing sequences deleted from the viral genome (Kamimura et al., 2011). Many viral vectors have been utilized for transgene delivery including adenovirus, adenoassociated virus, lentivirus, retrovirus, Semlinki forest virus, baculovirus, pox virus, herpes simplex virus, Epstein-Bar virus, etc. Each of these viruses has its advantages and disadvantages, so which to use for a particular experiment may depend on multiple factors, including insert size, titer required, target gene expression level and target cell type (Walter and Stein, 2000). The primary feature of RNA-based viral vectors, such as retroviruses, is that they are capable of long-term transgene expression through gene integration. DNA-based viral vectors normally result in transgene expression in episomal form without integration (Kamimura et al., 2011). However with the use of viral vectors higher demands on the laboratories (Biosafety Level 2) are encountered. The viral constructs are not trivial to obtain and many of the viral systems have high maintenance costs.

3.1. BACMAM TECHNOLOGY

Baculovirus infects insects in nature and is non-pathogenic to humans (Biosafety Level 1), but can transduce a broad range of mammalian and avian cells. Baculoviruses are a diverse group of DNA viruses capable of infecting more than 600 insects, among which *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) is the best characterized and most extensively employed. AcMNPV contains a circular double stranded DNA genome of approximately 134 kBP (Chen et al., 2011). Baculovirus cloning capacity is as large as 38 kBP, thus allowing for the insertion of multiple genes and regulatory elements. Baculovirus–insect cell expression system has been one of the most widely used systems for routine production of recombinant proteins during the past 30 years (Kost et al., 2005; Chen et al., 2011). Many advances have been achieved during this time. Baculoviruses have been used in pseudotyped viral vaccine development (Wu et al., 2009; Cox, 2012) and been implemented as gene delivery vehicles for diverse biomedical applications (Paul et al., 2014). The possibility to display foreign proteins on the surface particles improved GPCR binding studies (Veiksina et al., 2014) and the insertion of mammalian expression cassettes in baculoviruses has eanbled efficient transgene expression in different mammalian cells (Kost and Condreay, 2002; **Papers I–IV**).

The so-called BacMam system and employs a baculovirus vector with the cytomegalovirus promoter to drive the expression of proteins in mammalian cells. This approach provides many advantages such as high transduction rates; protein expression levels that can be adjusted by viral dose; low cytotoxicity to host cells; safety in production and handling; compatibility with a broad range of cells, including primary and stem cells; and, importantly, the ease and convenience of use (Kost and Condreay, 2002; Kost et al., 2005).

4. AIMS OF THE STUDY

The GPCRs regulate thousands of vital processes in the human organism. Many of the rare and the common diseases are caused by distortions in cellular signaling or metabolism: Some of these illnesses can be treated or the symptoms alleviated by GPCR-targeted compounds. Thus, the search for GPCR-specific drugs is constantly ongoing and the need for reliable GPCR activation assays to test for biological activity of novel compounds is evident.

The general aim of this work was the development and validation of cAMP biosensor assay for characterization of activities of different GPCR ligands. Several particular tasks were raised within the study:

- Generation, optimization and validation of the BacMam expression system for the cAMP biosensor. Achieving reliable protein expression levels in different cell lines.
- Optimization of the transduction of mammalian cells with the generated BacMam virus.
- Generation of cAMP biosensor based assay system for screening of biological activities of novel ligands of different GPCRs stably expressed in particular cell lines.
- Implementation of the cAMP biosensor assay for quantification of active forms of gonadotropins in various preparations.

5. MATERIALS AND METHODS

Cell lines and cell culture

Spodoptera frugiperda cells (Sf9) (Invitrogen Life Technologies) were cultured in suspension with EX-CELL® 420 growth medium (Sigma-Aldrich GmbH) in 27 °C incubator in non-humidified environment.

All mammalian cell lines were grown as an adherent monolayer and maintained at 37 °C and 5 % CO_2 in a humidified incubator in the appropriate growth medium supplemented with 10 % fetal bovine serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin. All media, Dulbecco's Phosphate-Buffered Saline (DPBS) and trypsin were also from PAA Laboratories. Fetal bovine serum was form Gibco, Invitrogen Life Technologies.

B16F10 mouse melanoma cell line (Invitrogen Life Technologies) was cultured in Roswell Park Memorial Institute (RPMI) medium. Human embryonic kidney HEK293 cell line (American Type Culture Collection, ATCC® CRL-1573™) was cultured in Dulbecco's Modified Eagle's Medium (DMEM). JEG-3, human choriocarcinoma cell line clone 3 (Kohler and Bridson, 1971) (American Type Culture Collection, ATCC® HTB-36™) was cultured in Minimum Essential Medium (MEM). African green monkey kidney fibroblast-like COS-7 cell line expressing the recombinant human LH receptor (Müller et al. 2003) was kindly provided by Manuela Simoni, University of Modena and Reggio Emilia, Italy. COS-7 cells were cultured in DMEM/Ham's F12 Medium.

GPCR ligands

Dopamine was from Sigma-Aldrich GmbH. Human, highly purified recombinant LH (r-hLH; Luveris) and CG (r-hCG; Ovitrelle), human urinary CG (u-hCG; Pregnyl) and recombinant follitropin alfa (r-FSH; Gonal-F) were from Merck Serono Europe Limited. Initial concentrations: Luveirs 187.5 IU/ml = 8.7 μg/ml; Ovitrelle 5780 IU/ml = 500 μg/ml; Pregnyl 5000 IU/ml = 432.5 μg/ml; Gonal-F 600 IU/ml = 44 μg/ml. All MC_1 receptor peptidic ligands were from Bachem, Switzerland:

α-MSH: Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2;
β-MSH: Asp-Glu-Gly-Pro-Tyr-Arg-Met-Glu-His-Phe-Arg-Trp-Gly-Ser-Pro-Pro-Lys-Asp-OH;
NDP-α-MSH: Ac-Ser-Tyr-Ser-Nle-Glu-His-DPhe-Arg-Trp-Gly-Lys-Pro-Val-NH_2;
MS-05: Ser-Ser-Ile-Ile-Ser-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2;
SHU-9119: Ac-Nle-c[Asp-His-D-Nal(2')-Arg-Trp-Lys]-NH_2;
HS-024: Ac-c[Cys-Nle-Arg-His-D-Nal(2')-Arg-Trp-Gly-Cys]-NH_2.

3-isobutyl-1-methylxanthine (IBMX) used to inhibit phosphodiesterase activity was from Sigma-Aldrich GmbH. Forskolin used to directly activate adenylate cyclases was from Tocris Bioscience.

Plasmid construction and generation of BacMam virus

The expression vector Epac2-camps (Nikolaev et al. 2004) was kindly provided by Professor Marin J. Lohse from University of Würzburg. The expression vector mTurqDel-EPAC(dDEPCD)-cp173Venus(d)-Venus(d) (H74 or $^{T}Epac^{VV}$) (Klarenbeek et al. 2011) was kindly provided by Dr. Kees Jalink from The Netherlands Cancer Institute. Both biosensor constructs under the control of the cytomegalovirus promoter were cloned from the pcDNA3.1(+) vector into the pFastBac™1 vector (Invitrogen Life Technologies) using the restriction enzymes (Thermo Scientific) Bst1107I (BstZ17I) and Bsp68I (NruI) for pcDNA3.1(+) and Eco105I (SnaBI) and KspAI (HpaI) for pFastBac™1, respectively. The polyhedrin promoter was removed from the pFastBac™1 vector to ensure low promoter interference during virus amplification. The obtained pFastBac-cAMP-biosensor constructs were transformed into DH10Bac™ competent cells (Invitrogen Life Technologies) for the production of recombinant bacmid DNA. PCR-verified bacmid DNA was then transfected into Sf9 insect cells using 4 equivalents of ExGen 500 (Fermentas) to prepare BacMam virus stocks according to the Invitrogen Life Technologies Bac-to-Bac® expression system manual. P1 viral stocks were amplified and the titers were determined by cell size based assay using Sf9 cells. P1 viral stocks were stored at -80 ºC until further amplification. P2 and P3 viral stocks were aliquoted and stored at -80 ºC or concentrated 10 times by centrifugation at 40000 × g and stored at 4 ºC until the day of the experiment.

Determination of viral titers by a cell size based assay

The viral titers were determined using a cell size-based assay (**Paper III**). Sf9 cells were seeded on 24-well cell culture plates at 0.2×10^{6} cells/well in 250 µl of EX-CELL 420 cell culture medium 30–60 minutes prior to infection. 250 µl of dilution samples were added to wells (3-fold serial dilutions of harvested virus supernatant or of concentrated virus in EX-CELL 420 medium). The cells were incubated in the presence of the virus for 24 h and thereafter the average cell diameter was determined using a Beckman Coulter cell counter. To calculate the titer, the average cell diameter was plotted versus the log (virus dilution) to obtain the sigmoidal dose-response curve. The virus concentration in infectious units per ml (IU/ml) was calculated using equation (1):

$$Virus\ concentration\ (IU/ml) = \frac{\frac{1}{ED_{50}} \times 50\%\ of\ infected\ cells}{V} \quad (1),$$

where V – sample volume in wells in milliliters (here 0.5 ml); ED_{50} – 50% effective virus dilution corresponding to dilution at which the average cell diameter has changed 50%; *50% of infected cells* – 50% of the cells in wells at the time of infection given that the number of cells is roughly equal to the number of infective viral particles and the proportion of secondary infection is minimal (Janakiraman et al., 2006).

[^{3}H]cAMP competition binding assay

80–90 % confluent cells on 100 mm polylysine coated petri dishes (Nunc) were detached and the medium was replaced by DPBS. Cells were divided to aliquots of 0.3 × 10^6 cells / assay tube and stimulated upon addition of 10 X ligand solution in the final assay volume of 200 µl. The reaction was carried out for 30 min at 37 ºC and was stopped by adding 200 µl of 1 M ice-cold $HClO_4$ solution. The lysates were stored on ice for 60 minutes and then neutralized with 200 µl of 1 M ice-cold KOH solution.

The content of accumulated cAMP in the samples was measured by competition binding with [^{3}H]cAMP to cAMP binding protein (extracted from bovine adrenal glands) as previously described by (Nordstedt et al. 1990, Vonk et al. 2008). Samples or calibration standards + [^{3}H]cAMP + cAMP binding protein were incubated for 60 min at +4 ºC. All solutions prepared in Brown Buffer (50 mM Tris-HCl, pH 7.4; 100 mM NaCl, 10 mM EDTA, 8 mM Theophylline and 6 mM DTT) in the final assay volume of 200 µl. All determinations were performed in triplicates. The bound [^{3}H]cAMP was determined by fast filtration through thick GF/B glass fiber filters (Whatman) using a FilterMate Harvester (Model D961962, Perkin Elmer). After five washes with ice-cold phosphate buffer (20 mM K-phosphate, 100 mM NaCl, pH 7.4), filter mats were dried in a microwave oven and impregnated with a MeltiLex™ B/HS scintillant using a MeltiLex® Heatsealer. Filter-bound radioactivity was counted using a PerkinElmer/Wallac MicroBeta TriLux 1450 LSC Luminescence Counter. The cAMP concentrations in the samples were determined by interpolating the detected values using the calibration curve obtained in the same assay.

cAMP biosensor assay

60–80 % confluent mammalian cells on 100 mm polylysine coated petri dishes (Nunc) were transduced with 250–500 µl/dish of 10 × viral stock (multiplicity of infection: 100–400) in 4 ml serum free growth medium for 3 h at 37 °C. Thereafter the medium was replaced with complete growth medium containing 10 mM sodium butyrate (Sigma-Aldrich GmbH) and the cells were incubated for another 21 h at 30 °C for enhanced recombinant protein production. All incubations were carried out in a humidified incubator set to 5 % CO_2. The following day the cells were detached and the medium was replaced by DPBS. The cells from one dish were plated on one black 96-well clear-bottom cell culture plate (Corning B.V. Life Sciences) about an hour prior to the assay. The functional assays were performed on a PHERAstar plate reader (BMG LABTECH GmbH), with excitation at 427 nm and simultaneous dual emission at 480 and 530 nm. The cells were assayed in the final volume of 100 µl DPBS upon addition of 10 X ligand solution.

Data analysis

The change in FRET (ΔFRET) was calculated using equation (2):

$$\Delta\text{FRET} = \frac{\frac{530\ nm_0}{480\ nm_0} - \frac{530\ nm}{480\ nm}}{\frac{530\ nm_0}{480\ nm_0}} \qquad \text{Eq. (2),}$$

where 480 nm_0 and 530 nm_0 refer to the fluorescence emissions at the corresponding wavelengths before and 480 nm and 530 nm after the ligand treatment, respectively. Experiments were performed in at least 3 independent determinations in triplicates. Data analysis was performed using three parameter logistic function Y=Bottom+(Top-Bottom)/(1+10^((LogEC_{50}-X))) in GraphPad PRISM™ 5.04 (GraphPad Software). Data are presented as mean pEC_{50} ± S.E.M.

6. RESULTS AND DISCUSSION

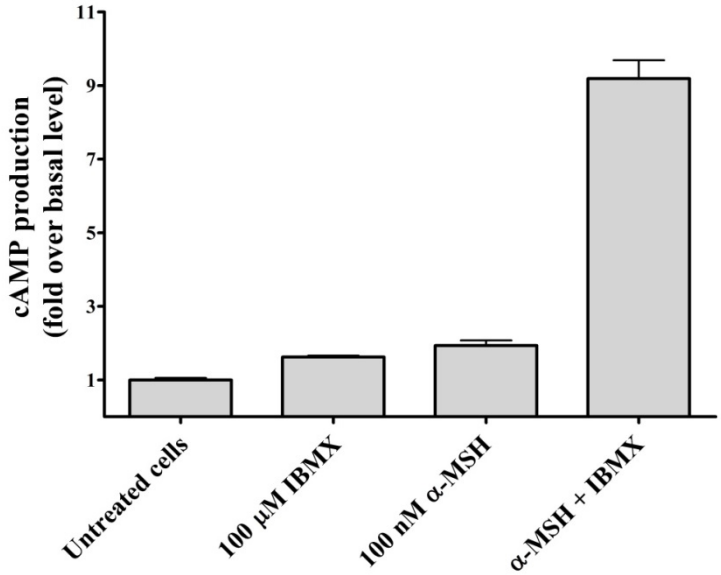
[^{3}H]cAMP competition binding assay (Nordstedt et al., 1990) was implemented in our laboratory by Vonk et al. (2008, 2009). This assay served as standard for cAMP detection and is still in use today, as a reference method at the beginning of projects with new GPCRs or new recombinant cell lines. However [^{3}H]cAMP competition binding assay is an *in vitro* endpoint assay only allowing detection of the analyte in cell lysates.

The use of cAMP biosensors provides significant advantages for monitoring of cAMP production in real time without the need for cell lysis. Due to suitable affinity for cAMP (~ 1 μM), the monomolecular structure of the biosensor construct and its uniform distribution throughout the cytosol Epac2-camps biosensor (Nikolaev et al., 2004) we selected for detection of cAMP upon GPCR stimulation.

For biosensor expression we first used common transfection reagents Lipofectamine 2000 (Invitrogen Life Technologies) and ExGen 500 (Fermentas). Inconsistent expression levels between independent assays posed problems for our assay development. Increases in the amount of DNA and lipophilic reagent caused increased cytotoxicity but did not result in higher protein expression. Also to perform dose-response experiments with a reasonable amount of data points we needed high number of cells expressing the biosensor. It was clear that the use of transfection reagents poorly suits the development of a high-throughput screening assay due to their relatively high cost and the constant need for large amounts of purified plasmid DNA.

To overcome these limitations we implemented the BacMam technology. Epac2-camps gene under the human cytomegalovirus promotor was cloned into the baculovirus vector and the polyhedrin promoter sequence driving protein expression in insect cells was eliminated. After obtaining the virus the conditions for cell transduction were optimized for the B16F10 cell line expressing the melanocortin MC_1 receptor. We found that suitable sensor expression levels were achieved at multiplicity of infection of 200 to 400 and by incubation of the mammalian cells with baculovirus for 2 to 4 h. To enhance protein expression after the transduction step, sodium butyrate, a histone deacetylase inhibitor was used at 10 mM. Sufficient protein expression for functional assays was achieved 24 h post-transduction and remained such for a further 24 h. It was demonstrated that the assay was flexible and had a convenient time frame between the transduction of cells and the functional measurements themselves (**Paper I**).

To verify expression and functionality of the MC1 receptor in B16F10 the cells were stimulated with MC1R endogenous agonist melanocyte stimulating hormone α-MSH. The accumulated cAMP was measured using the [^{3}H]cAMP competition binding assay (Figure 1). Upon 100 nM α-MSH a twofold increase in cAMP level was detected. Receptor activation upon stimulation with α-MSH alone and in presence of 100 μM IBMX (phosphodiesterase inhibitor) was much higher (ninefold increase over the basal cAMP level). These results showed that B16F10 cells are suitable for MC1R activation studies.



Firgure 1: Influence of a-MSH and IBMX on cAMP levels in B16F10 cells. Cells were treated with the ligands for 20 min at 37 °C. The accumulated cAMP was detected using the [^{3}H]cAMP competition binding assay. Graph showing data from a representative experiment carried out in sextuplicates (n=2).

After verification of MC1R expression and functionality in B16F10 cells, we started to develop the cAMP biosensor assay. Commonly for the cAMP accumulation assays, phosphodiesterase inhibitors are used to amplify the signal. Accounting for phosphodiesterases, enzymes that hydrolyze cAMP to a non-signaling AMP, is significant, because they determine the lifetime of the "effective pool" of cAMP measured in the assay (Willoughby and Cooper, 2008; Hill et al., 2010). Therefore we first investigated the role of inclusion of IBMX in the biosensor assay. It was determined that 100 μM IBMX did not affect EC_{50} values for α-MSH or forskolin (a direct activator of adenylate cyclase) but the inhibitor did also not improve the biosensor based detection system. IBMX decreased the dynamic range of the assay by elevating the basal level of cAMP (the change in FRET signal was smaller, compared to cells assayed without addition of phosphodiesterase inhibitor). Compared to the *in vitro* assay, where the accumulated cAMP is measured after cell lysis, the biosensor responds to immediate changes in cAMP. With the use of the biosensor it was now possible to detect changes in cAMP concentrations in real time, without the need for additional signal enhancement.

Bivalent metal cations are known to play an important role in ligand binding to GPCRs but the exact mechanisms still remain elusive and vary depending on the ion and on the receptor in question. It has been shown that Zn^{2+} and Ca^{2+} ions modulate binding of ligands to the MC1R (Holst et al., 2002; Kopanchuk et

al., 2005). To determine the effect of bivalent cations on receptor activation in B16F10 cells, cells were first treated with EGTA or EDTA, washed with phosphate buffered saline and thereafter, fixed concentrations of Ca^{2+} or Mg^{2+} were added to the reaction mixture. Upon removal of bivalent cations with 1 mM EDTA, the potency of the MC1R agonists (pEC_{50} = 6.8 ± 0.6 for α-MSH) was significantly lower than in presence of bivalent cations (Figure 2). Addition of 1 mM Ca^{2+} was found to be necessary for high-potency agonist effect (pEC_{50} = 9.2 ± 0.4), whereas 1 mM Mg^{2+} also increased the potency of agonists but to a lesser extent (pEC_{50} = 7.9 ± 0.1). Similar effects were observed for all MC_1R agonist tested (**Paper I**). After treatment with EGTA, which binds Ca^{2+} with higher affinity than Mg^{2+}, the obtained results were comparable to data observed after EDTA treatment. These results together demonstrated that the pEC_{50} values strongly depend on the ionic composition of the assay buffer. The effect of the bivalent cations must be caused at the step of ligand binding to receptor, because activation of cAMP signaling cascade is not abolished by their removal. In this light, all of the following experiments we carried out in phosphate buffered saline in the presence of 1 mM Ca^{2+}, if not stated otherwise.

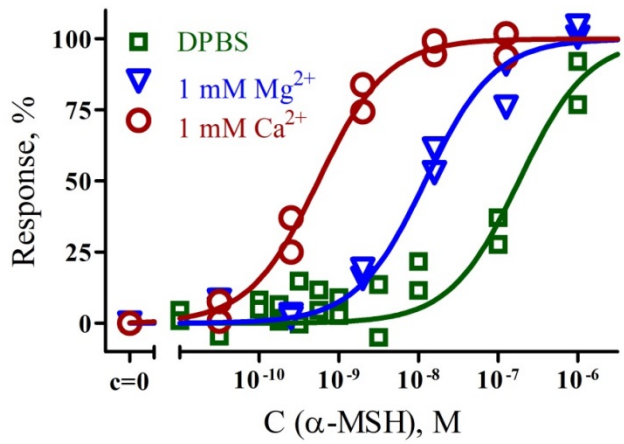


Figure 2: The effect of Ca^{2+} and Mg^{2+} on MC1R activation by α-MSH. B16F10 murine melanoma cells were transduced with BacMam-Epac2-camps virus for 3 h and further incubated for 21 h in complete growth medium supplemented with 10 mM sodium butyrate. Cells were washed with DPBS containing 1 mM EDTA prior to experiment. Chelating agents were removed and cells were assayed in DPBS (with or without 1 mM Ca^{2+} or Mg^{2+}) upon 10 min treatment with a full agonist, α-MSH. The maximal FRET change was normalized to 100 % response. Without Ca^{2+} and Mg^{2+}: pEC_{50} = 6.8 ± 0.6; in presence of 1 mM Mg^{2+}: pEC_{50} = 7.9 ± 0.1; in presence of 1 mM Ca^{2+}: pEC_{50} = 9.2 ± 0.4; (n=4). Graph showing data from a representative experiment.

The MC1 receptor activation was further characterized by a set of known MC1R ligands (Figure 3). In B16F10 cells all studied MC1R agonists caused a concentration dependent increase in cAMP concentration. α-MSH, β-MSH, NDP-α-MSH behaved as full agonists for MC1R with similar sensor activation profiles, while their activation level remained 75 % of the level achieved by forskolin. Partial agonists (SHU-9119, MS-05, HS-024) achieved a level of 75% of full agonist activation (**Paper I**). The selective low molecular weight MC4R agonist I-THIQ had no effect on MC1R activation in our system. These data are in agreement with previously published efficacies for these ligands. It was demonstrated that the cAMP biosensor assay is able to distinguish between full and partial agonists, which is important for characterization of newly synthesized ligands. *In vitro* cAMP accumulation assays and reporter gene assays are sometimes insensitive to detection of partial agonism, because of the signal amplification along the signaling cascade or due to measurements of the amplified readout.

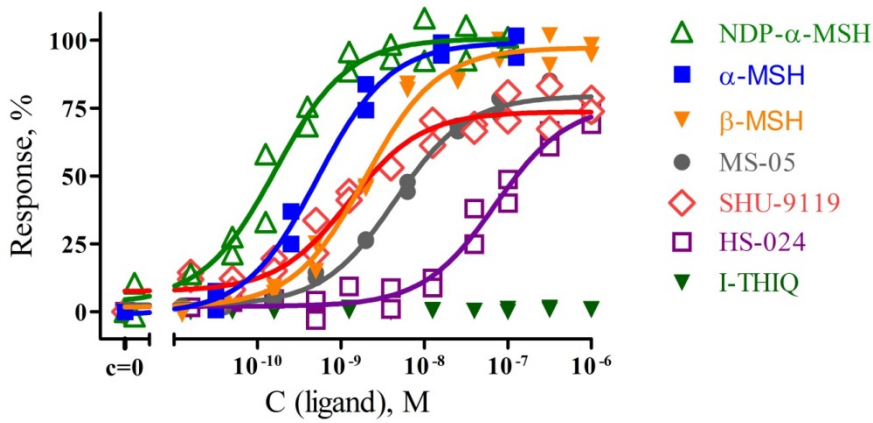


Figure 3: Influence of different concentrations of MC1R ligands on cAMP biosensor signal in B16F10 cells. Cells were transduced with BacMam-Epac2-camps virus for 3 h and further incubated for 21 h in complete growth medium supplemented with 10 mM sodium butyrate. Cells were treated with ligands for 10 min at 37 ºC. Measurements were performed in DPBS + 1 mM Ca^{2+} for full receptor activation. The maximal FRET change was normalized to 100 % of the full agonist NDP-α-MSH response. pEC_{50} ± S.E.M. (n=4): NDP-α-MSH: 9.9 ± 0.4; α-MSH: 9.2 ± 0.4; β-MSH: 8.74 ± 0.06; SHU-9119: 8.9 ± 0.3; MS-05: 7.96 ± 0.03; HS-024: 7.27 ± 0.13; I-THIQ showed no activation (n=2). Graph showing data from a representative experiment.

The study on MC1R in B16F10 cells demonstrated that the developed BacMam system for cAMP biosensor expression is suitable for GPCR activation studies and the calculated Z′-factor values > 0.6 for all MC_1R specific agonists and forskolin confirmed its suitability for high throughput screening (**Paper I**).

In 2011 a third generation $^{T}Epac^{VV}$ biosensor was constructed and characterized by Klarenbeek et al. The sensor consists of a part of Epac1 protein fused between a bright fluorescent protein mTurquoise and a tandem acceptor consisting of two Venus proteins. The new sensor was shown to have better signal-to-noise ratio and dynamic range compared to previous versions of Epac based cAMP biosensors (Klarenbeek et al., 2011).

The group kindly provided us with the new cDNA construct that we cloned into the BacMam expression vector. The BacMam viruses containing the $^{T}Epac^{VV}$ biosensor gene were generated analogues to the work with Epac2-camp biosensor. After generation and optimization of the expression system, $^{T}Epac^{VV}$ biosensor was compared the former, Epac2-camp biosensor. HEK293 cells stably expressing dopamine D1 receptor were transduced with BacMam viruses containing the gene of either of the biosensors and the cells were assayed on the next day. The dynamic range (FRET change window) was almost two times wider for the $^{T}Epac^{VV}$ biosensor, but no differences in the potencies were determined from the dose-response curves obtained with two different biosensors. The calculated EC_{50} values were approximately 1µM for forskolin and 1 nM for dopamine (Figure 4). The small difference in the affinities of the cAMP-binding domains of Epac1 and Epac2 proteins used in the biosensors (K_d ~ 4 and 1.2 µM, respectively; De Rooij et al., 2000) did not show discrepancies in the measured potencies for the known compounds. These data confirmed that switching from Epac2-based sensor to the Epac1-based biosensor for higher sensitivity in terms of FRET span is safe and justified. From then on $^{T}Epac^{VV}$ biosensor was used in all of the following studies.

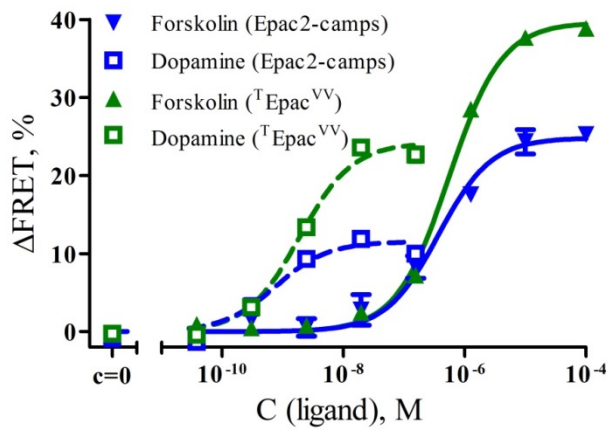


Figure 4: Cellular responses to forskolin and dopamine measured with Epac2-camps and $^{T}Epac^{VV}$ biosensors. HEK293 cells stably expressing dopamine D1 receptor were transduced with BacMam virus for 3 h and further incubated for 21 h in complete growth medium supplemented with 10 mM sodium butyrate. Cells were treated with adenylate cyclase activator forskolin or D1R agonist dopamine for 10 min at 37 ºC. Graph showing data from a representative experiment (n=2) performed in triplicates.

Dopamine has several functions in central nervous system including voluntary movement, feeding, affect, reward, sleep, attention, working memory, and learning (Beaulieu and Gainetdinov, 2011). Dopamine receptors are thus targets for many drugs. Although many dopaminergic ligands have been on the market for a long time (agonists mainly in the treatment of Parkinson's disease and antagonists as antipsychotics in the treatment of schizophrenia), development of more selective and potent dopaminergic compounds is still of great importance (Reinart-Okugbeni, 2012; Beaulieu et al., 2015). It is therefore important to have a functional assay for characterization of potencies of dopaminergic ligands in parallel to ligand binding experiments. All five subtypes of dopamine receptors signal via the adenylate cyclase pathway. D1 and D5 receptors are coupled to the Gs family proteins, hence agonist binding activates cAMP synthesis. D2, D3 and D4 receptors are coupled to the Gi family proteins and upon receptor activation cAMP synthesis is inhibited. We had successfully used the cAMP biosensor to monitor Gs coupled receptor activation (MC1 and D1 receptors), but now the assay system needed to be adapted for measurements of decreases in cAMP levels induced by Gi coupled receptor activation.

To monitor the decrease in cellular cAMP, its cellular level is increased by pretreatment with a direct AC activator forskolin. The assay conditions were optimized by varying the concentrations of forskolin between 1 and 50 μM. Upon cell stimulation with 10 μM forskolin together with receptor agonist, the dose dependent inhibition of cAMP production was detected in the widest dynamic range (15–20% of FRET change). Gi coupled receptor agonist potency in this type of assay is described by the IC_{50} value (Figure 5; **Paper II**).

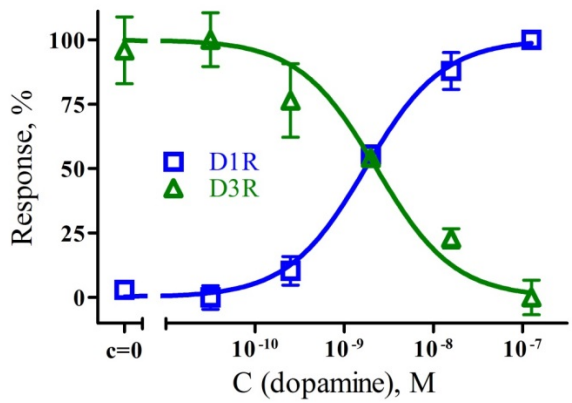


Figure 5: Inhibition and activation of cAMP synthesis. HEK293 cells stably expressing dopamine D1 or D3 receptor were transduced with BacMam virus for 3 h and further incubated for 21 h in complete growth medium supplemented with 10 mM sodium butyrate. HEK293-D1R cells were treated with serial dilutions of dopamine and the response was measured 10 minutes after agonist treatment (blue). For D3R stimulation cells were treated with serial dilutions of dopamine together with 10 μM forskolin (direct activator of adenylate cyclase). The response was measured 10 minutes after agonist treatment (green). Graph showing data from a representative experiment (n=3).

Our general aim is to approach and study GPCR systems from different directions. In **Paper II** we have introduced an approach to gather and integrate experimental data from ligand binding experiments, G protein activation studies and measurements of the second messenger levels. The strategy relies on the two basic properties, experiments are being performed using baculovirus constructs and the detected signals are based on molecular fluorescence. In the future, we aim to combine different experimental readouts into new meaningful information about the signal transduction mechanism of GPCRs.

Characterization of the biological activity of novel compounds requires precision and good assay reproducibility. We have provided a step-by-step protocol for generation and application of the BacMam based cAMP biosensor assay (**Paper III**). We also present a thorough description of a novel protocol for determination of virus titer with a cell size-based assay adapted and modified in our laboratory. To help other groups trying to set up the BacMam based expression system tips and observations have been included as notes at the end of the paper. This publication is a tool to disseminate our cumulative knowledge and observations in the field on cAMP detection and GPCR ligand characterization.

In addition to the functional characterization of low-molecule weight compounds and peptide ligands, the developed cAMP biosensor based assay system could also be applicable for studies of hormone receptors and their ligands, glycoproteins (**Paper IV**). The LH receptor is known to bind two proteins of the gonadotropin family, human luteinizing hormone (hLH) and human chorionic gonadotropin (hCG). Despite the high level of structural homology and action at a shared receptor, hLH and hCG have vital and unique roles in human development and reproduction. Our objective was to examine the differences or similarities of LH receptor activation by hLH and hCG. No significant differences in receptor activation and formation of cAMP in COS-7 cells expressing the recombinant LH receptor (Müller et al. 2003) were determined in this study during the first 120 minutes from the addition of hormones (**Paper IV**). The specificity of gonadotropin action at the LH receptor must be achieved by another mechanism or as a result of differences in the long-term signaling (Casarini et al. 2012; Choi and Smitz 2014).

Next, the biological activity of hCG preparations of different origins were compared. A four times lower potency for urinary hCG (EC_{50}=100 pM) compared to the recombinant hCG (EC_{50}=25 pM) was determined (Figure 6; **Paper IV**). The difference between the measured potencies may be explained by the different manufacturing and the subsequent stability of the urinary purified hormone compared to the recombinant preparation. Also, different patterns of glycosylation of recombinant and urinary preparations may influence the detected biological activity of the hormone. Today the produced hormone preparations are calibrated against the international reference preparations using immunoassays. These assays are based on epitope detection and generally do not account for the proper folding of the protein. The cAMP detection based assay system could be an important addition for quantification of active

amounts/concentrations of the hormones in pharmaceutical preparations, where the information about the fraction of biologically active form of the protein is of highest importance.

Since for evaluation of the biological activity of hormones LH receptor activation was used as the mediator of the biological signal (a natural biosensor), it opened a possibility to use the developed system for quantification of active concentrations of hCG, which is of special interest in the field of *in vitro* fertilization.

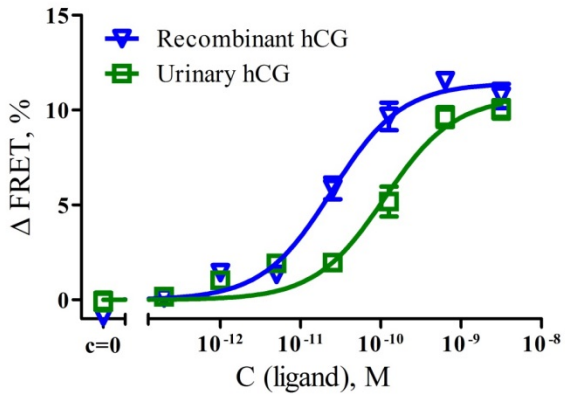


Figure 6: Influence of different concentrations of hCG preparations on cAMP biosensor signal. COS-7 cells stably expressing LH receptor were transduced with BacMam-^{T}Epac^{VV} virus for 3 h and further incubated for 21 h in complete growth medium supplemented with 10 mM sodium butyrate. Cells were treated with ligands for 30 min at 37 ºC. For urinary hCG (green): EC_{50}=100 pM; for recombinant hCG (blue): EC_{50}=25 pM. Graph showing data from a representative experiment (n=4) performed in triplicates.

Human chorionic gonadotropin is produced by trophoblasts, the cells that surround the growing human embryo and later form the placenta. Different isoforms of hCG (primarily hyperglycosylated hCG and the β-subunit of hCG) have been shown to be useful as biomarkers for embryo selection to improve the pregnancy chances after embryo transfer (Ramu et al. 2011; Butler et al. 2013). Today the transplanted embryos are selected based on their morphology and physicians' expertise and experience using grading systems based on the stage and morphological appearance of the embryo, with the success rate of implantation still under 50%. The high sensitivity (LOD: 5 pM or 4 mIU/mL) determined from a calibration curve using recombinant hCG, suggests the possibility to use cAMP biosensor assay for detection of hCG as a biomarker in the analysis of embryo spent culture media (**Paper IV**). The LODs of immunoassays used in previous quantitative studies are in the low mIU/mL range (Butler et al. 2013; Stenman et al. 2013; Xiao-Yan et al. 2013). However,

since in the cAMP biosensor assay only the active portion of the total hormone concentration is eliciting a measurable response, the possibly higher total hormone concentration of the probe (detectable by immunoassays) may result in some trade-off between signal specificity and sensitivity. Addressing the applicability of the developed BacMam based cAMP biosensor assay for challenging tasks as unbiased embryo selection is subject for future studies.

7. CONCLUSIONS

In summary, our studies on development and application of cAMP assays for monitoring of biological activity of various GPCR agonists led to the following conclusions:

- FRET based biosensors for cAMP detection in living cells proved advantageous over *in vitro* assays measuring the endpoint readout of accumulated cAMP in cell lysates. Receptor activation can be measured quantitatively in real time.
- BacMam gene delivery enables reliable and dose-adjustable expression of the recombinant cAMP biosensor protein in different cell lines.
- FRET based cAMP biosensor assay is well suited for characterization of biological activity of GPCR ligands as it can distinguish between the full and partial agonists as well as antagonists in competition experiments. The determined Z′ factor values (>0.6) confirm assay suitability for cell-based high-throughput screening.
- Bivalent metal cations are known to mediate ligand binding to melanocortin receptors. GPCR-mediated signal transduction (activation of cAMP signaling cascade) is unaffected by the use of bivalent metal cations, however Ca^{2+} was found to be required for high-potency agonist effect on MC1R. Mg^{2+} also increased the potency of agonists but to a lesser extent.
- It was demonstrated that cAMP biosensor assay was suitable for monitoring both, Gs and Gi coupled receptor activation.
- No significant differences between LH and hCG were found in kinetics nor in potencies of cAMP production in COS-7 cells stably expressing the LH receptor.
- Only the biologically active portion of the total hormone concentration is eliciting the measured response in the cAMP biosensor assay.
- The high sensitivity of the biosensor assay allows quantitative detection of gonadotropins in picomolar concentration.
- Our developed cAMP biosensor assay is a reliable tool for studies of different GPCRs and characterization of their ligands with various structural and functional properties.

8. SUMMARY IN ESTONIAN

Tsüklilise adenosiinmonofosfaadi biosensori arendamine ja rakendamine G valguga seotud retseptorite signaaliülekande uurimiseks

Maailma rahvastik on pidevas kasvus kuid ka vananemises ning sellest on tingitud mõnd haigust või sündroomi põdevate patsientide üha suurenev hulk. Enamik haigustest on põhjustatud häiretest organismi metabolismi või rakkude signaaliülekande regulatsioonis. G valguga seotud retseptorid (GPCR) moodustavad suure rakkudevaheliste signaalide ülekannet reguleeriva valkude perekonna. Inimese genoomist on leitud ligi 800 erineva GPCR-i geenijärjestused. Keemilise signaaliülekande moduleerimine on levinud ravistrateegia, mida kinnitab ka tõsiasi, et ligi kolmandik kõigist tänapäeva retseptiravimitest on suunatud just GPCR-tele. Ravimid ehk bioaktiivsed ühendid on seega muutunud tänapäeva maailma asendamatuks osaks.

Uute molekulide otsinguil on väga olulisteks ühendite molekulaarne disain, farmakokineetiliste parameetrite ja bioloogilise aktiivsuse määramine ning katsetulemuste võrdlus struktuurselt sarnaste või kehaomaste signaalimolekulide omadustega. Ühendite süstemaatiliseks testimiseks on loodud mitmeid katsesüsteeme ning nende arendamine on väga aktuaalne ka täna. Käesolevas doktoritöös arendati välja katsesüsteem GPCR-te ligandide iseloomustamiseks lähtudes tsüklilise adenosiinmonofosfaadi (cAMP) signaaliraja aktivatsiooni jälgimisest.

cAMP on signaalimolekul, mis kannab rakus edasi paljude erinevate GPCR-de poolt vahendatud bioloogilisi teateid. Kui retseptorile seondunud ligand on aktiveeriva iseloomuga, suureneb või väheneb rakus cAMP sünteesi eest vastutavate ensüümide adenülaadi tsüklaaside aktiivsus ning muutus cAMP kontsentratsioonis on võrdeline retseptori aktivatsiooniga. Rakendades cAMP suhtes tundlikke ja spetsiifilisi sensoreid, on võimalik jälgida retseptorile seostuvate ligandide bioloogilist aktiivsust elusrakkudes reaalajas. Käesolevas töös kasutatud biosensorid koosnevad cAMP siduvast peptiidijärjestusest ning kahest erinevate spektraalsete omadustega fluorestsentsvalgust. Puhkeolekus toimub kahe fluorofoori vahel Försteri resonantsenergia ülekanne (FRET). Ergastades biosensori fluorofoore kindla lainepikkusega valgusega (430 nm) on resonantsenergia ülekande tulemusena võimalik määrata mõlema fluorofoori poolt kiiratava valguse intensiivsust lainepikkustel 480 ja 530 nm. cAMP taseme suurenemisel rakus seostub signaalimolekul sensorile ning muudab fluorofooride omavahelist kaugust ja orientatsiooni võrreldes puhkeolekuga. Selle tulemusena väheneb fluorestsentsvalkude vaheline FRET ning muutub vastavatel lainepikkustel detekteeritava valguse intensiivsuste suhe. FRET-l põhineva biosensoriga on seega võimalik mõõta cAMP muutust elusrakkudes vastusena raku pinnal asuvate retseptorite aktivatsioonile.

Biosensorite kasutamiseks on vajalik nende ekspressioon (rakud peaksid tootma sensorvalku ise, lähtudes seda kodeerivast geenijärjestusest). Saavuta-

maks biosensori ühtlast ekspressiooni erinevates uuritavates rakkudes loodi käesolevas töös bakuloviirustel põhinev BacMam ekspressioonisüsteem. Inimesele ohutud putukaviirused transpordivad sensorit kodeeriva geenijärjestuse uuritavasse rakku, kus sellest sünteesitakse cAMP biosensorvalk. BacMam süsteem võimaldas reguleerida biosensori ekspressioonitaset, mille tulemuseks oli ühtlane ja hea korratavusega ekspressioon, mis lõi eelduse usaldusväärse katsesüsteemi loomiseks.

Töö käigus ekspresseeriti cAMP biosensorit mitmetes erinevates rakuliinides ning uuriti kolme erineva GPCR-i aktivatsiooni (melanokortiinne retseptor MC1R, dopamiini retseptor D1R ning inimese koorioni gonadotropiini ja luteniseeriva hormooni LH retseptor). Need retseptorid seovad struktuurselt väga erinevaid ligande. MC1 retseptori ligandideks on peptiidid, D1 retseptor seob madalmolekulaarseid ühendeid ja LH retseptori ligandideks on mitmekümne kilodaltoni suurused glükoproteiinidest hormoonid. Leiti, et FRET sensoril põhinev katsesüsteem on sobilik kõigi nende GPCR-de ligandide bioloogilise aktiivsuse iseloomustamiseks, kusjuures süsteem võimaldab eristada nii osalisi kui ka täisagoniste (aktivaatoreid). Kuna loodud katsesüsteem on rakendatav nii cAMP tõusu kui languse määramiseks, siis võimaldas see määrata ka erinevate dopamiini retseptorite alatüüpe, mis nii aktiveerivad kui ka inhibeerivad ensüümi adenülaadi tsüklaas. Lisaks ligandide endi aktivatsioonile võimaldas katsesüsteem määrata ka ligandi seostumist moduleerivate komponentide mõju. Näidati, et 1 mM Ca^{2+} või Mg^{2+} on vajalikud, et peptiidsed agonistid oleksid MC1 retseptoril efektiivsed. Katsesüsteemi tundlikkus (avastamispiir 5 pM) võimaldab seda kasutada aktiivsete hormoonide hulga määramiseks erinevates bioloogilistes proovides.

Käesolevas töös arendati välja FRET biosensoril põhinev katsesüsteem G valguga seotud retseptorite poolt reguleeritava cAMP taseme määramiseks elusrakkudes. Töö tulemusena on loodud tööriist, mille abil on võimalik määrata erinevate struktuursete omadustega ühendite bioloogilist aktiivsust vastavate retseptorite aktivatsiooni kaudu.

REFERENCES

Adams, Stephen R., Alec T. Harootunian, Ying Ji Buechler, Susan S. Taylor, and Roger Y. Tsien. "Fluorescence ratio imaging of cyclic AMP in single cells." *Nature* 349, no. 6311 (1991): 694–697.

Ascoli, Mario, Francesca Fanelli, and Deborah L. Segaloff. "The lutropin/choriogonadotropin receptor, a 2002 perspective." *Endocrine reviews* 23, no. 2 (2002): 141–174.

Baker, Jillian G., Ian P. Hall, and Stephen J. Hill. "Agonist and inverse agonist actions of β-blockers at the human β2-adrenoceptor provide evidence for agonist-directed signaling." *Molecular pharmacology* 64, no. 6 (2003): 1357–1369.

Biosciences, Amersham. "High-throughput screening for cAMP formation by scintillation proximity radioimmunoassay." *Proximity News* 23 (1996).

Beaulieu, Jean-Martin, and Raul R. Gainetdinov. "The physiology, signaling, and pharmacology of dopamine receptors." *Pharmacological reviews* 63, no. 1 (2011): 182–217.

Beaulieu, Jean-Martin, Stefano Espinoza, and Raul R. Gainetdinov. "Dopamine receptors–IUPHAR Review 13." *British Journal of Pharmacology* 172, no. 1 (2015): 1–23.

Beavo, Joseph A., and Laurence L. Brunton. "Cyclic nucleotide research – still expanding after half a century." *Nature Reviews Molecular Cell Biology* 3, no. 9 (2002): 710–718.

Becker, W. "Fluorescence lifetime imaging–techniques and applications." *Journal of microscopy* 247, no. 2 (2012): 119–136.

Bender, Andrew T., and Joseph A. Beavo. "Cyclic nucleotide phosphodiesterases: molecular regulation to clinical use." *Pharmacological reviews* 58, no. 3 (2006): 488–520.

Boussif, Otmane, Frank Lezoualc'h, MARLA ANTONIETrA Zanta, MOJGAN DJAVAHERI Mergny, Daniel Scherman, Barbara Demeneix, and Jean-Paul Behr. "A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine." *Proceedings of the National Academy of Sciences* 92, no. 16 (1995): 7297–7301.

Boute, Nicolas, Jockers, Ralf, and Tarik Issad. "The use of resonance energy transfer in high-throughput screening: BRET versus FRET." *Trends in pharmacological sciences* 23, no. 8 (2002): 351–354.

Brady, Scott, George Siegel, R. Wayne Albers, and Donald Price, eds. *Basic neurochemistry: principles of molecular, cellular, and medical neurobiology*. Academic Press, 2011.

Bradley, Jonathan, Johannes Reisert, and Stephan Frings. "Regulation of cyclic nucleotide-gated channels." *Current opinion in neurobiology* 15, no. 3 (2005): 343–349.

Brooker, Gary, J. F. Harper, W. L. Terasaki, and R. D. Moylan. "Radioimmunoassay of cyclic AMP and cyclic GMP." *Advances in cyclic nucleotide research* 10 (1978): 1–33.

Butler, Stephen A., Jameel Luttoo, Maísa OT Freire, Thomas K. Abban, Paola TA Borrelli, and Ray K. Iles. "Human Chorionic Gonadotropin (hCG) in the Secretome of Cultured Embryos Hyperglycosylated hCG and hCG-Free Beta Subunit Are Potential Markers for Infertility Management and Treatment." *Reproductive Sciences* 20, no. 9 (2013): 1038–1045.

Carlsson, Arvid. “A paradigm shift in brain research.” *Science* 294, no. 5544 (2001): 1021–1024.

Casarini, Livio, Monica Lispi, Salvatore Longobardi, Fabiola Milosa, Antonio La Marca, Daniela Tagliasacchi, Elisa Pignatti, and Manuela Simoni. “LH and hCG action on the same receptor results in quantitatively and qualitatively different intracellular signalling.” *PloS one* 7, no. 10 (2012): e46682.

Chen, Chi-Yuan, Chin-Yu Lin, Guan-Yu Chen, and Yu-Chen Hu. “Baculovirus as a gene delivery vector: recent understandings of molecular alterations in transduced cells and latest applications.” *Biotechnology advances* 29, no. 6 (2011): 618–631.

Chen, Linjie, Lili Jin, and Naiming Zhou. “An update of novel screening methods for GPCR in drug discovery.” *Expert opinion on drug discovery* 7, no. 9 (2012): 791–806.

Choi, Janet, and Johan Smitz. “Luteinizing hormone and human chorionic gonadotropin: origins of difference.” *Molecular and cellular endocrinology* 383, no. 1 (2014): 203–213.

Corbin, J. D., and E. G. Krebs. “A cyclic AMP-stimulated protein kinase in adipose tissue.” *Biochemical and biophysical research communications* 36, no. 2 (1969): 328–336.

Cox, Manon MJ. “Recombinant protein vaccines produced in insect cells.” *Vaccine* 30, no. 10 (2012): 1759–1766.

De Rooij, Johan, Fried JT Zwartkruis, Mark HG Verheijen, Robbert H. Cool, Sebastian MB Nijman, Alfred Wittinghofer, and Johannes L. Bos. “Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP.” *Nature* 396, no. 6710 (1998): 474–477.

De Rooij, Johan, Holger Rehmann, Miranda van Triest, Robert H. Cool, Alfred Wittinghofer, and Johannes L. Bos. “Mechanism of regulation of the Epac family of cAMP-dependent RapGEFs.” *Journal of Biological Chemistry* 275, no. 27 (2000): 20829–20836.

DiPilato, Lisa M., Xiaodong Cheng, and Jin Zhang. “Fluorescent indicators of cAMP and Epac activation reveal differential dynamics of cAMP signaling within discrete subcellular compartments.” *Proceedings of the National Academy of Sciences of the United States of America* 101, no. 47 (2004): 16513–16518.

Donaldson, J. I. L. L., STEPHEN J. Hill, and ANTHONY M. Brown. “Kinetic studies on the mechanism by which histamine H1 receptors potentiate cyclic AMP accumulation in guinea pig cerebral cortical slices.” *Molecular pharmacology* 33, no. 6 (1988): 626–633.

Fagan, Kent A., Jerome Schaack, Adam Zweifach, and Dermot MF Cooper. “Adenovirus encoded cyclic nucleotide-gated channels: a new methodology for monitoring cAMP in living cells.” *FEBS letters* 500, no. 1 (2001): 85–90.

Fesenko, Evgeniy E., Stanislav S. Kolesnikov, and Arkadiy L. Lyubarsky. “Induction by cyclic GMP of cationic conductance in plasma membrane of retinal rod outer segment.” (1985): 310–313.

Fredriksson, Robert, Malin C. Lagerström, Lars-Gustav Lundin, and Helgi B. Schiöth. “The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints.” *Molecular pharmacology* 63, no. 6 (2003): 1256–1272.

Förster, Th. “Zwischenmolekulare energiewanderung und fluoreszenz.” *Annalen der physik* 437, no. 1-2 (1948): 55–75.

Garland, Stephen L. “Are GPCRs still a source of new targets?.” *Journal of biomolecular screening* (2013): 1087057113498418.
Gilman, Alfred G. “A protein binding assay for adenosine 3′: 5′-cyclic monophosphate.” *Proceedings of the National Academy of Sciences* 67, no. 1 (1970): 305–312.
Gilman, Alfred G. “G proteins: transducers of receptor-generated signals.” *Annual review of biochemistry* 56, no. 1 (1987): 615–649.
Gloerich, Martijn, and Johannes L. Bos. “Epac: defining a new mechanism for cAMP action.” *Annual review of pharmacology and toxicology* 50 (2010): 355–375.
Godinho, Rosely O., Thiago Duarte, and Enio SA Pacini. “New perspectives in signaling mediated by receptors coupled to stimulatory G protein: the emerging significance of cAMP efflux and extracellular cAMP-adenosine pathway.” *Frontiers in pharmacology* 6 (2015).
Goedhart, Joachim, Laura van Weeren, Mark A. Hink, Norbert OE Vischer, Kees Jalink, and Theodorus WJ Gadella. “Bright cyan fluorescent protein variants identified by fluorescence lifetime screening.” *Nature methods* 7, no. 2 (2010): 137–139.
Gonzalez, Gustavo A., and Marc R. Montminy. “Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133.” *Cell* 59, no. 4 (1989): 675–680.
Graham, F. L., and A. J. Van der Eb. “A new technique for the assay of infectivity of human adenovirus 5 DNA.” *Virology* 52, no. 2 (1973): 456–467.
Gurevich, Eugenia V., John JG Tesmer, Arcady Mushegian, and Vsevolod V. Gurevich. “G protein-coupled receptor kinases: more than just kinases and not only for GPCRs.” *Pharmacology & therapeutics* 133, no. 1 (2012): 40–69.
Harper, J. F., and G. Brooker. “Femtomole sensitive radioimmunoassay for cyclic AMP and cyclic GMP after 2'0 acetylation by acetic anhydride in aqueous solution.” *Journal of cyclic nucleotide research* 1, no. 4 (1974): 207–218.
Heim, Roger, and Roger Y. Tsien. “Engineering green fluorescent protein for improved brightness, longer wavelengths and fluorescence resonance energy transfer.” *Current Biology* 6, no. 2 (1996): 178–182.
Heng, Boon Chin, Dominique Aubel, and Martin Fussenegger. “An overview of the diverse roles of G-protein coupled receptors (GPCRs) in the pathophysiology of various human diseases.” *Biotechnology advances* 31, no. 8 (2013): 1676–1694.
Hill, Stephen J., Christine Williams, and Lauren T. May. “Insights into GPCR pharmacology from the measurement of changes in intracellular cyclic AMP; advantages and pitfalls of differing methodologies.” *British journal of pharmacology* 161, no. 6 (2010): 1266–1275.
Holst, Birgitte, Christian E. Elling, and Thue W. Schwartz. “Metal ion-mediated agonism and agonist enhancement in melanocortin MC1 and MC4 receptors.” *Journal of Biological Chemistry* 277, no. 49 (2002): 47662–47670.
Houslay, Miles D. “Underpinning compartmentalised cAMP signalling through targeted cAMP breakdown.” *Trends in biochemical sciences* 35, no. 2 (2010): 91–100.
Hrubu, Victor J., Dongsi Lu, Shubh D. Sharma, Ana de L. Castrucci, Robert A. Kesterson, Fahad A. Al-Obeidi, Mac E. Hadley, and Roger D. Cone. “Cyclic lactam. alpha.-melanotropin analogs of Ac-Nle4-cyclo [Asp5, D-Phe7, Lys10]-. alpha.-melanocyte-stimulating hormone-(4–10)-NH2 with bulky aromatic amino acids at position 7 show high antagonist potency and selectivity at specific melanocortin receptors.” *Journal of medicinal chemistry* 38, no. 18 (1995): 3454–3461.

Huang, M., H. Shimizu, and J. Daly. “Regulation of Adenosine Cyclic 3', 5'-Phosphate Formation in Cerebral Cortical Slices Interaction among Norepinephrine, Histamine, Serotonin.” *Molecular pharmacology* 7, no. 2 (1971): 155–162.

Janakiraman, Vasantharajan, William F. Forrest, Bernard Chow, and Somasekar Seshagiri. “A rapid method for estimation of baculovirus titer based on viable cell size.” *Journal of virological methods* 132, no. 1 (2006): 48–58.

Jares-Erijman, Elizabeth A., and Thomas M. Jovin. “FRET imaging.” *Nature biotechnology* 21, no. 11 (2003): 1387–1395.

Jiang, Lily I., Julie Collins, Richard Davis, Keng-Mean Lin, Dianne DeCamp, Tamara Roach, Robert Hsueh et al. “Use of a cAMP BRET sensor to characterize a novel regulation of cAMP by the sphingosine 1-phosphate/G13 pathway.” *Journal of Biological Chemistry* 282, no. 14 (2007): 10576–10584.

Kamimura, Kenya, Takeshi Suda, Guisheng Zhang, and Dexi Liu. “Advances in gene delivery systems.” *Pharmaceutical medicine* 25, no. 5 (2011): 293–306.

Kandel, Eric R. “The molecular biology of memory: cAMP, PKA, CRE, CREB-1, CREB-2, and CPEB.” *Mol Brain* 5, no. 1 (2012): 14.

Kask, Ants, Felikss Mutulis, Ruta Muceniece, Rein Pähkla, Ilze Mutule, Jarl ES Wikberg, Lembit Rägo, and Helgi B. Schiöth. “Discovery of a Novel Superpotent and Selective Melanocortin-4 Receptor Antagonist (HS024): Evaluation in Vitro and in Vivo 1.” *Endocrinology* 139, no. 12 (1998): 5006–5014.

Klarenbeek, Jeffrey B., Joachim Goedhart, Mark A. Hink, Theodorus WJ Gadella, and Kees Jalink. “A mTurquoise-based cAMP sensor for both FLIM and ratiometric read-out has improved dynamic range.” *PLoS One* 6, no. 4 (2011): e19170.

Klarenbeek, Jeffrey, Joachim Goedhart, Aernoud van Batenburg, Daniella Groenewald, and Kees Jalink. “Fourth-Generation Epac-Based FRET Sensors for cAMP Feature Exceptional Brightness, Photostability and Dynamic Range: Characterization of Dedicated Sensors for FLIM, for Ratiometry and with High Affinity.” *PloS one* 10, no. 4 (2015).

Kopanchuk, Sergei, Santa Veiksina, Ramona Petrovska, Ilze Mutule, Michael Szardenings, Ago Rinken, and Jarl ES Wikberg. “Co-operative regulation of ligand binding to melanocortin receptor subtypes: evidence for interacting binding sites.” *European journal of pharmacology* 512, no. 2 (2005): 85–95.

Kost, Thomas A., and J. Patrick Condreay. “Recombinant baculoviruses as mammalian cell gene-delivery vectors.” *Trends in biotechnology* 20, no. 4 (2002): 173–180.

Kost, Thomas A., J. Patrick Condreay, and Donald L. Jarvis. “Baculovirus as versatile vectors for protein expression in insect and mammalian cells.” *Nature biotechnology* 23, no. 5 (2005): 567–575.

Lakowicz, Joseph R. *Principles of fluorescence spectroscopy*. Third Edition. Springer Science & Business Media, 2007.

Lam, Amy J., François St-Pierre, Yiyang Gong, Jesse D. Marshall, Paula J. Cranfill, Michelle A. Baird, Michael R. McKeown et al. “Improving FRET dynamic range with bright green and red fluorescent proteins.” *Nature methods* 9, no. 10 (2012): 1005–1012.

Lambert Instruments BV, 2015, http://www.lambertinstruments.com/technologies-1/2014/12/4/frequency-domain-flim-for-beginners

Lin, H. H. “G-protein-coupled receptors and their (Bio) chemical significance win 2012 Nobel Prize in Chemistry.” *Biomedical journal* 36, no. 3 (2013): 118.

Lohse, Martin J., Moritz Bünemann, Carsten Hoffmann, Jean-Pierre Vilardaga, and Viacheslav O. Nikolaev. “Monitoring receptor signaling by intramolecular FRET.” *Current opinion in pharmacology* 7, no. 5 (2007): 547–553.

Lohse, Martin J., Susanne Nuber, and Carsten Hoffmann. “Fluorescence/bioluminescence resonance energy transfer techniques to study G-protein-coupled receptor activation and signaling.” *Pharmacological reviews* 64, no. 2 (2012): 299–336.

Matz, Mikhail V., Arkady F. Fradkov, Yulii A. Labas, Aleksandr P. Savitsky, Andrey G. Zaraisky, Mikhail L. Markelov, and Sergey A. Lukyanov. “Fluorescent proteins from nonbioluminescent Anthozoa species.” *Nature biotechnology* 17, no. 10 (1999): 969–973.

Mayr, Bernhard, and Marc Montminy. “Transcriptional regulation by the phosphorylation-dependent factor CREB.” *Nature Reviews Molecular Cell Biology* 2, no. 8 (2001): 599–609.

McLoughlin, D. J., F. Bertelli, and C. Williams. “The A, B, Cs of G-protein-coupled receptor pharmacology in assay development for HTS.” (2007): 603–619.

Miyano, Kanako, Yuka Sudo, Akinobu Yokoyama, Kazue Hisaoka-Nakashima, Norimitsu Morioka, Minoru Takebayashi, Yoshihiro Nakata, Yoshikazu Higami, and Yasuhito Uezono. “History of the G Protein–Coupled Receptor (GPCR) Assays From Traditional to a State-of-the-Art Biosensor Assay.” *Journal of pharmacological sciences* 126, no. 4 (2014): 302–309.

Mutulis, Felikss, Sviatlana Yahorava, Ilze Mutule, Aleh Yahorau, Edvards Liepinsh, Sergei Kopantshuk, Santa Veiksina et al. “New substituted piperazines as ligands for melanocortin receptors. Correlation to the X-ray structure of “THIQ”.” *Journal of medicinal chemistry* 47, no. 18 (2004): 4613–4626.

Müller, Thomas, Jörg Gromoll, and Manuela Simoni. “Absence of exon 10 of the human luteinizing hormone (LH) receptor impairs LH, but not human chorionic gonadotropin action.” *The Journal of Clinical Endocrinology & Metabolism* 88, no. 5 (2003): 2242–2249.

Naylor, Louise H. “Reporter gene technology: the future looks bright.” *Biochemical pharmacology* 58, no. 5 (1999): 749–757.

NEN Life Science Products. A novel adenylyl cyclase activation assay on FlashPlate (Flasplate File #1, Application Note). (NEN Life Science Products Inc., Boston, Massachusetts, 1998).

Newman, Robert H., Matthew D. Fosbrink, and Jin Zhang. “Genetically encodable fluorescent biosensors for tracking signaling dynamics in living cells.” *Chemical reviews* 111, no. 5 (2011): 3614–3666.

Nikolaev, Viacheslav O., Moritz Bünemann, Lutz Hein, Annette Hannawacker, and Martin J. Lohse. “Novel single chain cAMP sensors for receptor-induced signal propagation.” *Journal of Biological Chemistry* 279, no. 36 (2004): 37215–37218.

Nikolaev, Viacheslav O., and Martin J. Lohse. “Monitoring of cAMP synthesis and degradation in living cells.” *Physiology* 21, no. 2 (2006): 86–92.

Nikolaev, Viacheslav O., Stepan Gambaryan, and Martin J. Lohse. “Fluorescent sensors for rapid monitoring of intracellular cGMP.” *Nature methods* 3, no. 1 (2006): 23–25.

Nordstedt, Christer, and Bertil B. Fredholm. “A modification of a protein-binding method for rapid quantification of cAMP in cell-culture supernatants and body fluid.” *Analytical biochemistry* 189, no. 2 (1990): 231–234.

Okumoto, Sakiko, Alexander Jones, and Wolf B. Frommer. “Quantitative imaging with fluorescent biosensors.” *Annual review of plant biology* 63 (2012): 663–706.

Paul, Arghya, Anwarul Hasan, Laetitia Rodes, Mugundhine Sangaralingam, and Satya Prakash. “Bioengineered baculoviruses as new class of therapeutics using micro and nanotechnologies: principles, prospects and challenges.” *Advanced drug delivery reviews* 71 (2014): 115–130.

Pierce, Kristen L., Richard T. Premont, and Robert J. Lefkowitz. “Seven-transmembrane receptors.” *Nature Reviews Molecular Cell Biology* 3, no. 9 (2002): 639–650.

Ponsioen, Bas, Jun Zhao, Jurgen Riedl, Fried Zwartkruis, Gerard van der Krogt, Manuela Zaccolo, Wouter H. Moolenaar, Johannes L. Bos, and Kees Jalink. “Detecting cAMP-induced Epac activation by fluorescence resonance energy transfer: Epac as a novel cAMP indicator.” *EMBO reports* 5, no. 12 (2004): 1176–1180.

Prinz, Anke, Mandy Diskar, Andrea Erlbruch, and Friedrich W. Herberg. “Novel, isotype-specific sensors for protein kinase A subunit interaction based on bioluminescence resonance energy transfer (BRET).” *Cellular signalling* 18, no. 10 (2006): 1616–1625.

Prystay, Linda, Annie Gagne, Patricia Kasila, Li-An Yeh, and Peter Banks. “Homogeneous cell-based fluorescence polarization assay for the direct detection of cAMP.” *Journal of biomolecular screening* 6, no. 2 (2001): 75–82.

Rall, T. W., Earl W. Sutherland, and Jacques Berthet. “The relationship of epinephrine and glucagon to liver phosphorylase IV. Effect of epinephrine and glucagon on the reactivation of phosphorylase in liver homogenates.” *Journal of Biological Chemistry* 224, no. 1 (1957): 463–475.

Ramu, Sivakumar, Brian Acacio, Mark Adamowicz, Sylvia Parrett, and Rajasingam S. Jeyendran. “Human chorionic gonadotropin from day 2 spent embryo culture media and its relationship to embryo development.” *Fertility and sterility* 96, no. 3 (2011): 615–617.

Reinart-Okugbeni, Reet. “Assay systems for characterisation of subtype-selective binding and functional activity of ligands on dopamine receptors.” PhD diss., 2012.

Reiter, Eric, and Robert J. Lefkowitz. “GRKs and β-arrestins: roles in receptor silencing, trafficking and signaling.” *Trends in endocrinology & metabolism* 17, no. 4 (2006): 159–165.

Rich, Thomas C., E. Tse Tonia, Joyce G. Rohan, Jerome Schaack, and Jeffrey W. Karpen. “In vivo assessment of local phosphodiesterase activity using tailored cyclic nucleotide–gated channels as cAMP sensors.” *The Journal of general physiology* 118, no. 1 (2001): 63–78.

Rodrigues, Adriana R., Henrique Almeida, and Alexandra M. Gouveia. “Intracellular signaling mechanisms of the melanocortin receptors: current state of the art.” *Cellular and Molecular Life Sciences* (2014): 1–15.

Rosenbaum, Daniel M., Søren GF Rasmussen, and Brian K. Kobilka. “The structure and function of G-protein-coupled receptors.” *Nature* 459, no. 7245 (2009): 356–363.

Roy, Rahul, Sungchul Hohng, and Taekjip Ha. “A practical guide to single-molecule FRET.” *Nature methods* 5, no. 6 (2008): 507–516.

Rusanov, Alexander L., Tatiana V. Ivashina, Leonid M. Vinokurov, Ilya I. Fiks, Anna G. Orlova, Ilya V. Turchin, Irina G. Meerovich, Victorya V. Zherdeva, and Alexander P. Savitsky. “Lifetime imaging of FRET between red fluorescent proteins.” *Journal of biophotonics* 3, no. 12 (2010): 774–783.

Sadana, Rachna, and Carmen W. Dessauer. “Physiological roles for G protein-regulated adenylyl cyclase isoforms: insights from knockout and overexpression studies.” *Neuro-signals* 17, no. 1 (2009): 5.

Sawyer, Tomi K., Pauline J. Sanfilippo, Victor J. Hruby, Michael H. Engel, Christopher B. Heward, Jean B. Burnett, and Mac E. Hadley. "4-Norleucine, 7-D-phenylalanine-alpha-melanocyte-stimulating hormone: a highly potent alpha-melanotropin with ultralong biological activity." *Proceedings of the National Academy of Sciences* 77, no. 10 (1980): 5754–5758.

Schenborn, Elaine T., and Virginia Goiffon. "DEAE-dextran transfection of mammalian cultured cells." In *Transcription Factor Protocols*, pp. 147–153. Humana Press, 2000.

Seifert, Roland. "Functional selectivity of G-protein-coupled receptors: from recombinant systems to native human cells." *Biochemical pharmacology* 86, no. 7 (2013): 853–861.

Sprenger, Julia U., and Viacheslav O. Nikolaev. "Biophysical techniques for detection of cAMP and cGMP in living cells." *International journal of molecular sciences* 14, no. 4 (2013): 8025–8046.

Steiner, A. L., Ch W. Parker, and D. M. Kipnis. "The measurement of cyclic nucleotides by radioimmunoassay." *Advances in biochemical psychopharmacology* 3 (1969): 89–111.

Stenman, Ulf-Håkan, and Henrik Alfthan. "Determination of human chorionic gonadotropin." *Best Practice & Research Clinical Endocrinology & Metabolism* 27, no. 6 (2013): 783–793.

Sutherland, Earl W. "Studies on the mechanism of hormone action." American Association for the Advancement of Science, 1972.

Szardenings, Michael, Ruta Muceniece, Ilze Mutule, Felikss Mutulis, and Jarl ES Wikberg. "New highly specific agonistic peptides for human melanocortin MC 1 receptor☆." *Peptides* 21, no. 2 (2000): 239–243.

Tasken, Kjetil, and Einar Martin Aandahl. "Localized effects of cAMP mediated by distinct routes of protein kinase A." *Physiological reviews* 84, no. 1 (2004): 137–167.

van der Krogt, Gerard NM, Janneke Ogink, Bas Ponsioen, and Kees Jalink. "A comparison of donor-acceptor pairs for genetically encoded FRET sensors: application to the Epac cAMP sensor as an example." *PloS one* 3, no. 4 (2008): e1916.

Veiksina, Santa, Sergei Kopanchuk, and Ago Rinken. "Budded baculoviruses as a tool for a homogeneous fluorescence anisotropy-based assay of ligand binding to G protein-coupled receptors: the case of melanocortin 4 receptors." *Biochimica et Biophysica Acta (BBA)-Biomembranes* 1838, no. 1 (2014): 372–381.

Vonk, Argo, Reet Reinart, and Ago Rinken. "Modulation of adenylyl cyclase activity in rat striatal homogenate by dopaminergic receptors." *Journal of pharmacological sciences* 108, no. 1 (2008): 63–70.

Vonk, Argo. "Determination of adenosine A2A-and dopamine D1 receptor-specific modulation of adenylate cyclase activity in rat striatum." PhD diss., 2009.

Walsh, D. A., John P. Perkins, and Edwin G. Krebs. "An adenosine 3', 5'-monophosphate-dependant protein kinase from rabbit skeletal muscle." *Journal of Biological Chemistry* 243, no. 13 (1968): 3763–3765.

Walter, W., and U. Stein. "Viral vectors for gene transfer a review of their use in the treatment of human disease." *Drugs* 60 (2000): 249–71.

Wicks, Wesley D. "Induction of hepatic enzymes by adenosine 3', 5'-monophosphate in organ culture." *Journal of Biological Chemistry* 244, no. 14 (1969): 3941–3950.

Wikberg, Jarl ES, Ruta Muceniece, Ilona Mandrika, Peteris Prusis, Jonas Lindblom, Claes Post, and Anna Skottner. “New aspects on the melanocortins and their receptors.” *Pharmacological research* 42, no. 5 (2000): 393–420.

Williams, Christine. “cAMP detection methods in HTS: selecting the best from the rest.” *Nature Reviews Drug Discovery* 3, no. 2 (2004): 125–135.

Willoughby, Debbie, and Dermot MF Cooper. “Live-cell imaging of cAMP dynamics.” *Nature methods* 5, no. 1 (2008): 29–36.

Wu, Qunfeng, Liurong Fang, Xuebao Wu, Bin Li, Rui Luo, Zhengjun Yu, Meilin Jin, Huanchun Chen, and Shaobo Xiao. “A pseudotype baculovirus-mediated vaccine confers protective immunity against lethal challenge with H5N1 avian influenza virus in mice and chickens.” *Molecular immunology* 46, no. 11 (2009): 2210–2217.

Xiao-yan, Chen, Li Jie, Jiang Dang, Li Tao, Liu Xin-ru, and Zhuang Guang-lun. “A highly sensitive electrochemiluminescence immunoassay for detecting human embryonic human chorionic gonadotropin in spent embryo culture media during IVF-ET cycle.” *Journal of assisted reproduction and genetics* 30, no. 3 (2013): 377–382.

Zaccolo, Manuela, and Tullio Pozzan. “Discrete microdomains with high concentration of cAMP in stimulated rat neonatal cardiac myocytes.” *Science* 295, no. 5560 (2002): 1711–1715.

Zhang, Jin, Yuliang Ma, Susan S. Taylor, and Roger Y. Tsien. “Genetically encoded reporters of protein kinase A activity reveal impact of substrate tethering.” *Proceedings of the National Academy of Sciences* 98, no. 26 (2001): 14997–15002.

ACKNOWLEDGEMENTS

The journey to completing my university education with writing this thesis began a long time ago. My high school teacher for biology and chemistry is one of the most inspiring and devoted people I know. Frau Schnell was the one to introduce me to the fascinating world of biochemistry and chemical signal transduction. She was also the first person to foresee my future career. To her, my mentor and a dear Friend, I owe all the gratitude I can express.

During the last ten years, I have had the honor to learn from many brilliant professors and lecturers of Tartu University. Yet there are three teachers, who have influenced and inspired me the most. Ago, Sergei and Reet have taught me how to ask and solve questions that no one else asks, they have helped me to become a scientist.

To Ago I am exceptionally thankful for inviting me to join his workgroup during my first year of studies. I hold in high regard how he has managed to guide me to become a confident and methodical person by providing me with so many different interesting or necessary tasks. I thank Ago for building a constructive working atmosphere, where I could always speak my mind.

Sergei is my true scientific role model. Confident and widely skilled, incredibly well read, Sergei is also a very warm person and a fascinating conversationalist. It has been a true privilege to work side by side with Sergei, to learn from his experience and benefit from his ideas and advice.

I want to thank Reet, my very first supervisor, for teaching me how to be and what to do in the laboratory. We made a great team. Working with Reet has resulted in a strong professional and personal friendship that I treasure very much.

I would like to acknowledge all the dedicated people who I have had the pleasure to work with during these years. Laura was the true sun of our laboratory. She was and still is an inspiration to me – always fully devoted to anything she takes on. My dear friends Anni and Santa have been my support system during the past years. New ideas along with scientific enthusiasm were born from our countless discussions. I want to thank everyone in our lab, who have helped me out and supported me during the very busy times. The opportunity to supervise and guide bachelor students Laura, Juuli, Tanel, Rainer and Maris has taught me quite a lot. Thank You for always asking the tough questions, and thank you for the easy ones that I actually knew how to answer!

I am very lucky to have so many friends in different fields of science. From our institute special thanks go to all my friends from chairs of analytical and bioorganic chemistry. I want to send my regards to all of Imaginaarkuivikud and to Karl, who has been like a brother to me during the ten years at the university.

My family has always supported the decisions I have made, even if choosing chemistry sounded a little “too hard” at first. Thank You for being there for me.

I am happy to have Siim in my life. He makes me want be the best.

PUBLICATIONS

CURRICULUM VITAE

Name: Olga Mazina
Date of birth: November 20, 1986
Citizenship: Estonian
Address: University of Tartu, Institute of Chemistry
Ravila 14a, 50411, Tartu, Estonia
E-mail: olga.mazina@ut.ee
Language skills: Estonian, English, Russian, German

Education:

1993–2005	German Gymnasium Tallinn
2005–2009	University of Tartu, BSc in chemistry
2009–2011	University of Tartu, MSc in chemistry, *cum laude*
2011…	University of Tartu, PhD student in chemistry

Professional employment:

2010 …	University of Tartu, Institute of Chemistry, chemist
2015 …	Competence Centre on Health Technologies, specialist
2010–2014	Competence Centre on Reproductive Medicine and Biology, specialist

Scientific publications:

1. **Mazina, O.**, Reinart-Okugbeni, R., Kopanchuk, S., & Rinken, A. (2012). BacMam system for FRET-based cAMP sensor expression in studies of melanocortin MC1 receptor activation. *Journal of biomolecular screening*, *17*(8), 1096–1101.
2. **Mazina, O.**, Tõntson, L., Veiksina, S., Kopanchuk, S., & Rinken, A. (2013). Application of Baculovirus Technology for Studies of G Protein-Coupled Receptor Signaling. In *Nanomaterials Imaging Techniques, Surface Studies, and Applications* (pp. 339–348). Springer New York.
3. Dzirkale, Z., Rumaks, J., Svirskis, S., **Mazina, O.**, Allikalt, A., Rinken, A., ... & Klusa, V. (2013). Lunasin-induced behavioural effects in mice: focus on the dopaminergic system. *Behavioural brain research*, *256*, 5–9.
4. **Mazina, O.**, Allikalt, A., Heinloo, A., Reinart-Okugbeni, R., Kopanchuk, S., & Rinken, A. (2015). cAMP Assay for GPCR Ligand Characterization: Application of BacMam Expression System. In *G Protein-Coupled Receptor Screening Assays* (pp. 65–77). Springer New York.
5. Veiksina, S., Kopanchuk, S., **Mazina, O.**, Link, R., Lille, A., & Rinken, A. (2015). Homogeneous Fluorescence Anisotropy-Based Assay for Characterization of Ligand Binding Dynamics to GPCRs in Budded Baculoviruses: The Case of Cy3B-NDP-α-MSH Binding to MC4 Receptors. In *G Protein-Coupled Receptor Screening Assays* (pp. 37–50). Springer New York.
6. **Mazina, O.**, Luik, T., Kopanchuk, S., Salumets, A., & Rinken, A. (2015). Characterization of the Biological Activities of Human Luteinizing Hormone and Chorionic Gonadotropin by a Förster Resonance Energy Transfer-based Biosensor Assay. Accepted for publication in *Analytical Letters*.

ELULOOKIRJELDUS

Nimi: Olga Mazina
Sünniaeg: 20. november, 1986
Kodakondsus: eesti
Aadress: Tartu Ülikool, Keemia Instituut
Ravila 14a, 50411, Tartu, Estonia
E-post: olga.mazina@ut.ee
Keelteoskus: eesti, inglise, vene, saksa

Haridus:

1993–2005	Tallinna Saksa Gümnaasium
2005–2009	Tartu Ülikool, BSc keemias
2009–2011	Tartu Ülikool, MSc keemias, *cum laude*
2011…	Tartu Ülikool, doktoriõpe keemias

Teenistuskäik:

2010 …	Tartu Ülikool, Keemia Instituut, keemik
2015 …	Tervisetehnoloogiate Arenduskeskus AS, spetsialist
2010–2014	Reproduktiivmeditsiini Teadusarenduskeskus AS, spetsialist

Teaduspublikatsioonid:

1. **Mazina, O.**, Reinart-Okugbeni, R., Kopanchuk, S., & Rinken, A. (2012). BacMam system for FRET-based cAMP sensor expression in studies of melanocortin MC1 receptor activation. *Journal of biomolecular screening*, *17*(8), 1096–1101.
2. **Mazina, O.**, Tõntson, L., Veiksina, S., Kopanchuk, S., & Rinken, A. (2013). Application of Baculovirus Technology for Studies of G Protein-Coupled Receptor Signaling. In *Nanomaterials Imaging Techniques, Surface Studies, and Applications* (pp. 339–348). Springer New York.
3. Dzirkale, Z., Rumaks, J., Svirskis, S., **Mazina, O.**, Allikalt, A., Rinken, A., ... & Klusa, V. (2013). Lunasin-induced behavioural effects in mice: focus on the dopaminergic system. *Behavioural brain research*, *256*, 5–9.
4. **Mazina, O.**, Allikalt, A., Heinloo, A., Reinart-Okugbeni, R., Kopanchuk, S., & Rinken, A. (2015). cAMP Assay for GPCR Ligand Characterization: Application of BacMam Expression System. In *G Protein-Coupled Receptor Screening Assays* (pp. 65–77). Springer New York.
5. Veiksina, S., Kopanchuk, S., **Mazina, O.**, Link, R., Lille, A., & Rinken, A. (2015). Homogeneous Fluorescence Anisotropy-Based Assay for Characterization of Ligand Binding Dynamics to GPCRs in Budded Baculoviruses: The Case of Cy3B-NDP-α-MSH Binding to MC4 Receptors. In *G Protein-Coupled Receptor Screening Assays* (pp. 37–50). Springer New York.
6. **Mazina, O.**, Luik, T., Kopanchuk, S., Salumets, A., & Rinken, A. (2015). Characterization of the Biological Activities of Human Luteinizing Hormone and Chorionic Gonadotropin by a Förster Resonance Energy Transfer-based Biosensor Assay. Accepted for publication in *Analytical Letters*.

DISSERTATIONES CHIMICAE UNIVERSITATIS TARTUENSIS

1. **Toomas Tamm.** Quantum-chemical simulation of solvent effects. Tartu, 1993, 110 p.
2. **Peeter Burk.** Theoretical study of gas-phase acid-base equilibria. Tartu, 1994, 96 p.
3. **Victor Lobanov.** Quantitative structure-property relationships in large descriptor spaces. Tartu, 1995, 135 p.
4. **Vahur Mäemets.** The ^{17}O and ^{1}H nuclear magnetic resonance study of H_2O in individual solvents and its charged clusters in aqueous solutions of electrolytes. Tartu, 1997, 140 p.
5. **Andrus Metsala.** Microcanonical rate constant in nonequilibrium distribution of vibrational energy and in restricted intramolecular vibrational energy redistribution on the basis of slater's theory of unimolecular reactions. Tartu, 1997, 150 p.
6. **Uko Maran.** Quantum-mechanical study of potential energy surfaces in different environments. Tartu, 1997, 137 p.
7. **Alar Jänes.** Adsorption of organic compounds on antimony, bismuth and cadmium electrodes. Tartu, 1998, 219 p.
8. **Kaido Tammeveski.** Oxygen electroreduction on thin platinum films and the electrochemical detection of superoxide anion. Tartu, 1998, 139 p.
9. **Ivo Leito.** Studies of Brønsted acid-base equilibria in water and non-aqueous media. Tartu, 1998, 101 p.
10. **Jaan Leis.** Conformational dynamics and equilibria in amides. Tartu, 1998, 131 p.
11. **Toonika Rinken.** The modelling of amperometric biosensors based on oxidoreductases. Tartu, 2000, 108 p.
12. **Dmitri Panov.** Partially solvated Grignard reagents. Tartu, 2000, 64 p.
13. **Kaja Orupõld.** Treatment and analysis of phenolic wastewater with microorganisms. Tartu, 2000, 123 p.
14. **Jüri Ivask.** Ion Chromatographic determination of major anions and cations in polar ice core. Tartu, 2000, 85 p.
15. **Lauri Vares.** Stereoselective Synthesis of Tetrahydrofuran and Tetrahydropyran Derivatives by Use of Asymmetric Horner-Wadsworth-Emmons and Ring Closure Reactions. Tartu, 2000, 184 p.
16. **Martin Lepiku.** Kinetic aspects of dopamine D_2 receptor interactions with specific ligands. Tartu, 2000, 81 p.
17. **Katrin Sak.** Some aspects of ligand specificity of P2Y receptors. Tartu, 2000, 106 p.
18. **Vello Pällin.** The role of solvation in the formation of iotsitch complexes. Tartu, 2001, 95 p.

19. **Katrin Kollist.** Interactions between polycyclic aromatic compounds and humic substances. Tartu, 2001, 93 p.
20. **Ivar Koppel.** Quantum chemical study of acidity of strong and superstrong Brønsted acids. Tartu, 2001, 104 p.
21. **Viljar Pihl.** The study of the substituent and solvent effects on the acidity of OH and CH acids. Tartu, 2001, 132 p.
22. **Natalia Palm.** Specification of the minimum, sufficient and significant set of descriptors for general description of solvent effects. Tartu, 2001, 134 p.
23. **Sulev Sild.** QSPR/QSAR approaches for complex molecular systems. Tartu, 2001, 134 p.
24. **Ruslan Petrukhin.** Industrial applications of the quantitative structure-property relationships. Tartu, 2001, 162 p.
25. **Boris V. Rogovoy.** Synthesis of (benzotriazolyl)carboximidamides and their application in relations with *N*- and *S*-nucleophyles. Tartu, 2002, 84 p.
26. **Koit Herodes.** Solvent effects on UV-vis absorption spectra of some solvatochromic substances in binary solvent mixtures: the preferential solvation model. Tartu, 2002, 102 p.
27. **Anti Perkson.** Synthesis and characterisation of nanostructured carbon. Tartu, 2002, 152 p.
28. **Ivari Kaljurand.** Self-consistent acidity scales of neutral and cationic Brønsted acids in acetonitrile and tetrahydrofuran. Tartu, 2003, 108 p.
29. **Karmen Lust.** Adsorption of anions on bismuth single crystal electrodes. Tartu, 2003, 128 p.
30. **Mare Piirsalu.** Substituent, temperature and solvent effects on the alkaline hydrolysis of substituted phenyl and alkyl esters of benzoic acid. Tartu, 2003, 156 p.
31. **Meeri Sassian.** Reactions of partially solvated Grignard reagents. Tartu, 2003, 78 p.
32. **Tarmo Tamm.** Quantum chemical modelling of polypyrrole. Tartu, 2003. 100 p.
33. **Erik Teinemaa.** The environmental fate of the particulate matter and organic pollutants from an oil shale power plant. Tartu, 2003. 102 p.
34. **Jaana Tammiku-Taul.** Quantum chemical study of the properties of Grignard reagents. Tartu, 2003. 120 p.
35. **Andre Lomaka.** Biomedical applications of predictive computational chemistry. Tartu, 2003. 132 p.
36. **Kostyantyn Kirichenko.** Benzotriazole – Mediated Carbon–Carbon Bond Formation. Tartu, 2003. 132 p.
37. **Gunnar Nurk.** Adsorption kinetics of some organic compounds on bismuth single crystal electrodes. Tartu, 2003, 170 p.
38. **Mati Arulepp.** Electrochemical characteristics of porous carbon materials and electrical double layer capacitors. Tartu, 2003, 196 p.

39. **Dan Cornel Fara.** QSPR modeling of complexation and distribution of organic compounds. Tartu, 2004, 126 p.
40. **Riina Mahlapuu.** Signalling of galanin and amyloid precursor protein through adenylate cyclase. Tartu, 2004, 124 p.
41. **Mihkel Kerikmäe.** Some luminescent materials for dosimetric applications and physical research. Tartu, 2004, 143 p.
42. **Jaanus Kruusma.** Determination of some important trace metal ions in human blood. Tartu, 2004, 115 p.
43. **Urmas Johanson.** Investigations of the electrochemical properties of polypyrrole modified electrodes. Tartu, 2004, 91 p.
44. **Kaido Sillar.** Computational study of the acid sites in zeolite ZSM-5. Tartu, 2004, 80 p.
45. **Aldo Oras.** Kinetic aspects of dATPαS interaction with $P2Y_1$ receptor. Tartu, 2004, 75 p.
46. **Erik Mölder.** Measurement of the oxygen mass transfer through the air-water interface. Tartu, 2005, 73 p.
47. **Thomas Thomberg.** The kinetics of electroreduction of peroxodisulfate anion on cadmium (0001) single crystal electrode. Tartu, 2005, 95 p.
48. **Olavi Loog.** Aspects of condensations of carbonyl compounds and their imine analogues. Tartu, 2005, 83 p.
49. **Siim Salmar.** Effect of ultrasound on ester hydrolysis in aqueous ethanol. Tartu, 2006, 73 p.
50. **Ain Uustare.** Modulation of signal transduction of heptahelical receptors by other receptors and G proteins. Tartu, 2006, 121 p.
51. **Sergei Yurchenko.** Determination of some carcinogenic contaminants in food. Tartu, 2006, 143 p.
52. **Kaido Tämm.** QSPR modeling of some properties of organic compounds. Tartu, 2006, 67 p.
53. **Olga Tšubrik.** New methods in the synthesis of multisubstituted hydrazines. Tartu. 2006, 183 p.
54. **Lilli Sooväli.** Spectrophotometric measurements and their uncertainty in chemical analysis and dissociation constant measurements. Tartu, 2006, 125 p.
55. **Eve Koort.** Uncertainty estimation of potentiometrically measured ph and pK_a values. Tartu, 2006, 139 p.
56. **Sergei Kopanchuk.** Regulation of ligand binding to melanocortin receptor subtypes. Tartu, 2006, 119 p.
57. **Silvar Kallip.** Surface structure of some bismuth and antimony single crystal electrodes. Tartu, 2006, 107 p.
58. **Kristjan Saal.** Surface silanization and its application in biomolecule coupling. Tartu, 2006, 77 p.
59. **Tanel Tätte.** High viscosity $Sn(OBu)_4$ oligomeric concentrates and their applications in technology. Tartu, 2006, 91 p.

60. **Dimitar Atanasov Dobchev**. Robust QSAR methods for the prediction of properties from molecular structure. Tartu, 2006, 118 p.
61. **Hannes Hagu**. Impact of ultrasound on hydrophobic interactions in solutions. Tartu, 2007, 81 p.
62. **Rutha Jäger.** Electroreduction of peroxodisulfate anion on bismuth electrodes. Tartu, 2007, 142 p.
63. **Kaido Viht.** Immobilizable bisubstrate-analogue inhibitors of basophilic protein kinases: development and application in biosensors. Tartu, 2007, 88 p.
64. **Eva-Ingrid Rõõm.** Acid-base equilibria in nonpolar media. Tartu, 2007, 156 p.
65. **Sven Tamp.** DFT study of the cesium cation containing complexes relevant to the cesium cation binding by the humic acids. Tartu, 2007, 102 p.
66. **Jaak Nerut.** Electroreduction of hexacyanoferrate(III) anion on Cadmium (0001) single crystal electrode. Tartu, 2007, 180 p.
67. **Lauri Jalukse.** Measurement uncertainty estimation in amperometric dissolved oxygen concentration measurement. Tartu, 2007, 112 p.
68. **Aime Lust.** Charge state of dopants and ordered clusters formation in CaF_2:Mn and CaF_2:Eu luminophors. Tartu, 2007, 100 p.
69. **Iiris Kahn**. Quantitative Structure-Activity Relationships of environmentally relevant properties. Tartu, 2007, 98 p.
70. **Mari Reinik.** Nitrates, nitrites, N-nitrosamines and polycyclic aromatic hydrocarbons in food: analytical methods, occurrence and dietary intake. Tartu, 2007, 172 p.
71. **Heili Kasuk.** Thermodynamic parameters and adsorption kinetics of organic compounds forming the compact adsorption layer at Bi single crystal electrodes. Tartu, 2007, 212 p.
72. **Erki Enkvist.** Synthesis of adenosine-peptide conjugates for biological applications. Tartu, 2007, 114 p.
73. **Svetoslav Hristov Slavov**. Biomedical applications of the QSAR approach. Tartu, 2007, 146 p.
74. **Eneli Härk.** Electroreduction of complex cations on electrochemically polished Bi(*hkl*) single crystal electrodes. Tartu, 2008, 158 p.
75. **Priit Möller.** Electrochemical characteristics of some cathodes for medium temperature solid oxide fuel cells, synthesized by solid state reaction technique. Tartu, 2008, 90 p.
76. **Signe Viggor.** Impact of biochemical parameters of genetically different pseudomonads at the degradation of phenolic compounds. Tartu, 2008, 122 p.
77. **Ave Sarapuu.** Electrochemical reduction of oxygen on quinone-modified carbon electrodes and on thin films of platinum and gold. Tartu, 2008, 134 p.
78. **Agnes Kütt.** Studies of acid-base equilibria in non-aqueous media. Tartu, 2008, 198 p.

79. **Rouvim Kadis.** Evaluation of measurement uncertainty in analytical chemistry: related concepts and some points of misinterpretation. Tartu, 2008, 118 p.
80. **Valter Reedo.** Elaboration of IVB group metal oxide structures and their possible applications. Tartu, 2008, 98 p.
81. **Aleksei Kuznetsov.** Allosteric effects in reactions catalyzed by the cAMP-dependent protein kinase catalytic subunit. Tartu, 2009, 133 p.
82. **Aleksei Bredihhin.** Use of mono- and polyanions in the synthesis of multisubstituted hydrazine derivatives. Tartu, 2009, 105 p.
83. **Anu Ploom.** Quantitative structure-reactivity analysis in organosilicon chemistry. Tartu, 2009, 99 p.
84. **Argo Vonk.** Determination of adenosine A_{2A}- and dopamine D_1 receptor-specific modulation of adenylate cyclase activity in rat striatum. Tartu, 2009, 129 p.
85. **Indrek Kivi.** Synthesis and electrochemical characterization of porous cathode materials for intermediate temperature solid oxide fuel cells. Tartu, 2009, 177 p.
86. **Jaanus Eskusson.** Synthesis and characterisation of diamond-like carbon thin films prepared by pulsed laser deposition method. Tartu, 2009, 117 p.
87. **Marko Lätt.** Carbide derived microporous carbon and electrical double layer capacitors. Tartu, 2009, 107 p.
88. **Vladimir Stepanov.** Slow conformational changes in dopamine transporter interaction with its ligands. Tartu, 2009, 103 p.
89. **Aleksander Trummal.** Computational Study of Structural and Solvent Effects on Acidities of Some Brønsted Acids. Tartu, 2009, 103 p.
90. **Eerold Vellemäe.** Applications of mischmetal in organic synthesis. Tartu, 2009, 93 p.
91. **Sven Parkel.** Ligand binding to 5-HT_{1A} receptors and its regulation by Mg^{2+} and Mn^{2+}. Tartu, 2010, 99 p.
92. **Signe Vahur.** Expanding the possibilities of ATR-FT-IR spectroscopy in determination of inorganic pigments. Tartu, 2010, 184 p.
93. **Tavo Romann**. Preparation and surface modification of bismuth thin film, porous, and microelectrodes. Tartu, 2010, 155 p.
94. **Nadežda Aleksejeva.** Electrocatalytic reduction of oxygen on carbon nanotube-based nanocomposite materials. Tartu, 2010, 147 p.
95. **Marko Kullapere.** Electrochemical properties of glassy carbon, nickel and gold electrodes modified with aryl groups. Tartu, 2010, 233 p.
96. **Liis Siinor.** Adsorption kinetics of ions at Bi single crystal planes from aqueous electrolyte solutions and room-temperature ionic liquids. Tartu, 2010, 101 p.
97. **Angela Vaasa.** Development of fluorescence-based kinetic and binding assays for characterization of protein kinases and their inhibitors. Tartu 2010, 101 p.

98. **Indrek Tulp.** Multivariate analysis of chemical and biological properties. Tartu 2010, 105 p.
99. **Aare Selberg.** Evaluation of environmental quality in Northern Estonia by the analysis of leachate. Tartu 2010, 117 p.
100. **Darja Lavõgina.** Development of protein kinase inhibitors based on adenosine analogue-oligoarginine conjugates. Tartu 2010, 248 p.
101. **Laura Herm.** Biochemistry of dopamine D_2 receptors and its association with motivated behaviour. Tartu 2010, 156 p.
102. **Terje Raudsepp.** Influence of dopant anions on the electrochemical properties of polypyrrole films. Tartu 2010, 112 p.
103. **Margus Marandi.** Electroformation of Polypyrrole Films: *In-situ* AFM and STM Study. Tartu 2011, 116 p.
104. **Kairi Kivirand.** Diamine oxidase-based biosensors: construction and working principles. Tartu, 2011, 140 p.
105. **Anneli Kruve.** Matrix effects in liquid-chromatography electrospray mass-spectrometry. Tartu, 2011, 156 p.
106. **Gary Urb.** Assessment of environmental impact of oil shale fly ash from PF and CFB combustion. Tartu, 2011, 108 p.
107. **Nikita Oskolkov.** A novel strategy for peptide-mediated cellular delivery and induction of endosomal escape. Tartu, 2011, 106 p.
108. **Dana Martin.** The QSPR/QSAR approach for the prediction of properties of fullerene derivatives. Tartu, 2011, 98 p.
109. **Säde Viirlaid.** Novel glutathione analogues and their antioxidant activity. Tartu, 2011, 106 p.
110. **Ülis Sõukand.** Simultaneous adsorption of Cd^{2+}, Ni^{2+}, and Pb^{2+} on peat. Tartu, 2011, 124 p.
111. **Lauri Lipping.** The acidity of strong and superstrong Brønsted acids, an outreach for the "limits of growth": a quantum chemical study. Tartu, 2011, 124 p.
112. **Heisi Kurig.** Electrical double-layer capacitors based on ionic liquids as electrolytes. Tartu, 2011, 146 p.
113. **Marje Kasari.** Bisubstrate luminescent probes, optical sensors and affinity adsorbents for measurement of active protein kinases in biological samples. Tartu, 2012, 126 p.
114. **Kalev Takkis.** Virtual screening of chemical databases for bioactive molecules. Tartu, 2012, 122 p.
115. **Ksenija Kisseljova**. Synthesis of aza-β^3-amino acid containing peptides and kinetic study of their phosphorylation by protein kinase A. Tartu, 2012, 104 p.
116. **Riin Rebane.** Advanced method development strategy for derivatization LC/ESI/MS. Tartu, 2012, 184 p.

117. **Vladislav Ivaništšev.** Double layer structure and adsorption kinetics of ions at metal electrodes in room temperature ionic liquids. Tartu, 2012, 128 p.
118. **Irja Helm.** High accuracy gravimetric Winkler method for determination of dissolved oxygen. Tartu, 2012, 139 p.
119. **Karin Kipper.** Fluoroalcohols as Components of LC-ESI-MS Eluents: Usage and Applications. Tartu, 2012, 164 p.
120. **Arno Ratas.** Energy storage and transfer in dosimetric luminescent materials. Tartu, 2012, 163 p.
121. **Reet Reinart-Okugbeni**. Assay systems for characterisation of subtype-selective binding and functional activity of ligands on dopamine receptors. Tartu, 2012, 159 p.
122. **Lauri Sikk.** Computational study of the Sonogashira cross-coupling reaction. Tartu, 2012, 81 p.
123. **Karita Raudkivi.** Neurochemical studies on inter-individual differences in affect-related behaviour of the laboratory rat. Tartu, 2012, 161 p.
124. **Indrek Saar.** Design of GalR2 subtype specific ligands: their role in depression-like behavior and feeding regulation. Tartu, 2013, 126 p.
125. **Ann Laheäär.** Electrochemical characterization of alkali metal salt based non-aqueous electrolytes for supercapacitors. Tartu, 2013, 127 p.
126. **Kerli Tõnurist.** Influence of electrospun separator materials properties on electrochemical performance of electrical double-layer capacitors. Tartu, 2013, 147 p.
127. **Kaija Põhako-Esko.** Novel organic and inorganic ionogels: preparation and characterization. Tartu, 2013, 124 p.
128. **Ivar Kruusenberg.** Electroreduction of oxygen on carbon nanomaterial-based catalysts. Tartu, 2013, 191 p.
129. **Sander Piiskop.** Kinetic effects of ultrasound in aqueous acetonitrile solutions. Tartu, 2013, 95 p.
130. **Ilona Faustova**. Regulatory role of L-type pyruvate kinase N-terminal domain. Tartu, 2013, 109 p.
131. **Kadi Tamm.** Synthesis and characterization of the micro-mesoporous anode materials and testing of the medium temperature solid oxide fuel cell single cells. Tartu, 2013, 138 p.
132. **Iva Bozhidarova Stoyanova-Slavova.** Validation of QSAR/QSPR for regulatory purposes. Tartu, 2013, 109 p.
133. **Vitali Grozovski**. Adsorption of organic molecules at single crystal electrodes studied by *in situ* STM method. Tartu, 2014, 146 p.
134. **Santa Veikšina**. Development of assay systems for characterisation of ligand binding properties to melanocortin 4 receptors. Tartu, 2014, 151 p.
135. **Jüri Liiv.** PVDF (polyvinylidene difluoride) as material for active element of twisting-ball displays. Tartu, 2014, 111 p.

136. **Kersti Vaarmets.** Electrochemical and physical characterization of pristine and activated molybdenum carbide-derived carbon electrodes for the oxygen electroreduction reaction. Tartu, 2014, 131 p.
137. **Lauri Tõntson**. Regulation of G-protein subtypes by receptors, guanine nucleotides and Mn^{2+}. Tartu, 2014, 105 p.
138. **Aiko Adamson.** Properties of amine-boranes and phosphorus analogues in the gas phase. Tartu, 2014, 78 p.
139. **Elo Kibena**. Electrochemical grafting of glassy carbon, gold, highly oriented pyrolytic graphite and chemical vapour deposition-grown graphene electrodes by diazonium reduction method. Tartu, 2014, 184 p.
140. **Teemu Näykki.** Novel Tools for Water Quality Monitoring – From Field to Laboratory. Tartu, 2014, 202 p.
141. **Karl Kaupmees.** Acidity and basicity in non-aqueous media: importance of solvent properties and purity. Tartu, 2014, 128 p.
142. **Oleg Lebedev**. Hydrazine polyanions: different strategies in the synthesis of heterocycles. Tartu, 2015, 118 p.
143. **Geven Piir.** Environmental risk assessment of chemicals using QSAR methods. Tartu, 2015, 123 p.