Master thesis for the degree master of pharmacy

The assessment of the dynamics of cAMP hydrolysis

Changes in cAMP and cGMP in the presence of mutant PDE3A

university of groningen

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Abstract

Background: Cardiovascular diseases are the leading cause of death worldwide and the main risk factor is high blood pressure.(1) Current treatment options for heart failure involving phosphodiesterase inhibitors have shown increased mortality in patients.(2) Autosomal dominant hypertension, a salt-resistant form of hypertension with brachydactyly causes death by stroke before the age of 50.(3) Previous studies have elucidated that the genetic basis for this condition can be found in the PDE3A gene. However, how mutants of PDE3A affect cAMP levels and the influence on PKA activity has yet to be evaluated.

In order to characterized this in this study, immunoaffinity, purification, immunoblotting, Alphascreen analysis and protein-protein overlay were used. The purified PDE3A mutants as well as the wildtype show significant hydrolysing properties of cAMP after 6 and 30 minutes of incubation. The binding sites of PDE3A1 WT to PKA were identified. More research should be conducted to quantify the hydrolysing properties of the PDE3A isoforms PDE3A1 and PDE3A2 and compare these to the wildtype. A peptag assay could elucidate possible changes in kinase activity of the mutants. Differential regulation of catalytic activity by

We focused on 2 different isoforms: alternative phosporylation of PDE3A1 and PDE3A2 could gain more attention for development of alternative isoform selective PDE3 inhibitors that increase contractility in patients with heart failure without increasing mortality.

Preface

Cardiovascular disease is responsible for the largest fraction of deaths worldwide with high blood pressure as the main risk factor. The second messengers cyclic 3',5'- adenosine monophosphate and cyclic 3',5'-guanosine monophosphate, in this document further referred to as cAMP and cGMP, are important key factors in regulating blood pressure that are broken down by enzymes called phosphodiesterases (PDEs). Over the years, several families have been identified to present genetic mutations of these PDEs that cause them to have very high bloodpressure, that if left untreated, causes not rarely, death by stroke before the age of 50. This study focuses on assessing the hydrolysis of cAMP and cGMP by these mutants of PDE, specifically PDE3A and how this is different from the wild type PDE3A. Moreover, we will look into the protein overlay, to gain information about the dynamics of PDE3A and kinase binding.

Inhoudsopgave

Introduction

1.1 Hypertension and cardiovascular disease

Cardiovascular diseases (CVD) are responsible for the largest fraction of deaths worldwide. The main risk factor for cardiovascular disease is hypertension (HTN)(4) which is commonly known as high blood pressure (HP). Despite new clinical guidelines and a broad range of pharmaceuticals, it remains a mounting threat to modern society in terms of health(5).

The two main strategies to lower BP are lifestyle interventions and drug treatment. In some cases lifestyle interventions have been proven to be effective for lowering BP and sometimes even the risk of CVD. Most patients with hypertension will however require drug treatment, in addition to lifestyle interventions. The drug treatment of hypertension has been proven by very solid evidence and has been underpinned by the largest number of outcome based Randomised Control trials (RCTs) in clinical medicine. Meta analysis of these studies including thousands of patients have shown that a 10 mmHg reduction in systolic blood pressure (SBP), or a 5 mmHg reduction in diastolic blood pressure (DBP) reduces all major CV events by 20%. It reduces all-cause mortality by 10-15%, stroke by 35%, coronary events by 20% and heart failure by 40%. Moreover, these relative risk reductions are consistent and irrespective of baseline BP (within the hypertensive range), comorbidities (e.g. diabetes, CKD), the level of CV risk, age, sex and ethnicity(6). The European Guidelines for management of arterial hypertension provided by the European Society of Hypertension (ESH) and the European Society of Cardiology (ESC)(6), recommend five major drug classes for the treatment of hypertension: ACE (Angiotensin converting enzyme) inhibitors, ARBs (Angiotensin II receptor blockers), beta-blockers, CCBs (Calcium channel blockers) and diuretics. Placebo controlled studies have shown that they reduce BP and CV events and are associated with lower overall CV morbidity and mortality. Meta analyses have confirmed that this benefit predominantly derives from lowering BP. These analyses have reported some cause specific differences in outcome between drugs, but overall major CV outcomes and mortality were similar for initial therapies with all five major classes of drugs. Other classes of drugs (e.g. alpha blockers) have been studied less widely in RCTs until now and have shown to have a higher risk of adverse effects. However they have proven to be useful for patients whose BP cannot be controlled by combinations of the 5 classes mentioned previously(6).

The cause of hypertension is often not known and the traditional classification of hypertension therefore distinguishes between primary hypertension, in which the cause is not a known disease, and secondary hypertension where the hypertension is the result of a second diagnosed condition. Numerous studies(7)(8) have focused on finding the causes of primary hypertension, and several theories that could explain why BP increases with age have caught attention. One of the most prevalent hypotheses for the cause of hypertension is the relationship between excessive salt intake and increasing blood pressure with age. And however epidemiological studies have claimed that a direct association can be found between excessive sodium intake and the prevalence and incidence of hypertension, these cohort studies were often inconclusive and merely showed variable individual sensitivity to sodium loading(9).

Not only is hypertension the main risk factor for CVD, it's prevalence has been continuously increasing and regardless of advances in anti-hypertensive drug therapy, blood pressure control has been inadequate(5). The need for novel treatment options is therefore evident.

Introduction to hypertension and cardiovascular disease on a molecular level

The adenylyl cyclase/cAMP system

cAMP is a nucleotide that is synthesized in the cell from ATP by the action of soluble and membrane bound enzyme adenylyl cyclase after activation by G-protein coupled receptors (GPCRs). Various levels of cAMP are continuously present in the cell and cAMP is inactivated by phosphodiesterases (PDEs). Phospodiesterases catalyze the stereospecific hydrolysis of the second messengers adenosine 3',5' – cyclic monophosphate and guanosine 2',5' – cyclic monophosphate to respectively 5'- AMP and 5'-GMP by breaking the phosphodiester bond of the molecule.(10)

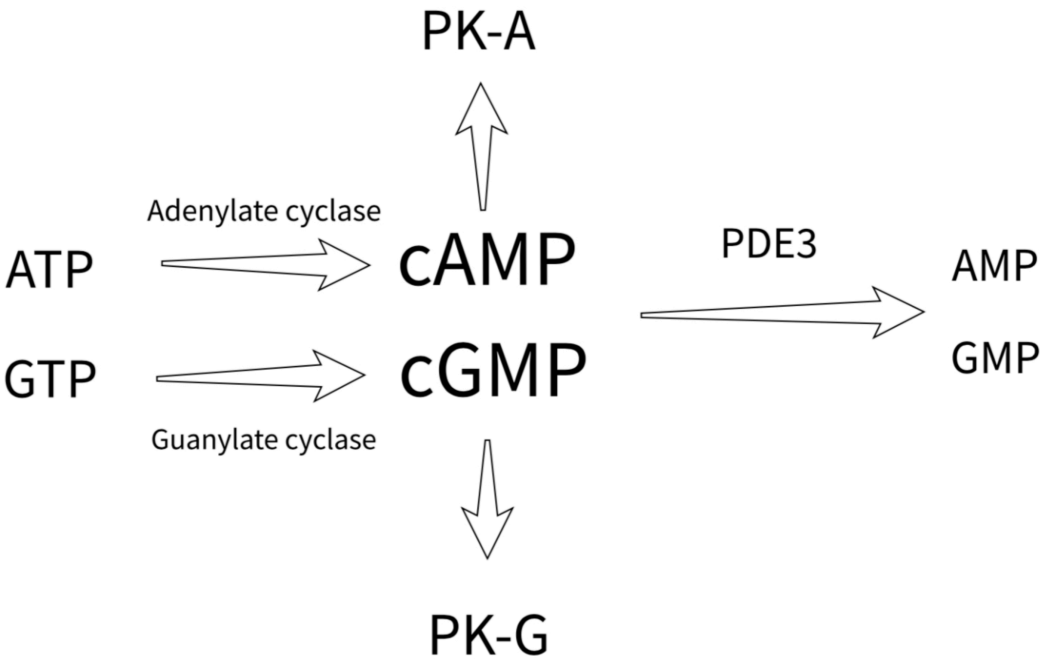


Figure 1 ***The role of PDE3 in cAMP- and cGMP-mediated signal transduction. PK-A: Protein kinase A (cAMP dependent). PK-G: Protein kinase G (cGMP-dependent).***

Excitation-contraction (EC) coupling and its key proteins in cardiomyocytes

The ubiquitous second messenger Ca^{2+} is essential for EC coupling of cardiac muscle cells as it directly activates the myofilaments that cause muscle contractions. During EC coupling the membrane of the cardiac muscle cell is depolarized which leads to the opening of voltage-

gated Ca^{2+} channels and Ca^{2+} influx, as illustrated in figure 2. The voltage-dependent L-type calcium channel (LTCC) physically accumulates at the transverse tubules (t-tubules) and allows the entry of Ca^{2+} into the myocyte. When the intracellular concentration of Ca^{2+} is increased, the opening of the ryanodine receptor 2 (RyR2) is initiated and the local release of Ca^{2+} from the sarcoplasmatic reticulum (SR) is triggered. The SR serves as the major Ca^{2+} storage of the cardiomyocyte and the release of Ca^{2+} from this organelle results in the contraction of the cardiac muscle. After the increase of cytosolic Ca^{2+}, the Ca^{2+} is then pumped back to the SR by SERCA, a protein on the membrane of the SR, or extruded by sarcolemnal sodium ion (Na+)/Ca^{2+} exchanger (NCX). This induces the relaxation of the muscle. (11) As described before, cardiac contractility is controlled by the amplitude and the kinetics of Ca^{2+} in cardiomyocytes. This is in turn regulated by the phosphorylation and dephosphorylation of key proteins involved in EC coupling as is shown schematically in figure 2.(11) Cyclic adenosine monophosphate (cAMP) is one of the most important second messengers for the regulation of cardiac muscle contraction. This is established primarily through the β-adrenergic signalling pathway, which is the pathway necessary for the effects of catecholamines on cardiac contractility. Catecholamines, (i.e. epinephrine and norepinephrine), bind primarily to the β_1-adrenoreceptors in cardiomyocytes.(12) These receptors are coupled to Gs-proteins that activate adenylyl cyclases which will stimulate the formation of cAMP from ATP. cAMP will activate protein kinase A (PKA). PKA being the main source of phosphorylation of various proteins involved (i.e. LTCC, RyR2 inositol 1,4,5-triphosphate receptor (IP_3R) and phospholamban (PLN). Phosporylation of these proteins, due to increased stimulation by cAMP increases contractility of the cardiac muscle (inotropy), heart rate (chronotropy) and conduction velocity (dromotropy). Therefore, PKA is sometimes referred to as an “on switch” for cardiac contractility while enzymes that hydrolyse cAMP can serve as an “off switch”. (11)

This degradation of cAMP to AMP is catalysed by phosphodiesterases (PDEs) that are regulated by the intracellular nucleotide concentrations, phosphorylation, Ca^{2+} /calmodulin binding and by other regulatory proteins. cAMP efflux out of the cell is mediated by so called cyclic nucleotide efflux transporters. Over time, different genetic models have been created to assess the role of cAMP synthesis and hydrolysis proteins in cardiac physiology.(12)

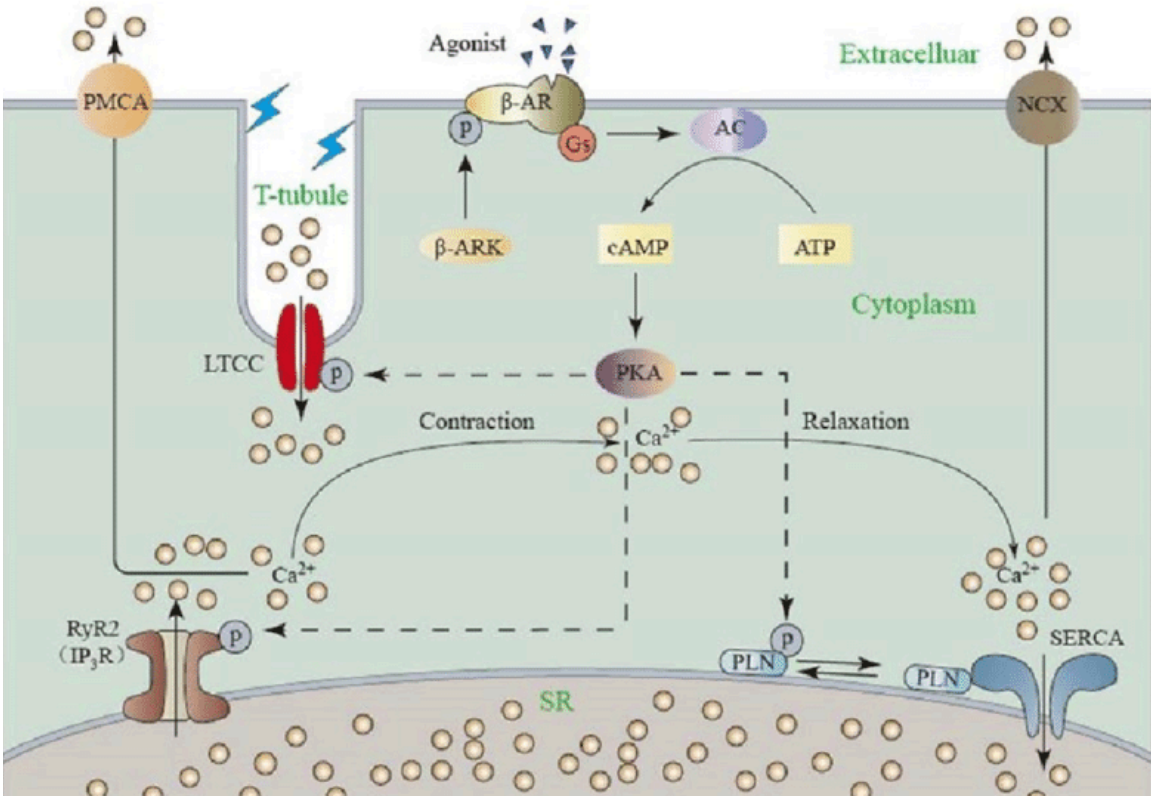


Figure 2 Schematic representation of Ca^{2+} handling in cardiomyocyte during excitation–contraction coupling. A large amount of calcium ions is released from the SR by RyR2 after a small uptake of calcium ions by the LTCC. This results in a contraction of the myofilaments. Relaxation is induced when calcium ions are pumped back into the SR by SERCA or NCX. LTCC: L-type calcium channel; RyR2: ryanodine receptor 2; IP3R: inositol 1,4,5-trisphosphate (IP3) receptor; NCX: Na+/Ca2+ exchange; PLN: phospholamban; SR: sarcoplasmic reticulum.(11)

Control of the vascular smooth muscle tone

Vascular smooth muscle cells (VSMCs) are controlled by mediators that are secreted by sympathetic nerves, vascular endothelium and circulating hormones. As is shown in figure 3, the contraction of a smooth muscle cell is initiated by a rise in calcium ions [Ca^{2+}] in the cell, which activates myosin light-chain kinase (MLCK) causing the phosphorylation of myosin. Logically, contraction can also be caused by a sensitization of the myofilaments to Ca^{2+} by inhibition of myosin phosphatase. The rise of [Ca^{2+}], can be established by the release of intracellular Ca^{2+} via inositol triphosphate (IP_3), or by depolarization of the membrane, which opens voltage gated calcium channels, causing the entry of Ca^{2+}. This will result in more contraction of the VSMCs. (13)

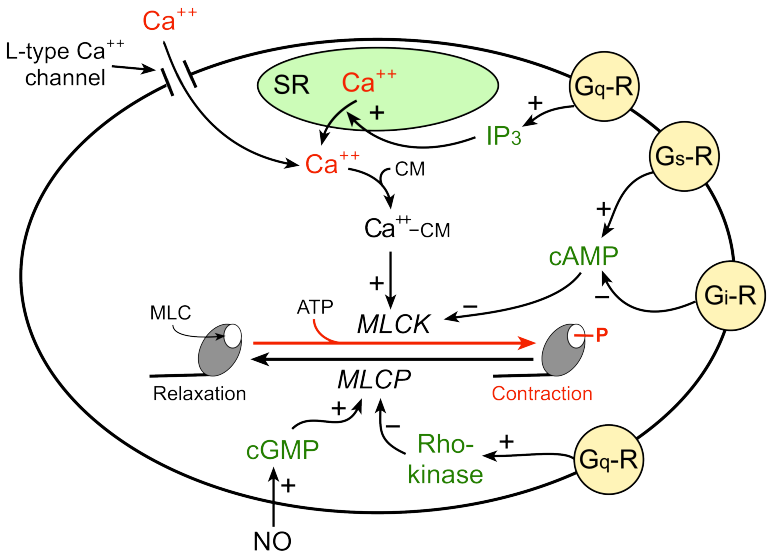


Figure 3 The mechanism by which an increase in intracellular calcium stimulates vascular smooth muscle contraction (schematically)(14)

As described previously, cAMP and cGMP are key in the regulation of cardiac muscle contraction and relaxation. But they also play an important role in the regulation of vascular smooth muscle tone. In cardiac myocytes, an increase in cAMP or cGMP leads to an increase in excitation contraction coupling, whereas in VSMCs, increasing cAMP leads to relaxation by inactivating MLCK and facilitating the efflux of Ca^{2+}, leading to relaxation of the VSMCs. In addition to that, an increase in cGMP levels opposes agonist-induced increases in intracellular [Ca^{2+}] which leads to relaxation of the VSMCs as well. In the VSMCs, as well as in the heart, cAMP and cGMP are broken down by PDE's, specifically PDE3. Therefore, inhibition of PDE(3) will increase intracellular cAMP which further inhibits MLCK and thereby producing less contractile force, promoting relaxation.(13)

cAMP and cGMP catabolism by phosphodiesterases (PDEs) and crosstalk

PDEs hydrolyze the cyclic nucleotides cGMP and cAMP to the inactive guanosine monophosphate (GMP) and adenosine monophosphate (AMP), respectively. They are therefore important for regulating cellular levels of the second messengers cAMP and cGMP. PDEs can be divided into 11 families, PDE1 to PDE11. 7 of these families have been investigated in the heart (i.e. PDE1, PDE2, PDE3, PDE4, PDE5, PDE8 and PDE9). PDE1, PDE2, PDE3, PDE10 and PDE11 are dual-substrate specific which means that they can hydrolyze both cAMP and cGMP. PDE4, PDE7 and PDE8 are specific for cAMP whereas PDE5, PDE6 and PDE9 only hydrolyze cGMP. Dual-substrate specific PDEs, especially PDE2 and PDE3, lead to crosstalk between cAMP and cGMP. PDE3 primarily hydrolyzes cAMP, with a Vmax for cAMP that is 4 to 10 times as high as the Vmax for cGMP), but it can be competitively inhibited by cGMP. PDE3 is therefore sometimes referred to as the cGMP inhibited PDE. This way, increasing levels of cGMP can cause an increase in cAMP levels by inhibiting PDE3. This mechanism is referred to as the so-called positive cGMP to cAMP crosstalk.(15)

Brachydactlyly type E with hypertension

Schuster et al. started elucidating the genetic basis for rare, monogenic inherited forms of hypertension. In a Turkish family, they found a particularly interesting 100% cosegregation between hypertension and shortness of fingers and toes, in medical terms: brachydactyly. Their data indicated that a single genetic defect on chromosome 12 p was responsible for an age-corrected BP difference of 30 mm Hg between affected and unaffected people by the age of 50 in this family with autosomal dominant hypertension and brachydactyly type E (HTNB). In a lot of cases, this condition causes, if left untreated, a stroke before the age of 50.(3) Further research was conducted by among others Maass et al. to find that the specific mutation causing HTNB was in the PDE3A gene. They reported 6 missense mutations in 6 unrelated families that cause this specific syndrome. (16)

Different mutations of PDE3A that cause HTNB and types of mutations

PDE3A exists in 3 different isoforms: PDE3A1, PDE3A2 and PDE3A3. They are all derived from a single gene and the same transcript. They also have the same catalytic domain and their activity and inhibitor sensitivity are similar. The difference however is in the N terminus, as can be seen in figure 4. PDE3A is located in different parts of the human body. PDE3A1 is the major form in the heart whereas PDE3A2 predominantly occurs in the vascular smooth muscle cells. PDE3A3 is mainly found in the placenta. (1)

Mutations that cause HTNB are amino acid substitutions in PDE3A1 and A2 enzymes, N-terminally of the catalytic domain. Ercu et al. and Maass et al. found that all HTNB mutations affect a region of 15 base pairs (bp) in exon 4 of the PDE3A gene that encodes a 5-amino-acid segment (amino acids 445-449), N-terminally of the catalytic domain. As this specific part of the region where this mutation is located is not present in the A3 enzyme, PDE3A3 which lacks this N-terminal extension, is not affected by this type of mutation. Mutants of the A1 and A2 enzyme are hyperactive and the 2 serine residues S428 and S438 are phosphorylated by respectively protein kinase 1 (PKA) and protein kinase C (PKC). Ercu et al. presented a new human PDE3A mutation that causes an amino acid substitution within a 5-amino-acid-long PDE3A region N-terminally of the catalytic domain.(1) They suggested that their findings are relevant to essential hypertension as found in the general population. Their suggestion is supported by the linkage they found in a subfamily of Chinese subjects, which have essential hypertension that coincided with this study's PDE3A locus. Furthermore, 4 independent genome-wide association studies identified this PDE3A locus, while a fifth study discusses its relevance(1).

The PDE3 family plays a prominent role in heart, vascular smooth muscle cells (VSMCs), oocytes, and platelets.(17) Hunter et al.(18) observed that platelet activation stimulates protein kinase C–dependent phosphorylation of PDE3A on multiple serine residues, among others Ser428 and Ser438, which leads to a subsequent increase in cAMP hydrolysis. The molecular causes for HTNB are heterozygous missense, gain-of-function mutations in the gene that encodes the cGMP-inhibited cAMP PDE3A. PDE3A belongs to the PDE3 family, which catalyzes the hydrolysis of intracellular, cAMP and cGMP.

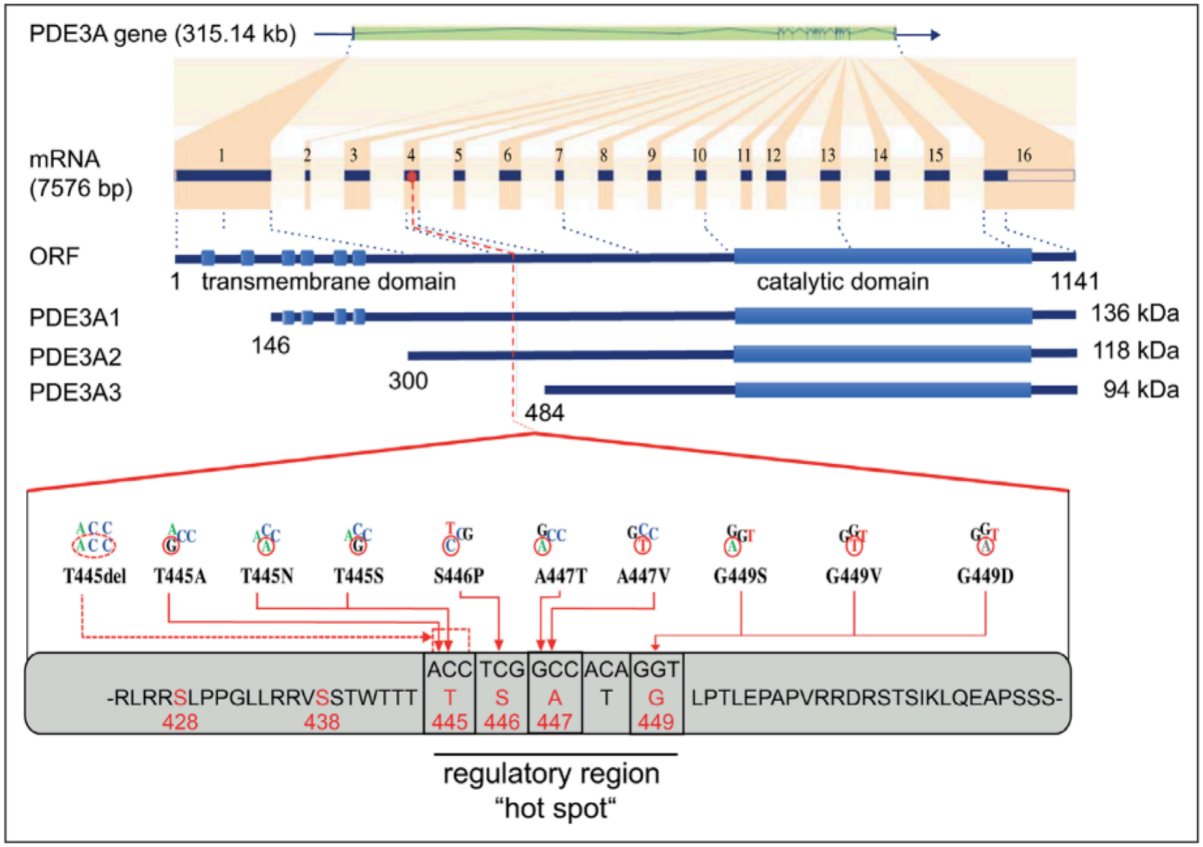


Figure 4 HNTB mutations are located in a 15 bp region of the PDE3A gene

Drugs that act via cyclic nucleotides

There are many drugs known to relax vascular smooth muscle by increasing cellular concentrations of cGMP or cAMP through cyclase activation. For instance, NO, nitrates and the natriuretic peptides act through cGMP. BAY41-2272, a pyrazolopyridine, activates soluble guanylyl cyclase via an NO-independent site. Adenosine and PGI2, 2 β agonists, increase cAMP in the cytoplasm.(13) Another class of drugs that affect vascular smooth muscle and cardiac muscle by regulating cAMP are the phosphodiesterase inhibitors. Inhibitors of cyclic nucleotide phosphodiesterase (PDE3A) increase cardiac contractility in patients with heart failure. Unfortunately, it has become clear that their long-term use increases mortality in these patients.(13)

Methods of measuring cAMP

For a long time, cAMP has been quantified through a radioactive functional assay. This assay is based on affinity chromatography purification using ^{3}H-cAMP over ^{3}H-ATP + ^{3}H-cAMP separated on an affinity column. Although this assay has a high sensitivity, the technique is not suitable for high throughput screening (HTS).

Most of HTS biochemical methods rely on the general principle that cAMP accumulation is being detected by competition for a specific cAMP antibody between free unlabelled cAMP present in the sample to evaluate the presence of a labelled form (radioactive, fluorescent or enzymatic). All these direct techniques require cells or tissue disruption which makes real-time and sub-cellular analysis of cAMP quantification impossible.

A method that can define the cellular localization of cAMP production is the Resonance Enyergy Transfer method (RET). This method was first described by Theodor Förster in the 1940's. The mechanism of RET relies on an energy transfer between a donor chromophore

that may transfer energy to another acceptor chromophore through non-radiative dipole-dipole coupling upon distances proximity conditions. The name FRET "Förster Resonance Energy Transfer" or more commonly used "Fluorescence Resonance Energy Transfer" was born. The efficiency of the energy transfer is inversely proportional to the sixth power of the distance between donor and acceptor, which makes RET extremely sensitive to minor changes in distance and interactions and hereby allowing an accurate sensing of changes in protein conformations for instance. RET based methods have been developed to detect cAMP production and it has been extensively reviewed over the years. All methods (i.e. Bioluminescence resonance energy transfer (BRET)) rely on the use of the downstream cAMP effectors (PKA, Exchange protein directly activated by cAMP, Cyclic nucleotide gated channels (GNGC) figure 5) that all directly bind cAMP molecules related to the specific cAMP binding motif. In the absence of cAMP production, either a full-length cAMP-effector probe or single cAMP domain sensor extracted from the different effectors are fused to an energy donor and acceptor, allowing the generation of a basal RET signal. Upon cAMP binding, a conformational rearrangement in the RET-based sensor will lead to a certain modification of the signal. Since the first pioneering studies where RET probes were used to study cAMP availibity in cardiac tissue, many efforts have been made to improve the signal to noise ratio, RET efficiency (optimizing donor-acceptor couple and linker optimization), cAMP binding affinity (mutagenesis on single domain or full length protein probes) and many other RET factors.(12)

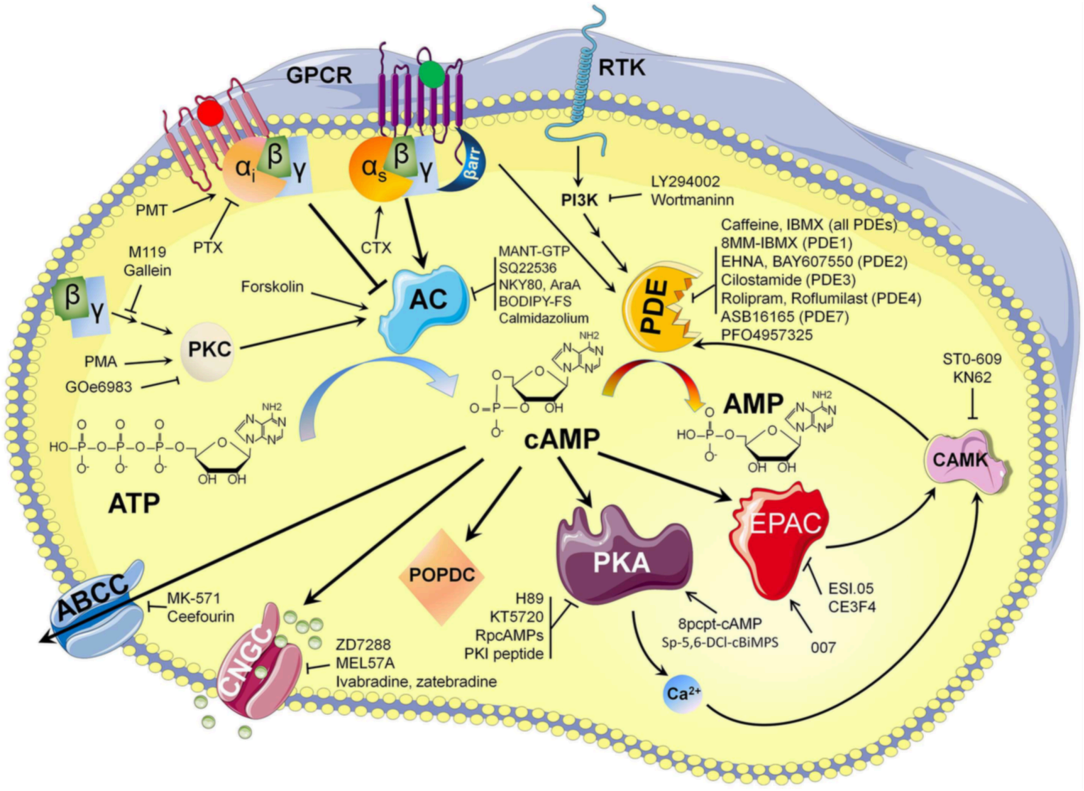


Figure 5 cAMP synthesis and hydrolysis: pharmacological ways to modulate its availability.(12) **An overview of cAMP signalling in living cells and the growing list of molecules available to modulate cAMP availability.**

Michaelis Menten kinetics for enzyme function

Enzymes are proteins that are essentially the catalysts of life Many functional attributes can be assigned to enzymes. At a molecular level, enzymes catalyze biochemical reactions by the accelaration of the conversion of substates to a product by burying it in a pocket within the so called active site of the enzyme. If it wasn't for enzyme catalysis, most reactions in the body would be too slow to be useful for life. However, it must be noted that not all reactions in nature require catalysis.(19)

To understand how enzymes function, and to differentiate between different enzymes properties, we need a kinetic description of their activity. The Michaelis-Menten model is one of the best models for enzyme kinetics. The michaelis-menten equation expresses the velocity (V) of the forming of the product as a function of the substrate concentration [Substrate], with the maximum speed V_{max} and the Michaelisconstant K_M as parameters:

$$V_0 = V_{Max} \frac{[\text{Substrate}]}{[\text{Substrate}] + K_M}$$

In this equation:
V_0 is the initial velocity of the reaction
V_{max} is the maximal rate of the reaction
[Substrate] is the concentration of the substrate
K_M is the Michaelis-Menten constant

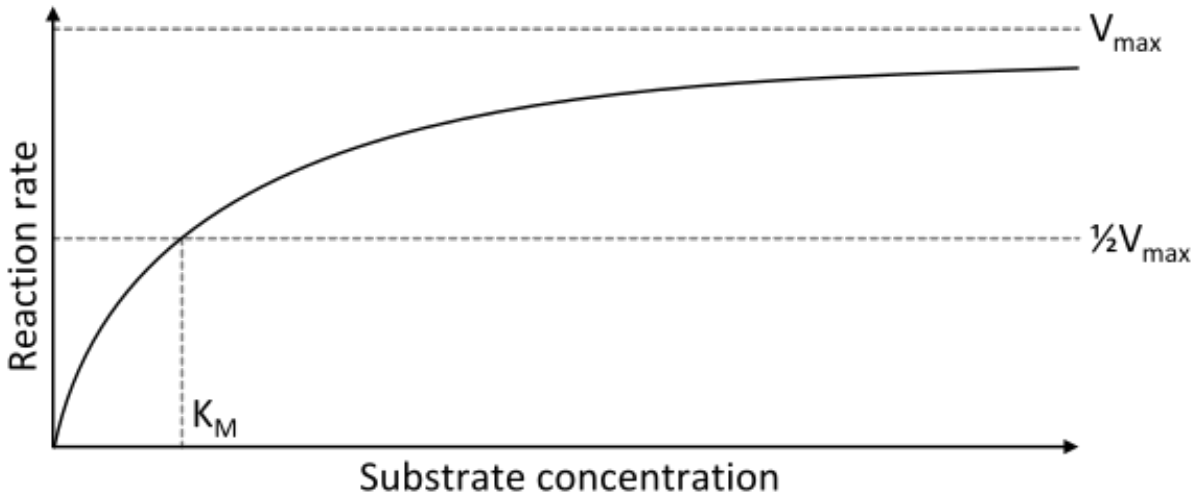


Figure 6 The Michaelis-Menten model for enzyme kinetics(20)

The constant K_M shows the concentration of substrate when the reaction velocity is equal to half the maximal velocity for the reaction. It can be thought of as a measure of how well a substrate complexes with an enzyme. It is otherwise known as the binding affinity. A low K_M indicates a large binding affinity as the reaction will approach the V_{max} faster. If the equation has a high K_M, it means that the enzyme does not bind as efficiently with the substrate and the V_{max} will only be reached if the substrate concentration is high enough to saturate the enzyme.

Effectively this means that general rule is that the lower the K_M, the higher the binding affinity.

An interesting issue is that an extremely high excess of substrate ([Substrate]>>K_M) the term ([Substrate]/ ([Substrate]+ K_M) essentially becomes 1 and the initial velocity approaches V_{max}. this resembles zero order reaction. (20) In this situation, the enzyme concentration is irrelevant for determining the maximal velocity of the enzyme turnover.

Aims of the project

The aim of this project is to elucidate whether there are global changes in cAMP levels in the presence of mutant PDE3A and whether this causes changes in kinase activity. In this project, an attempt to purifying the PDE3A1 isoform next to the already successfully purified PDE3A2 will be made. The mainly heart located PDE3A1 isoform could potentially show different hydrolysing properties as the PDE3A2 isoform found in the vascular smooth muscle cells, which is of interest for elucidating more about the mechanism of HTNB.

Method and materials

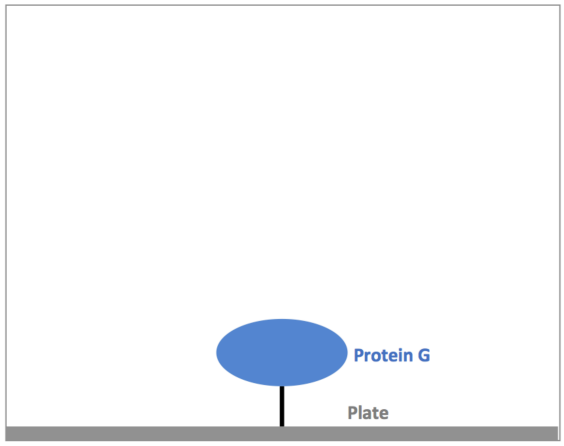
Culturing, seeding and transfection of HEK 293 cells

HEK 293 cells were used for the experiments and kept in culture in T75 Flasks. Cells were maintained in DMEM. Splitting of HEK 293 cells: Complete medium including penicillin and streptomyicin (P/S) (concentration) and 10% Fecal Calf Serum (FCS) is used at room temperature.

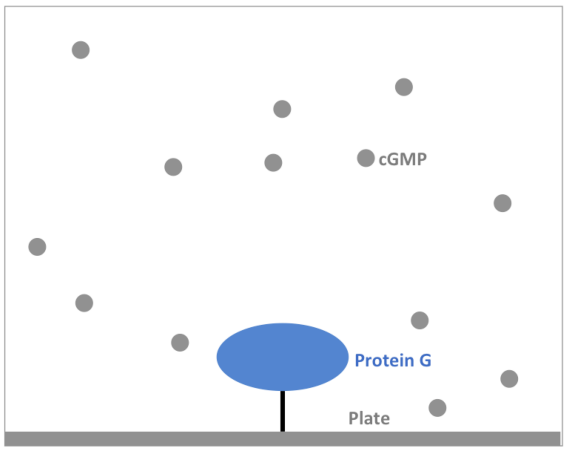
cAMP/cGMP direct immunoassay (colorimetric) by Biovision

The cAMP and cGMP direct immunoassay kits by Biovision were designed to measure levels of cAMP or cGMP in biological samples quantitatively and with high sensitivity. The 96-well plate strips that come with the kit can efficiently anchor cAMP or cGMP monoclonal antibody. cAMP or cGMP conjugated to HRP directly competes with the cAMP from sample or standards and after washing the amount of the HRP conjugate can be determined by reading the plate at 450 nm . Measuring at 450 nm will yield an inverse proportional intensity to concentration ratio. The kit can detect approximately between 0.02-2 µM of cAMP in a sample.(21) A more detailed description of the procedure can be found in the protocol in Appendix 2 Protocol Biovision cAMP/cGMP direct immunoassay kit (Colorimetric).

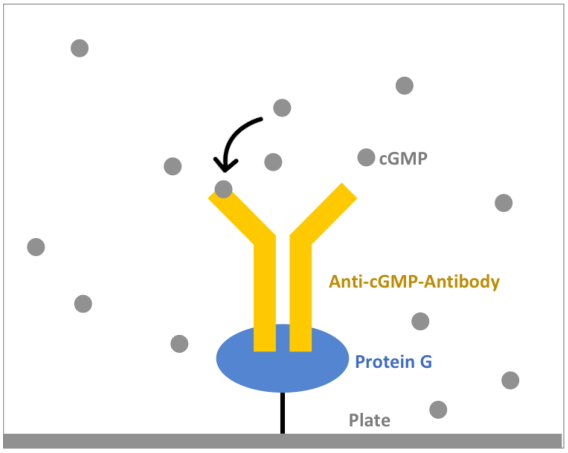
Biovision cAMP/cGMP direct immunoassay kit (Colorimetric) workflow



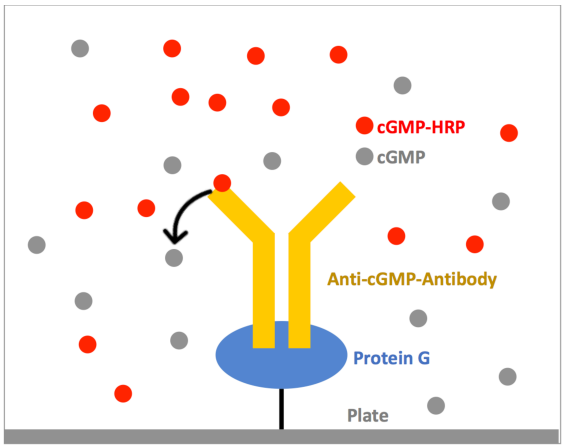
1. The kit comes with 96-well coated strips with recombinant protein G to anchor a cGMP antibody



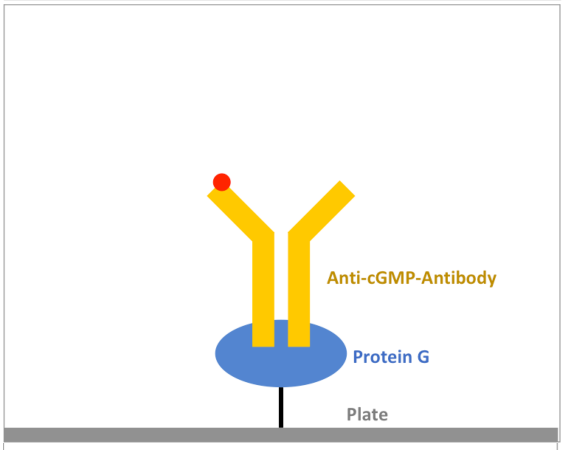
2. cGMP for either the samples or standard is added to the protein G coated wells.



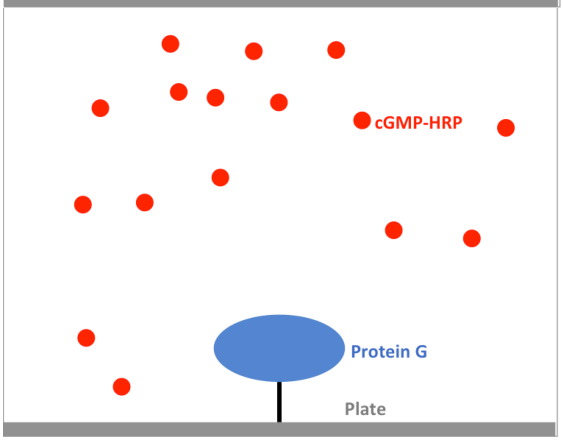
3. Anti-cGMP antibody is added to each of the wells following the addition of cGMP. This step is followed by a 1-hour incubation at room temperature. During this time the antibody binds to the protein G, as well as the cGMP



4. After the 1-hour incubation, HRP conjugated to cGMP is added at a fixed concentration. After this step, another 1 hour incubation at room temperature follows. During this incubation step, a proportion of the cell-derived or standard cGMP will be displaced by the cGMP-HRP conjugate in a concentration that is proportional to the ratio of cGMP to cGMP-HRP. This can be quantified using the standards.



5. The second incubation is followed by wash steps to remove the unbound cGMP and cGMP-HRP. After washing the wells are incubated for 1 hour at room temperature with HRP developer to allow for colorimetric detection of cGMP-HRP. The reaction is stopped by adding 1 M HCl (hydrochloric acid). The assay is finally measured at 450 nm. The standards are used to determine the concentration of cGMP in the experimental samples.



6. Among different controls for this assay, is the no-antibody condition. This control is intended to serve as the background binding control. By subtracting this value from all of the sample values, you normalize against off-target binding of the experiment.

Transfection of HEK 293 cells and PMA treatment

HEK 293 cells were seeded on day 1 and transfected with polyethylenimine (PEI) on day 2. The transfection was expressed for 48 hours after which the cells were lysed on day 4 and the protein of interest was purified. When PMA treatment was carried out, the HEK cells were incubated at 37 °C with 100 ng/mL phorbol 12-myristate 13-acetate (PMA) prior to lysis of the cells. A more detailed description of the protocol can be found in the protocol in Appendix 1 Transfection of HEK 293 cells with PEI .

Purification of PDE3A proteins

The purification of PDE3A proteins after expression in HEK293 cells is carried out by pulling down with the Anti-FLAG M2 magnetic beads. These beads are made of 4% agarose with the anti-FLAG M2 (mouse monoclonal) antibody attached to it. The M2 antibody recognizes the FLAG-sequence at the N-terminus, the Met-N-terminus and the C terminus . This allows for the capture of fusion proteins with the FLAG peptide sequence DYKDDDDK-tag. There are several options for the elution of a fusion protein after it is captured by the FLAG M2 magnetic beads. We used the peptide competition technique with FLAG peptide as this provides the most efficient elution conditions for the Anti-FLAG M2 magnetic beads. This elution technique requires a 30 minute incubation at 37 °C. A more detailed description of the procedure can be found in appendix 3: purification of PDE3A types.(22)

Bradford total protein assay

The Bradford assay for the determination of the total protein concentration of a sample is performed with the Thermo Scientific™ Coomassie Plus™ Kit. The kit provides a ready-to-use Coomassie-binding and colorimetric method for the quantitation of total protein. Unlike some other kits with reagents for the well-known Bradford method that tend to give non linear response curves, this formulation has substantially improved linearity for a defined range of protein concentration. Moreover, the Coomassie Plus Reagent has significantly less protein-to-protein variation than other Bradford type Coomassie formulations. The principle behind the assay is an immediate shirt in absorption maximum form 465 nm to 595 nm with a concomintant color change from brown to blue when the Coomassie dye binds the protein in an acidic medium. Protein sample concentrations can be estimated by reference to absorbance obtained with a standard dilution series of known protein concentrations. These standards should be assayed alongside the unknown samples.(23)

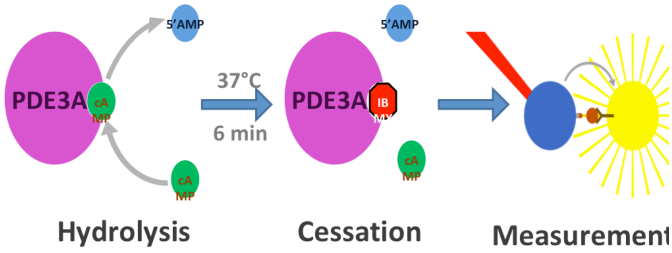
Western Blotting, coomassie gel and protein-protein overlay

Equal amounts of total protein were loaded into 8% SDS–polyacrylamide gel electrophoresis for both Coomassie stained gels and gels for Western blotting (Appendix 3). Then the gel was transferred to a nitrocellulose membrane and the membranes were incubated with the primary antibodies at 4°C overnight. Secondary antibodies (anti-mouse, 1:5000, anti-rabbit, 1:5000, or anti-rat) were incubated at room temperature for 1 h the next day. Protein bands were developed on film using Western detection ECL-plus kit (PerkinElmer, Waltman, MA). ImageJ software was used for band densitometry analysis. The protein-protein overlay of PKA with PDE3A1 WT was carried out with anti PDE3A antibodies according to Appendix

3.

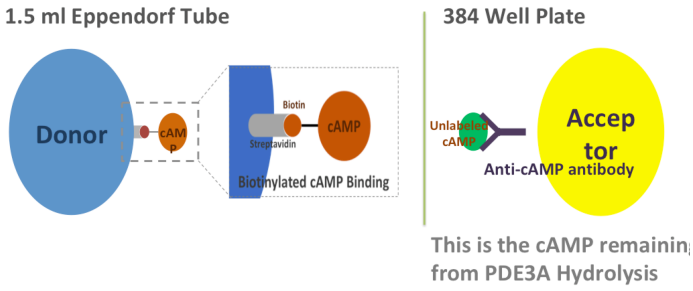
Hydrolysis pilot and total assay

PDE3A Kinetics Experimental Workflow

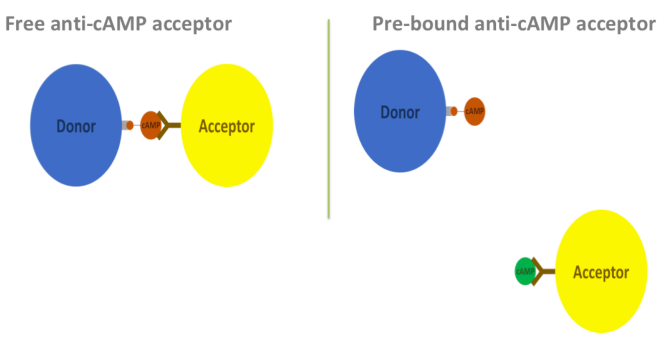


After purifying the PDE3A isoforms (so the different mutants and the wildtype) these proteins can be assessed for their catalytic hydrolyzing properties of cAMP with the AlphaScreen cAMP detection kit. In this so called hydrolyzing pilot The PDE3A is incubated with a fixed amount of cAMP for either 0, 6 or 30 minutes, at which point IBMX (a non selective PDE inhibitor) is added to essentially end the incubation. Detection with the Alphascreen then gives an impression of whether the cAMP in the sample has successfully been hydrolysed by the PDE to any extend.

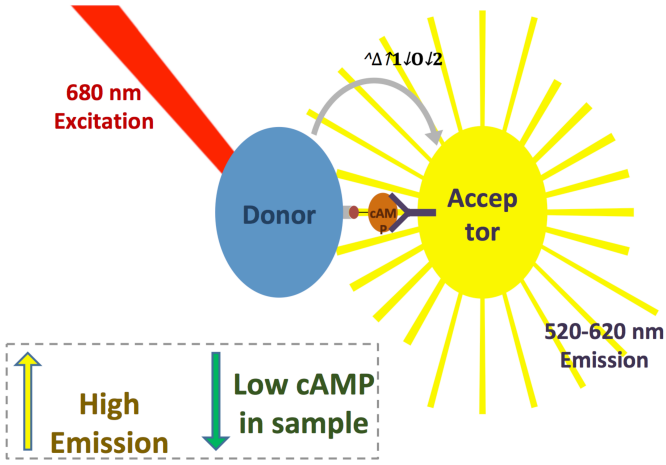
Assay principle



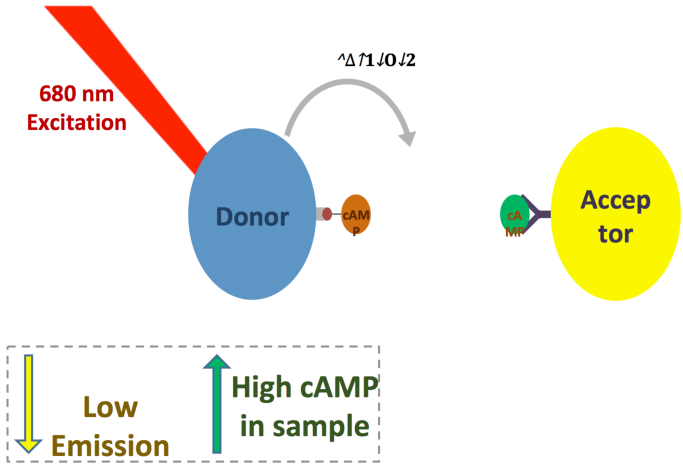
1.The main priniciple of this assay is that there are 2 different beads (donor and acceptor beads) that will release excitation energy in the form of reactive oxygen species when it's connected through a biotinylated cAMP tracer. The more free cAMP is in the sample the more this will compete with the biotinylated cAMP. So after the incubation, the cAMP left after PDE hydrolysis will bind to the Acceptor bead here on the right. And the donor bead connected to the biotinylated cAMP is shown on the left.



2. This figure shows the 2 possible states of the donor and acceptor beads. So they are either connected through the biotinylated cAMP or not connected as free cAMP has bound to the acceptor bead



3. In the absence of free cAMP, maximal Alpha signal is achieved



4. Free cAMP added to the sample competes with the biotin-cAMP tracer for the binding to the anti-cAMP Alphascreen acceptor beads, causing a decrease in Alpha signal.

Results

The first part of this project consisted of determining basal levels of cAMP and cGMP in untransfected HEK 293 cells with the colorimetric direct immunoassay kit. The first results section shows the obtained standard curves for these experiments and in case of the cGMP standard curve also the linear portion of the curve that is used to determine levels of cGMP in a sample. In the second part of the project, different mutants of the isoforms PDE3A2 and PDE3A1 as well as the wildtype were expressed in HEK cells and the results of the purifications at different stages of the process are shown in the second section by Western blotting. The protein concentrations (µg/ml) of the purified PDE3A elutions can be found in table 1. The third section shows the results of the cAMP hydrolysis assays by the purified PDE3A1 and PDE3A2 in column graphs. The fourth section of results is dedicated to the protein-protein overlay of PKA-Cα with PDE3A1 WT to study the binding sites of these proteins.

Results I Determining basal levels of cAMP/cGMP in HEK cells with the colorimetric direct immunoassay kit (Biovision)

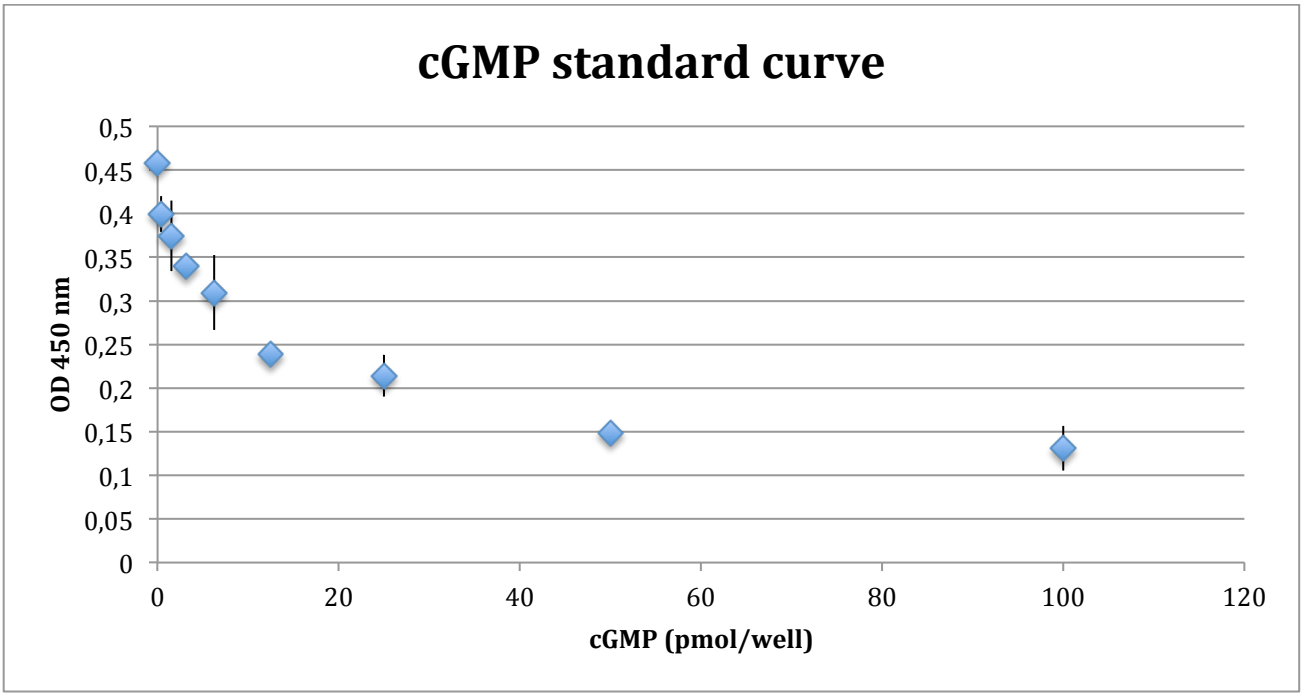


Figure 7 cGMP standard curve established according to the assay kit protocol by Biovision (first attempt)

Figure 7 shows the result of the cGMP standard curve that was established with the cGMP immunoassay kit (Biovision), the first time the procedure was carried out. The curve was not normalized as the negative control of 0,437 would not allow normalisation.

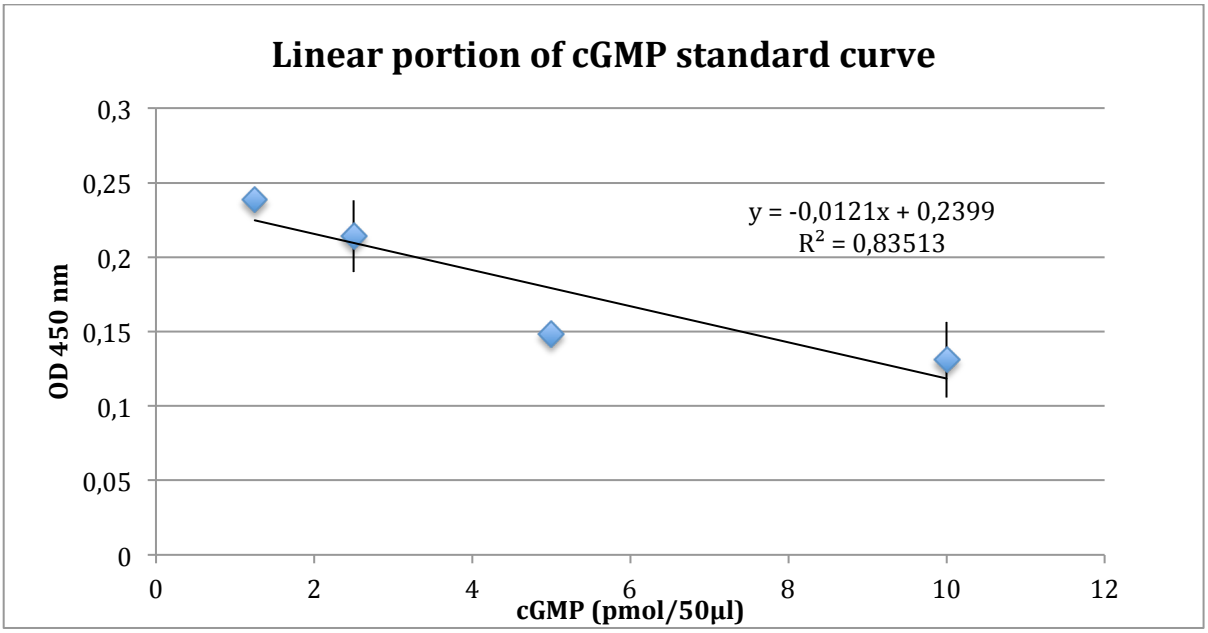


Figure 8 linear portion of the standard curve in figure 4

In figure 8 the linear portion of the cGMP standard curve is plotted to determine a cGMP concentration in the sample. This part of the curve can be used for the determination of the cGMP concentration in a sample of HEK 293 cells. The manufacturer expects this part to be linear but according to this result it is not linear enough to calculate a concentration from. Moreover the negative control of 0,437 is too high to normalize the data and calculate cGMP concentrations in the sample.

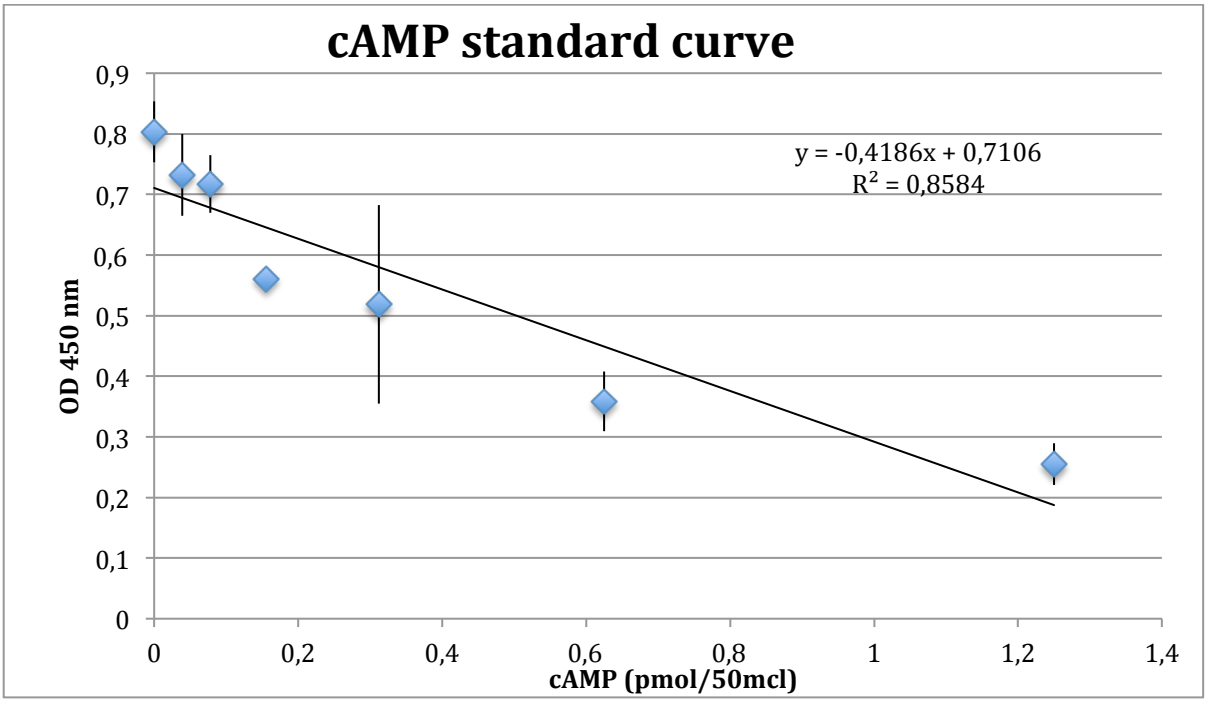


Figure 9 cAMP standard curve established according to the assay kit protocol by Biovision (first attempt)

In figure 9 the cAMP standard curve (normalised for the negative control) is plotted. The linearity (R^2) is 0,8584. Levels of cAMP in HEK 293 cells were calculated to be an average of 16.11 µM.

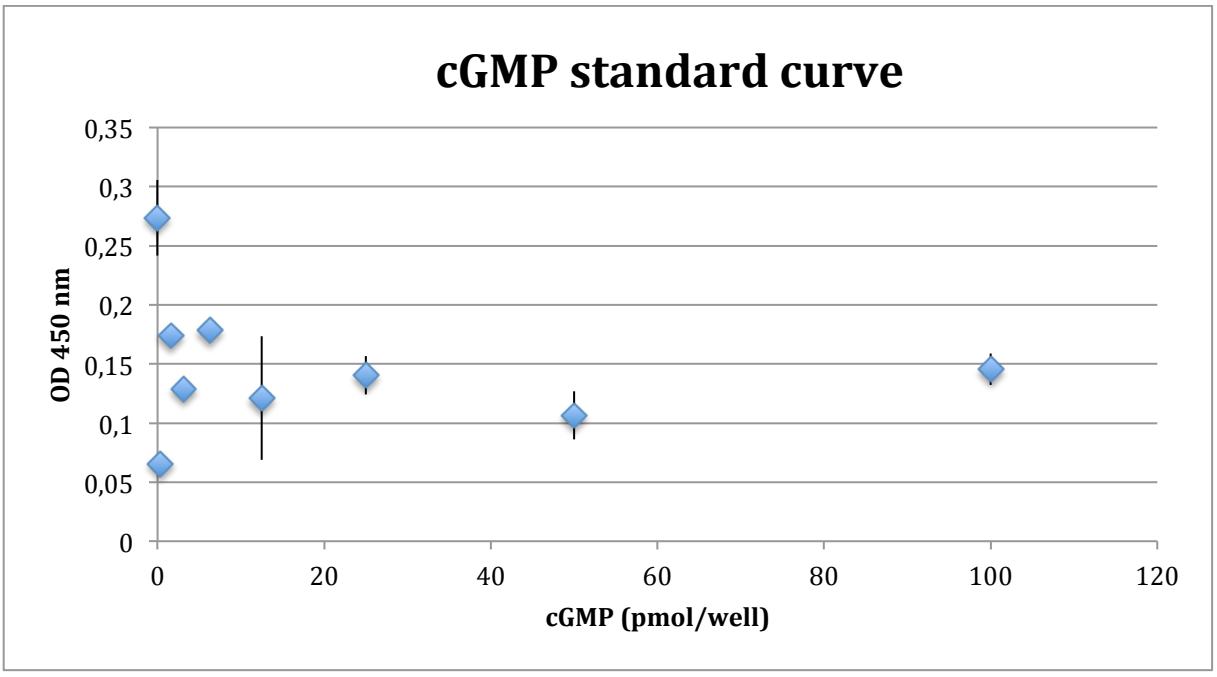


Figure 10 cGMP standard curve established according to the assay kit protocol by Biovision (Second attempt)

Figure 10 shows the result of the cGMP standard curve that was established with the cGMP immunoassay kit by Biovision, the second time the procedure was carried out. The curve was not normalised as the negative control of 0,172 makes normalisation no possible as most of the values in the curve will become negative values. A calculation of the cGMP levels in the HEK 293 cell samples was therefore not carried out.

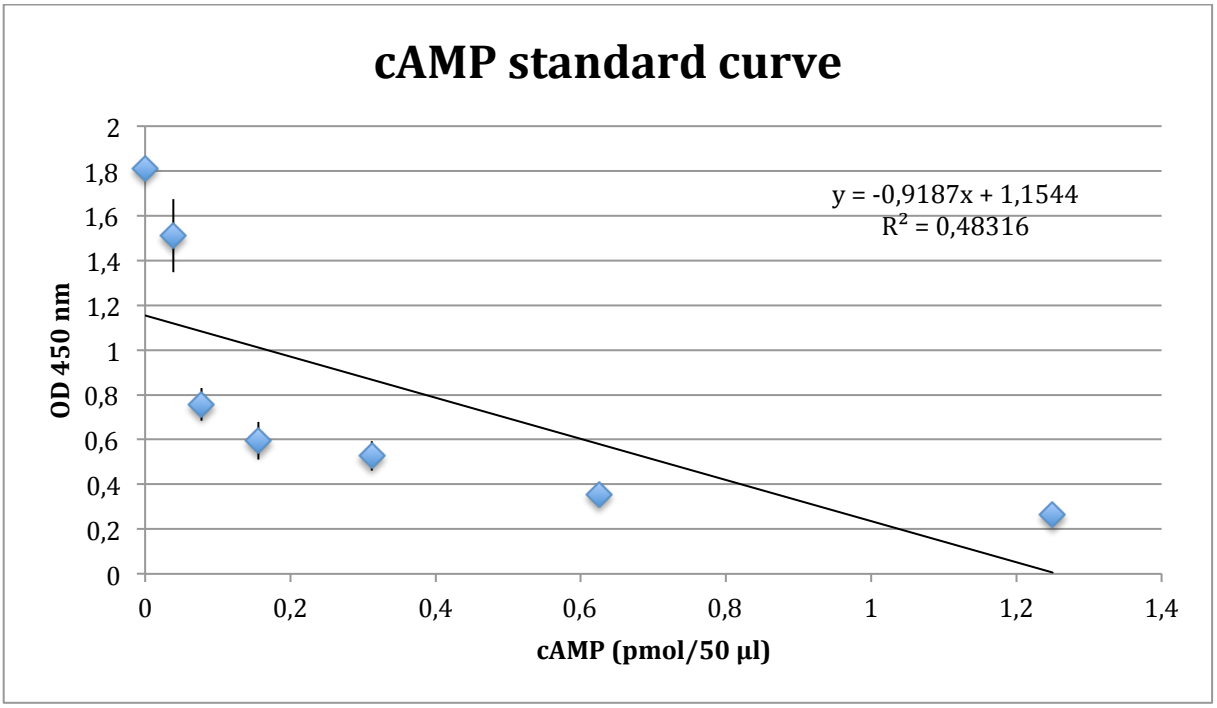


Figure 11 cAMP standard curve established according to the assay kit protocol by Biovision (second attempt)

Figure 11 shows the result of the cGMP standard curve that was established with the cGMP immunoassay kit by Biovision, the second time the procedure was carried out as the first attempt did not provide enough data points to draw a conclusion from. The curve was not normalised as the negative control of 0,172 makes normalisation no possible as most of the values in the curve will become negative values. A calculation of the cGMP levels in the HEK 293 cell samples was therefore not carried out.

Results II Purification and qualification of FLAG-tagged PDE3A

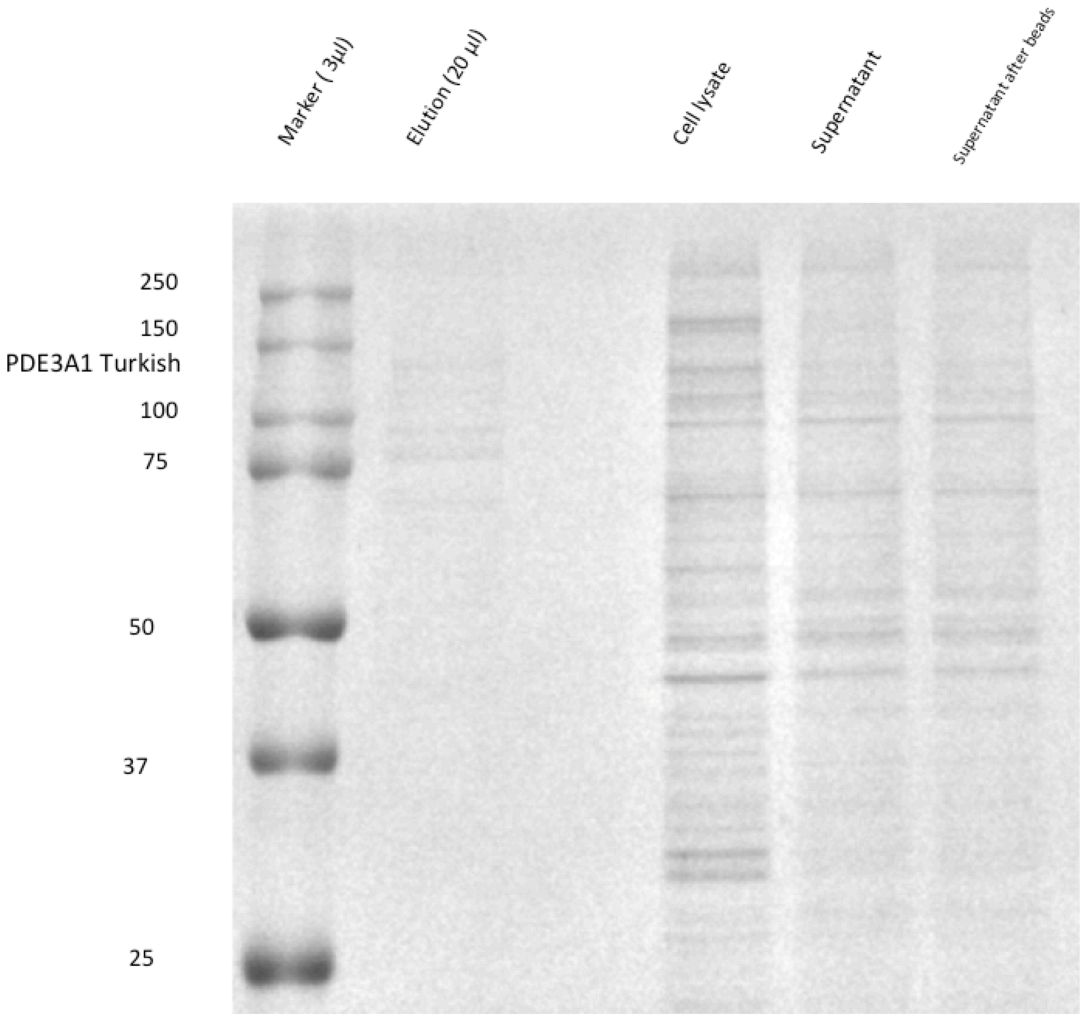


Figure 12 Coomassie stained gel of FLAG-tagged PDE3A1 Turkish from HEK cells Figure 12 shows a Coomassie stained 10% Acrylamide (AA) gel. After pulling down of the PDE3A1 Turkish, a Coomassie staining of 20 µl of the elution sample on a 10% AA gel was performed. The first lane shows the marker, the second lane shows the protein elution with the protein of interest, the third lane is empty, the fourth lane shows the cell lysate, the fifth lane shows the supernatant before beads and the sixth lane shows the supernatant after beads. PDE3A1 is reported at 136 kD(24)**. The elution shows the presence of a hazy lane around 136 kD in this figure. Image J software was used to clarifiy this band in figure 9.**

In Figure 12, a Coomassie stained gel is shown of the PDE3A1 Turkish derived from HEK cells at different stages of the elution and purification process. The band located at 136 kD indicates the presence of PDE3A1 in the elution sample.

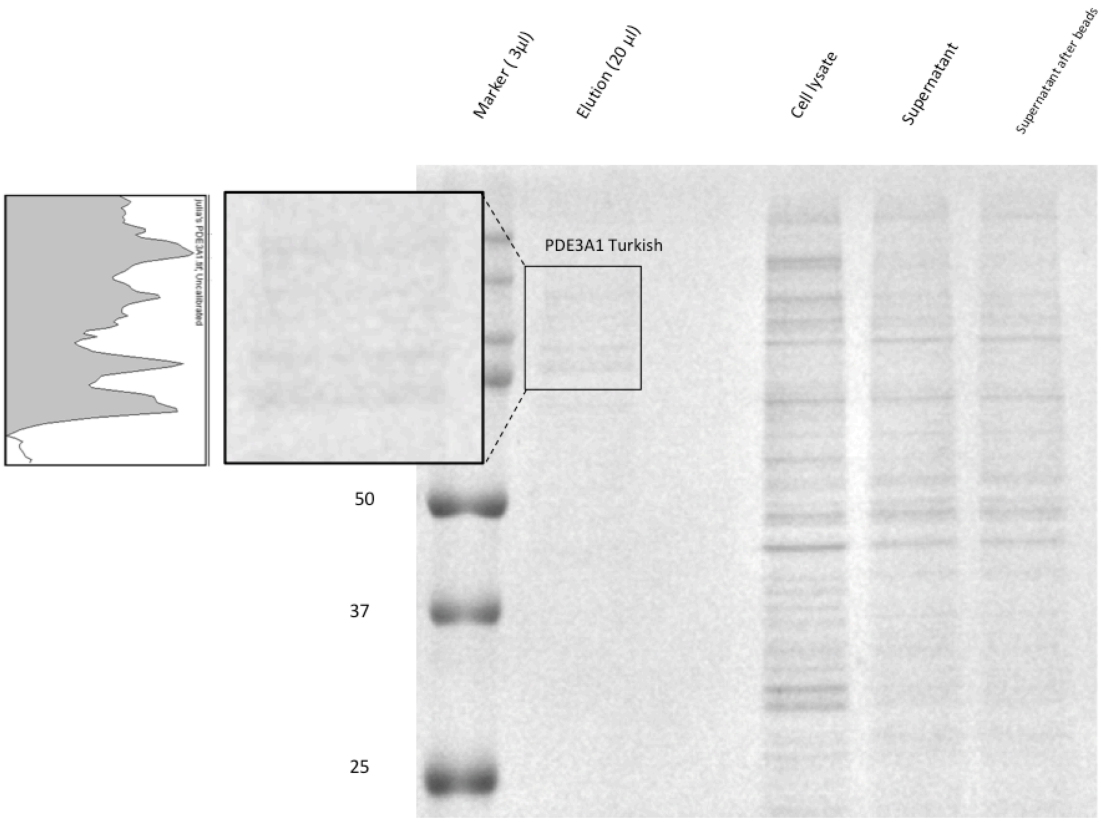


Figure 13 Coomassie stained gel of FLAG-tagged PDE3A1 Turkish from HEK cells with Image Studio analysis of elution bands

Figure 13 shows the same Coomassie stained gel as was presented in figure 12. However Image Studio software here clarifies the presence of bands in the elution lane around 136 kD. A Bradford total protein assay measured the elution to be a 129 µg/ml elution of total protein.

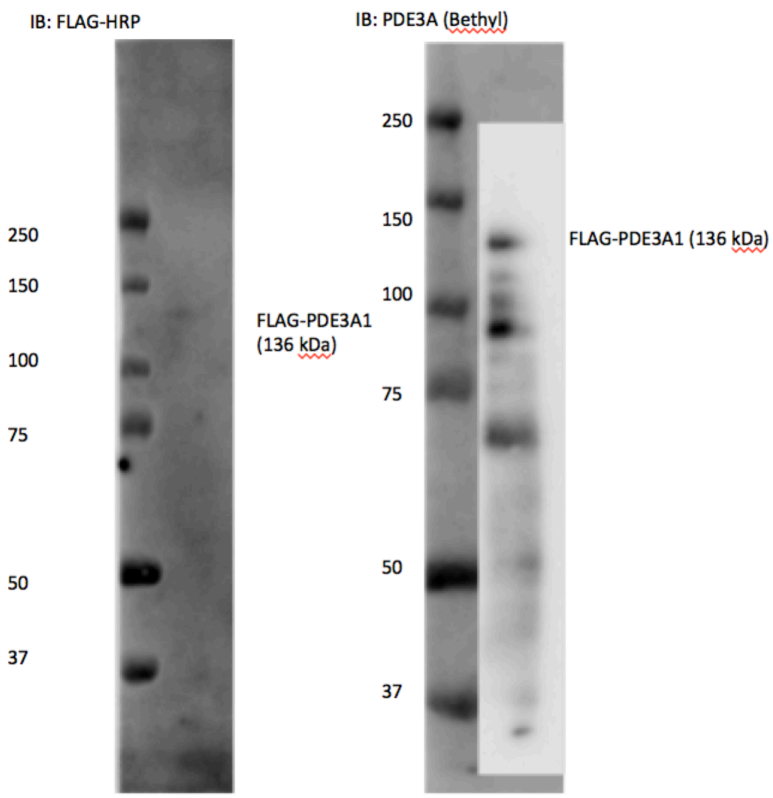


Figure 14 IB: FLAG-tagged PDE3A1 Turkish from HEK293 detected with FLAG-HRP antibodies (left) and anti-PDE3A antibodies (Right) To find a more specific and clear way of qualifying PDE3A1 Turkish, a Western blot of the FLAG-tagged PDE3A1 Turkish elution was performed. 2 different first antibodies were used to detect. In figure 14, The FLAG-HRP (blocking 5% milk in TBS-T, first antibody: FLAG-HRP 1:2500, second antibody: anti-rabbit 1:2500) incubated membrane shows a thin band around 136 kD where the PDE3A1 Turkish would be expected. The anti-PDE3A (by Bethyl) (blocking 5% milk in TBS-T, first antibody: Anti-PDE3A 1:1000, second antibody: anti-rabbit 1:2500)

Figure 14 shows the elution of the FLAG-tagged PDE3A1 Turkish from HEK cells detected with FLAG-HRP (left) and anti-PDE3A antibody. The incubated membrane shows a band around 136 kD where you would expect PDE3A1. The membrane shows the presence of proteins of different sizes in this elution.

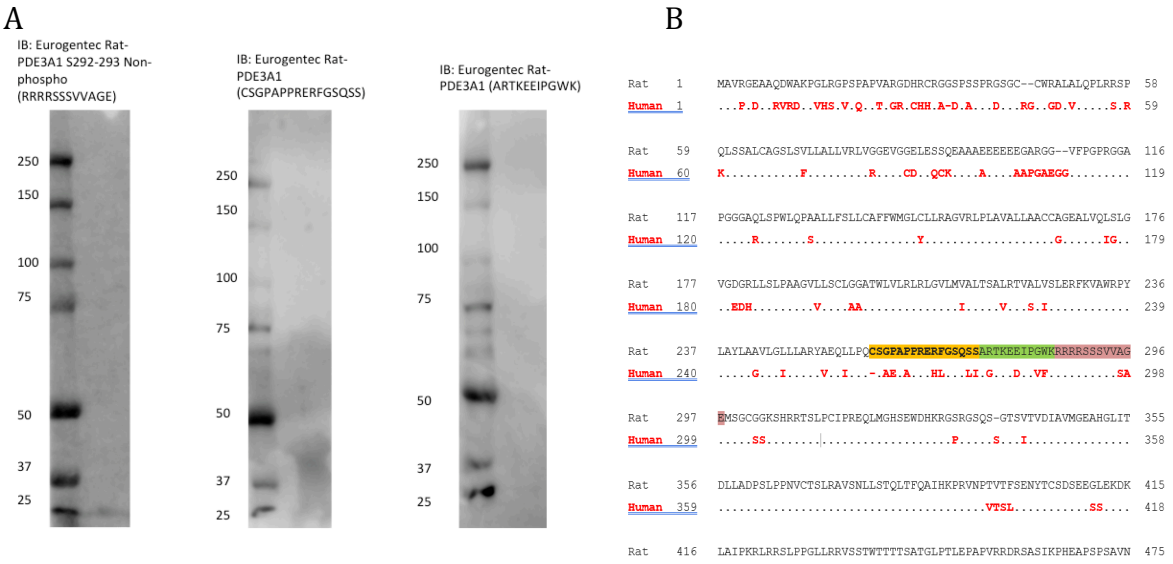


Figure 15 A: Western blot of PDE3A1 Turkish (100 ng) detected with (1) Eurogentec Rat-PDE3A1 S292-293 Non-phospho (RRRRSSSVVAGE), (2) Eurogentec Rat-PDE3A1 (CSGPAPPRERFGSQSS) and (3) Eurogentec Rat-PDE3A1 (ARTKEEIPGWK) B: Alignment of Human PDE3A and Rat PDE3A with the antibody sequences highlighted in (1) pink, (2) yellow and (3) green.

In figure 15, 3 Western blots are shown of the purified elution of the Turkish mutant of PDE3A1. The probe lanes incubated with 3 different first antibodies did not show the abundance of protein in this sample.

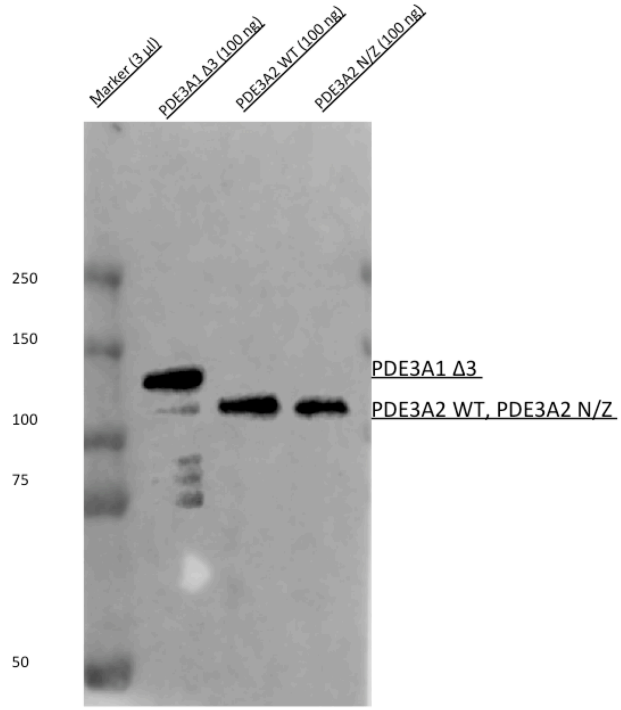


Figure 16 Western blot of FLAG-tagged PDE3A1 Δ3, PDE3A2 WT and PDE3A2 N/Z (all 100 ng) Detection with monoclonal anti-FLAG antibody.

Figure 16 shows a Western blot of FLAG-tagged PDE3A1 Δ3, PDE3A2 WT and PDE3A2 N/Z. 100 ng of total protein was loaded per well. Detection was carried out with

monoclonal anti-FLAG antibody.The concentrations of total protein according to the Bradford total protein assay were respectively 10,9, 94,6 and 59,7 µg/ml for PDE3A1 Δ3, PDE3A2 WT and PDE3A2 N/Z. In the lanes with PDE3A2 WT and PDE3A2 N/Z loaded, 1 band can be observed for each lane around 118 kD. The lane with loaded PDE3A1 Δ3 elution presents a band around 136 kD

FLAG-tagged PDE3A purified	Total protein concentration (µg/ml)
PDE3A1 WT no PMA	129,4
PDE3A1 WT + PMA	172,9
PDE3A1 Turkish no PMA	67,4
PDE3A1 Δ3 No PMA	10,9
PDE3A2 WT no PMA	94,6
PDE3A2 N/Z no PMA	59,7

Table 1 Bradford total protein assay measurements of the purified FLAG-tagged PDE3A types in (*µ*g/ml)

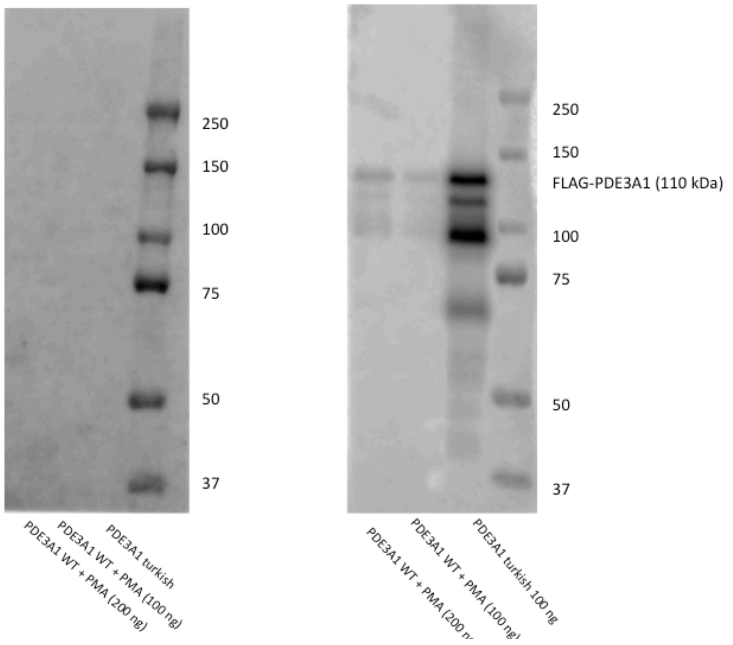


Figure 17 Western blot of FLAG-tagged PDE3A1 WT (200, 100 ng of total protein) and PDE3A1 Turkish (100 ng total protein). Detection with monoclonal Anti-FLAG antibody on the left anti-PDE3A antibody on the right hand side.

Figure 17 shows a Western blot of FLAG-tagged PDE3A1 WT and PDE3A1 Turkish. For the Turkish mutant 100 ng of total protein was loaded. The WT protein was loaded at 100 ng and 200 ng in different lanes. The anti-FLAG incubated membrane shows no bandst that indicate the presence of a protein. However, in the anti-PDE3A antibody incubated membrane, the turkish mutant shows a strong signal around 136 kDa as well as 2 strong bands at lower molecular weight. The WT protein at 200 ng shows the same bands but at a weaker signal. Loading of 100 ng of the WT protein results the same pattern but at a slightly weaker signal than the 200 ng PDE3A1 WT signal.

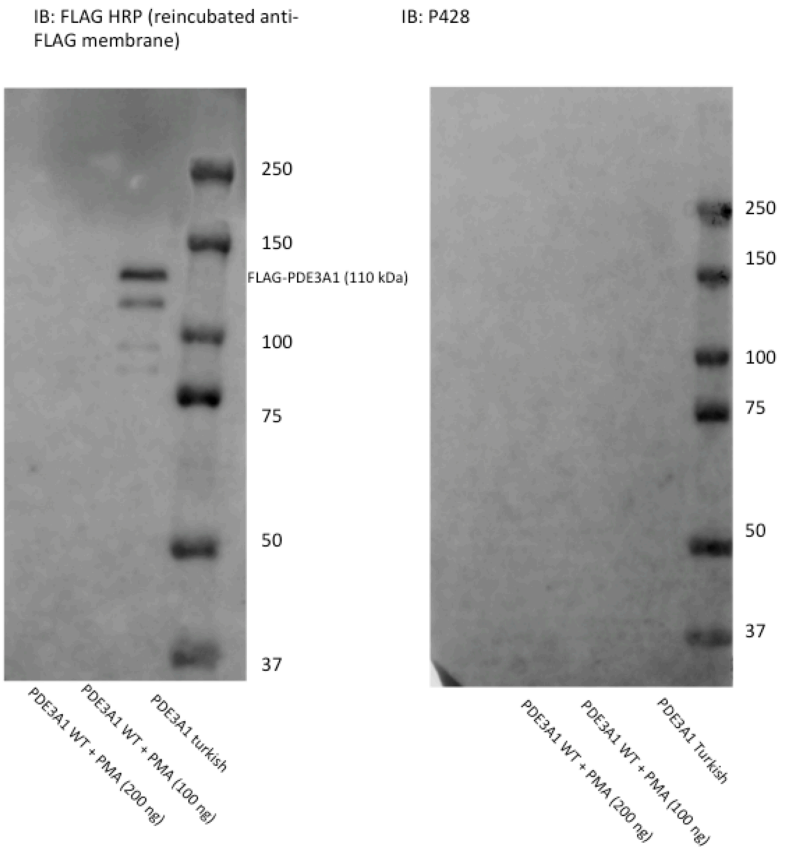


Figure 18 Western blot of FLAG-tagged PDE3A1 WT (200, 100 ng of total protein) and PDE3A1 Turkish (100 ng total protein)

Figure 18 shows 2 Western blots of FLAG-tagged PDE3A1 WT and PDE3A1 Turkish. For the Turkish mutant 100 ng of total protein was loaded for both blots. The WT protein was loaded at 100 ng and 200 ng in different lanes of both membranes. Detection was carried out with anti-FLAG HRP antibody on the left and (phospho) Ser428 antibody on the right on the The Turkish mutant shows a signal around 136 kDa as well as 2 less strong bands at lower molecular weight. The WT protein at either 100 or 200 ng shows no bands. Detection with the Ser428 antibody did not result in detection of any bands in this blot.

Results III: The assesment of the cAMP hydrolysis properties of purified PDE3A

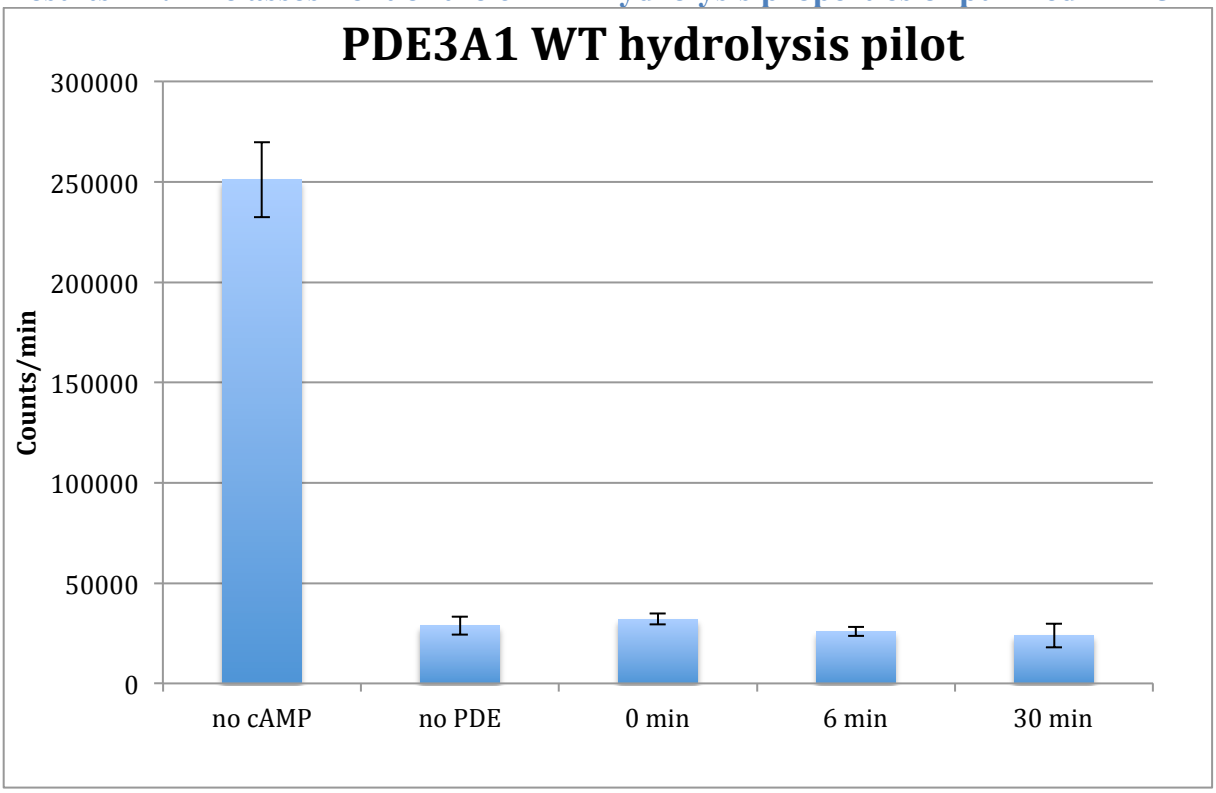


Figure 19 hydrolysis pilot of PDE3A1 WT with the 0, 6 and 30 minute incubation conditions (N = 3). Controls with no cAMP and no PDE conditions. Vertical axis: Alphascreen signal in counts/min .

Figure 19 shows the results of a hydrolysis pilot of the PDE3A1 WT that was purified previously. The no cAMP condition results in a signal around 250000 counts/min. The no PDE condition and 0, 6 and 30 minute incubation conditions with PDE3A1 WT show a signal around 3000 counts/min and no significant differences in signal between one another.

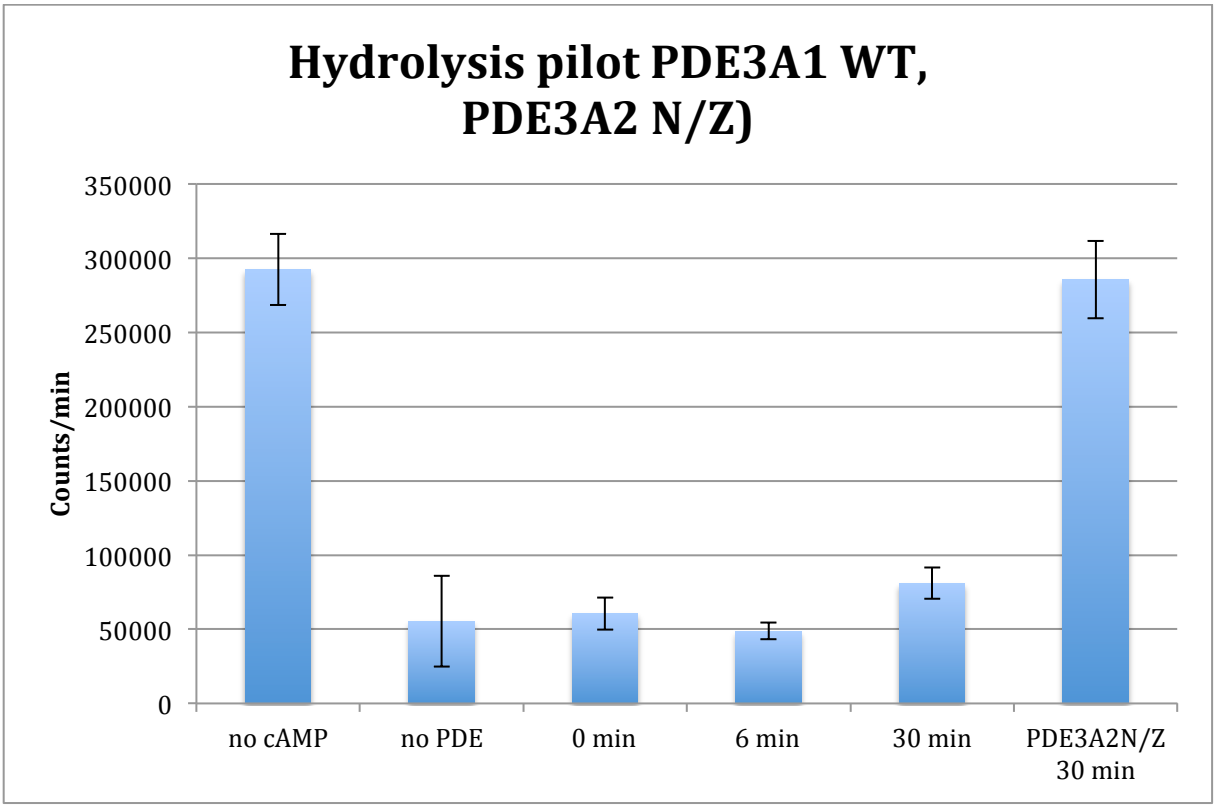


Figure 20 hydrolysis pilot of PDE3A1 WT with the 0, 6 and 30 minute incubation conditions and PDE3A2 N/Z 30 minute incubation. Controls with no cAMP and no PDE conditions. (N = 3) Vertical axis: Alphascreen signal in counts/min.

Figure 20 shows the results of a hydrolysis pilot of the PDE3A1 WT and PDE3A2 N/Z that were purified previously. The no cAMP condition and the 30 minute incubation with PDE3A2 N/Z condition result in a signal around 290000 counts/min. The no PDE condition and 0 and 6 minute incubation conditions with PDE3A1 WT all show a signal around 5000 counts/min and no significant differences in signal between one another. The 30 minute incubation condition with PDE3A1 WT shows a slightly elevated signal at around 75000 counts/min compared to the other incubation conditions with PDE3A1 WT but this elevation is not significant.

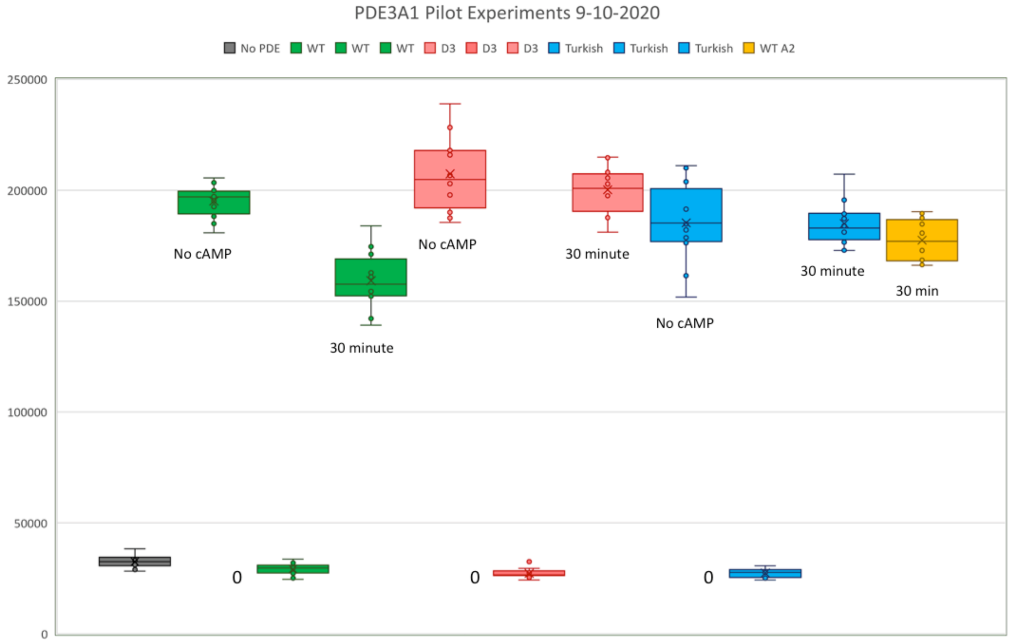


Figure 21 hydrolysis pilot of PDE3A1 WT PDE3A1 Δ3, PDE3A1 Turkish with the 0 and 30 minute incubation conditions and PDE3A2 N/Z 30 minute incubation. Controls with no cAMP and no PDE conditions. (N = 6)Vertical axis: Alphascreen signal in counts/min.

Figure 21 shows the hydrolysis pilot of PDE3A1 WT, PDE3A1 Δ3, PDE3A1 Turkish and for the no cAMP, 0 and 30 minute condition. PDE3A2 WT is shown for the 30 minute incubation condition. The control no PDE is shown on the left hand side of the figure in grey.

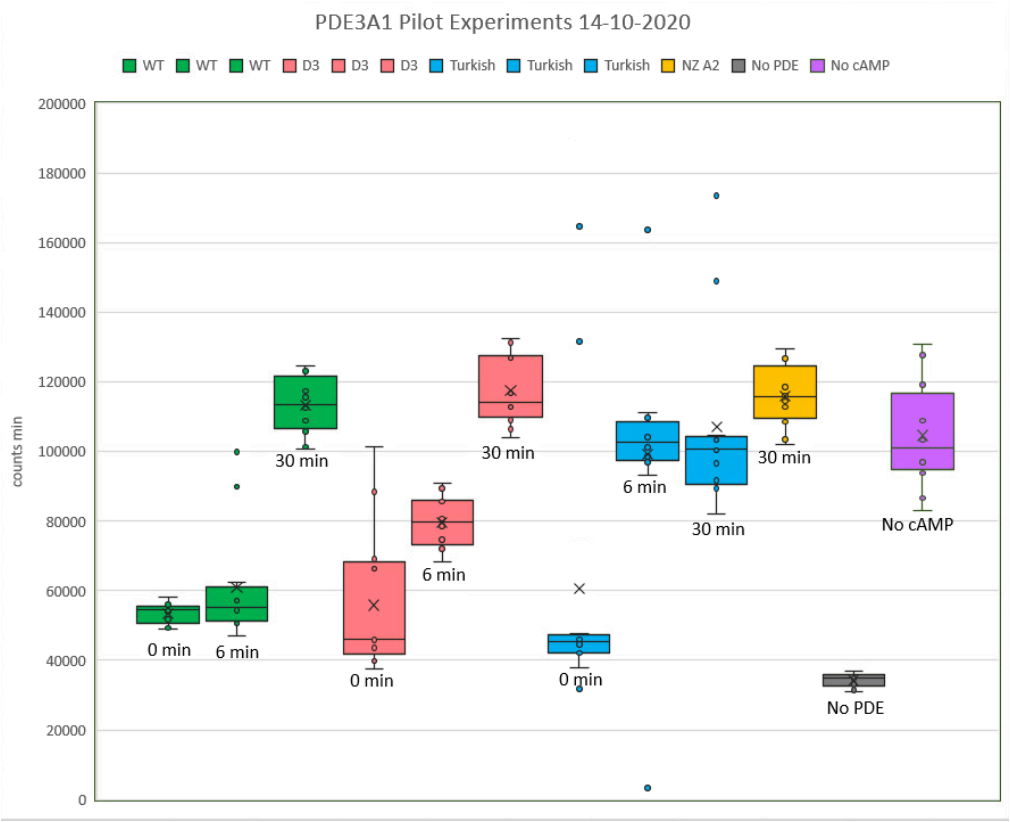


Figure 22 hydrolysis pilot of PDE3A1 WT PDE3A1 Δ3, PDE3A1 Turkish with the 0, 6 and 30 minute incubation conditions and PDE3A2 N/Z 30 minute incubation. Controls with no cAMP and no PDE conditions. (N = 6) Vertical axis: Alphascreen signal in counts/min.

Figure 22 shows the hydrolysis pilot of PDE3A1 WT, PDE3A1 Δ3, PDE3A1 Turkish and PDE3A2 N/Z is shown for the 0, 6 and 30 minute incubation conditions. The controls no cAMP and no PDE are shown on the right.

Results IV Overlay of PKA-Cα with PDE3A1 WT

This blot overlay was done to study the interaction between the PKA-Cα protein and PDE3A1 WT.

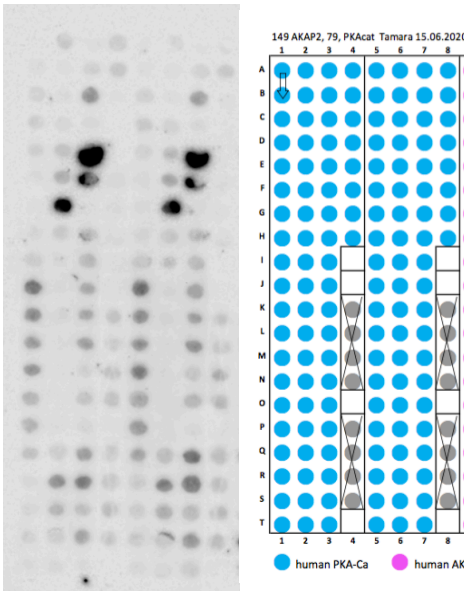


Figure 23 Overlay of the PKA-Cα protein with PDE3A1 WT (left) and the PKA- Cα sequence (per 25 aminoacids) map (right)

Figure 23 shows the result of the incubated PKA-Cα spotted membrane with PDE3A1 WT on the left. On the right a schematic figure of the membrane is given for reference.

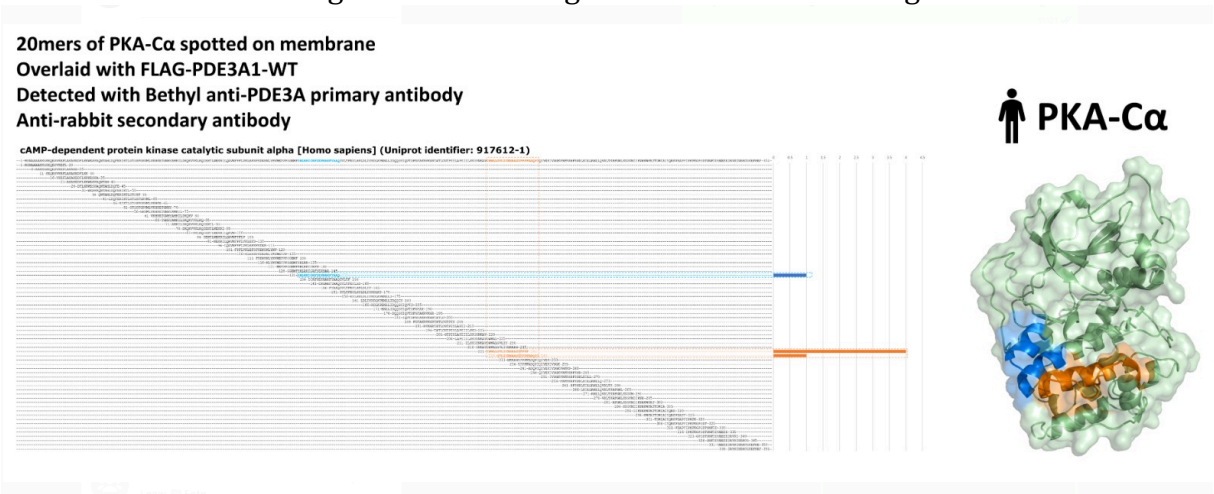


Figure 24 Overview of the binding sites of PDE3A1 WT to the PKA-Cα in the amino acid sequence.

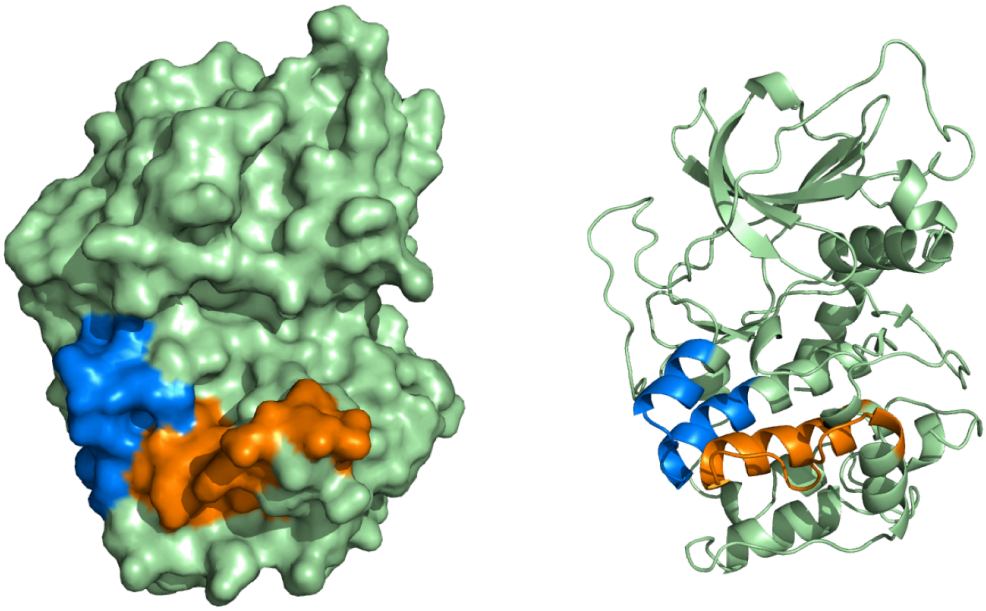


Figure 25 Visual represatation of the binding sites of PDE3A1 WT to PKA- Cα. Figure is of PKA Cα.

Discussion

Measuring basal levels of cAMP and cGMP with the cGMP and cAMP direct immunoassay kit (colorimetric) by Biovision

At the start of this project, it was attempted to determine basal levels of cAMP and cGMP with the respective direct immunoassay kits (Biovision) in HEK 293 cells. To determine basal levels of cAMP and cGMP in untransfected HEK cells, a total protein concentration of ≥1 mg/ml of the HEK cell lysate is recommended by the Biovision direct immunoassay kits protocol. Before determining the basal cAMP levels in untransfected HEK cells, a total protein concentration is therefore measured by the Bradford total protein assay. Often this requirement was not met due to a small portion of cells lysed, or use of in inadequate lysis buffer. A cAMP assay with HEK 293 cells was therefore not always carried out. However on several occasions, the protein levels were high enough to perform a cGMP or cAMP assay on the same day.

The first attempts with the cGMP kit resulted in a standard curve that showed enough similarities to the assay kits protocol standard curves, to continue improving this assays standard curve so basal levels of a sample could be calculated from these curves. From the first established cAMP assay standard curve, basal cAMP levels of HEK 293 cells were calculated to be an average of 16.11 µM. Basal levels of cAMP are rarely reported, especially in HEK293 cells, but Sudlow et al (1997) (25). reported 3.6 to 27.6 µM cAMP levels in molluscan central neurons. Jiang et al (2014)(26). reported 9 µM of cAMP in haemotacytes of scallop Chlamis Farreri. Jiang and his group found these results by using the same direct immunoassay kit by Biovision. However, in order to give a more firm and substantial answer to the question what levels of cAMP HEK293 cell lysate contains, the experiment with HEK293 cells should be repeated several times. Unfortunately, numerous attempts to establish a useable cGMP and cAMP standard curve did not result in a workable curve, and calculations of cGMP/cAMP levels in samples were not possible. The reoccurring setback of a very high negative control of the assays also limited the usability of the assay data.

The lack of reproducible results from this assay could be explained by the chronological order in which the assays were conducted. The more time passed after reconstitution of the assay kits components, the more inconsequent the results were. This is supported by the fact that 2 different people carried out the procedure with a kit that had been opened for a few weeks and didn't find any results resembling the assay kits protocol standard curves. However, immediately after opening a new kit, the results showed sufficient overlap with the kit's protocol standard curve and the results were reproducible between the 2 different people. This way it becomes clear that subsequent human error is less likely to be the source of the issues with this assay and there's a problem with the components of this kit that seem to be deteriorating over time. In addition to the poor results that were obtained with this assay, the assay kit is also too costly to misspend any more of its resources. No further investigations were made to specify the problems but the company (Biovision) was contacted and no further research was done with this assay.

Purification and qualification of FLAG-tagged PDE3A

HEK cells were transfected with PEI and different mutants of PDE3A1 and PDE3A2 were purified, as well as the WT. It was decided to attempt to purify a PDE3A1 type protein as until then, only the A2 enzyme had been purified by this research group, and it was not clear if pulling down of the A1 protein would be successful. Over the course of this project, different FLAG-PDE3A mutants and wildtype were pulled down with the M2 anti-FLAG magnetic beads. The availability of different constructs of the PDE3A1 and PDE3A2 types The specific type of mutant or whether it would be the A2 or A1 type of protein was decisive for the decision what protein was next to purify. The first FLAG-tagged PDE3A1 protein that was purified was the Turkish mutation of PDE3A1. Qualification was performed by Coomassie staining a 10% acrylamide gel with the protein of interest containing elution after gel electrophoresis. However Image Studio analysis of the band found at around 136 kD confirms the presence of a band and therefore the presence of a protein with the size of 136 kD, the pulled down PDE3A1 protein of the Turkish mutation. A Western blot was performed to make the presence of PDE3A1 even clearer. Different antibodies were assessed for thing binding properties to PDE3A and their usefulness for detecting PDE3A on a Western blot. The PDE3A antibody by Bethyl seemed to have good binding properties and gave a strong signal around 136 kD, as can be seen in figure 11. This band is therefore likely to be PDE3A1 Turkish. The elution however also contained smaller proteins that bind with the anti PDE3A antibody. There are multiple factors that can explain the presence of more than one band in this blot. It is likely that these bands below the 136 kD band can be assigned to digested proteins of the PDE3A1 protein that still bind to the anti PDE3A antibody but are smaller due to digestion. A way to overcome this problem could be to re-evaluate the protease inhibitors that are used in the lysis buffer. The efficacy and quality could be assessed and the concentration of certain inhibitors could be increased to keep the PDE3A protein from being digested. Decreasing the concentration of the anti PDE3A antibody could also help to decrease binding to digested proteins.

The FLAG-HRP incubated membrane shows a small possibly damaged band due to transfer difficulties around 136 kD that is likely to be the band representing the purified PDE3A1 of the Turkish mutation. The band could be damaged because of a transfer problem in the Western blot procedure, specifically when the 8% AA gel is transferred to the membrane.

To test whether human PDE3A1 could be detected with anti PDE3A antibodies against rat, 3 different custom made antibodies from Eurogentec were assessed and their results are presented in figure 13. The overlap of the Eurogentec Rat-PDE3A1 S292-293 Non-phospho (RRRRSSSVVAGE) sequence with the human PDE3A according to the sequence alignment is the larger than for the Eurogentec Rat-PDE3A1 (CSGPAPPRERFGSQSS) and the Eurogentec Rat-PDE3A1 (ARTKEEIPGWK) antibody. However none of these antibodies could be used for the detection of human PDE3A1. The anti-rat (RRRRSSSVVAGE) antibody was expected to work for the detection of human PDE3A1 as the sequence only differs by 2 aminoacids. The problem could be in the concentration of the first antibody. The solution might therefore be to increase the primary antibody to 1:1000 instead of the 1:2500 that was used for this membrane.

Figure 14 shows the membrane for the detection of FLAG-tagged PDE3A1 Δ3, PDE3A2 WT and PDE3A2 N/Z that were purified. The monoclonal anti-FLAG primary antibody, that binds to the FLAG-tag of the purified proteins shows adequate qualities for detecting FLAG-tagged PDE3A1 and PDE3A2. The PDE3A2 is detected around 118 kD, as can be expected from it's reported size of 118 kD by Yan et al.(24). PDE3A1 is detected at a slightly bigger size of 136 kD.

Over the course of the project, it became clear that the concentration of PDE3A1 WT in the respective elutions (No PMA and PMA treated) were not as high as the levels of PDE3A1 mutants and the PDE3A2 types. A Bradford total protein assay of the PDE3A1 WT showed a 129,4 µg/ml total protein elution (Table 1), but when these measurements were used for the calculations of the amount of protein to be loaded on the gel for electrophoresis, only very vague bands for PDE3A1 WT were obtained compared to the Turkish mutant of PDE3A1 (67,4 µg/ml total protein) (detected with the anti-PDE3A primary antibody) (figure 14), indicating that the portion of PDE3A1 WT in the 129,4 µg/ml total protein elution is relatively low compared to the portion of Turkish mutant in the respective 67,4 µg/ml total protein elution. Instead, it is more likely to be a mixture of proteins of different sizes, one of them being the FLAG-tagged PDE3A1 WT. In figure 15 it also becomes clear that the Anti-FLAG primary antibody incubated membrane does not show the presence of proteins of any kind. The chemiluminescense of membrane was detected by measuring emission at 700 nm for 30 seconds 1 minute and, 3 minutes in order to obtain a signal but this was not successful. The lack of signal from the anti-FLAG antibody this time after successful procedures in the past with the same antibody can be explained by contamination of the antibody, or inadequate storing in between usage, resulting in a deteriorated antibody and lower concentrations of the antibody. New anti-FLAG primary antibodies were therefore ordered and this particular membrane was re-incubated with a HRP antibody against FLAG as is showed in figure 16. After re-incubation the presence of the Turkish mutant of PDE3A1 became visible, although the signal is not as strong as on the anti-PDEA incubated membrane. In combination with the lower levels of the wildtype protein in the elution this explains why the WT protein was not detected with this antibody on this membrane. The Ser428 (P428) is a weaker binding antibody that will only detect proteins that are phosphorylated at the Ser428 site and are present in relative abundancy in the lanes. Of the Turkish mutant most certainly enough protein was loaded in the lane as this became clear from the other membranes detected with other antibodies. However, the Turkish mutant was not PMA treated and therefore not phosphorylated at the Ser428 site and was therefore not detected by the Ser428 antibody, as can be expected. Previous detection of the WT protein with strong binding anti-PDE3A antibodies already showed the relative small amount of protein in these membranes. The absence of a signal in the PDE3A1 WT lanes of this membrane is not surprising when the relatively poor binding properties of the Ser428 antibody are taken into consideration.

The Assesment of the cAMP hydrolysis properties of purified PDE3A

The cAMP hydrolysis properties of the pulled down PDE3A were assessed using the cAMP detection kit by Perkin Elmer. Figure 17 shows that the control conditions no cAMP and no PDE show a respective maximum and minimum signal as is expected from these conditions. In this assay, the presence of any hydrolysis properties of the previously purified PDE3A1 WT is assessed. The aim of this so called hydrolysis pilot is to determine whether the PDE3A is active at all, and whether it has the capacity to hydrolyse cAMP to any extend. From the results of this first hydrolysis pilot it became clear that the PDE3A1 WT that was used for the hydrolysis incubation did not result in a significantly higher signal than for the no PDE control condition. In other words, there was no indication for any hydrolysis properties of this PDE at any of the incubation conditions (0, 6 and 30 minutes).

Figure 18 shows the results of a subsequent assay with a control condition of 30 minutes PDE3A2 N/Z incubation. This PDE3A2 N/Z had been proven effective before and could therefore be seen as a positive control. The PDE3A2 N/Z elution showed hydrolysing properties again in this assay, but the hydrolysing properties of the PDE3A1 WT remained non existent according to this assay. This positive control ruled out the possibility of a systemic error with the protocol or assay and the suspected low concentration of PDE3A1 WT in the elution asked for a 3rd attempt to find hydrolysing capacity of the PDE3A1 WT where the concentration of the protein would be four folded. Figure 19 shows an overview of an hydrolysis pilot with the PDE3A1 WT, PDE3A1 Δ3, PDE3A1 Turkish and PDE3A2 WT for the conditions 0 and 30 minute incubation. Figure 19 essentially shows the results of a subsequent assay intended to compare the different 30 minute incubations to the no cAMP condition to be able to give an implication about the relative amount of cAMP that is hydrolysed by the purified PDE3A that it is incubated with. Not only did it become clear that increasing the amount of PDE3A1 WT used in the assay indicated the presence of hydrolyising properties of this PDE, the properties of the different mutants and wildtype seem to be similar after 30 minutes of incubation.

In figure 20 the results of a hydrolysis pilot with PDE3A1 WT, PDE3A1 Δ3, PDE3A1 Turkish and PDE3A2 N/Z for the 0, 6 and 30 minute incubation conditions. The 30 minute incubation conditions show strong similarities to the no cAMP condition control, with insignificant differences indicating that after 30 minutes, all cAMP added to the samples has been hydrolysed. According to this assay, the Turkish mutant has already hydrolysed all the cAMP in the sample after 6 minutes. The wildtype of PDE3A1 shows no significant hydrolysis of cAMP after 6 minutes as the there is no significant increase in Alphascreen signal compared to the 0 minute incubation condition. However, after 30 minutes it reaches a phase were all the cAMP has been hydrolysed, similar to the mutants of PDE3A1. The PDE3A1 Δ3 mutant shows a higher Alphascreen signal after 6 minutes compared to the 0 minute incubation condition, indicating that substantial cAMP has been hydrolysed at this point, however total hydrolysis of all the cAMP in the sample appears to happen after 30 minutes of incubation. Throughout the analysis of the purified PDE3A proteins, it became apparent that the however the total protein concentration of PDE3A1 WT elutions tended to be relatively high (>100 µg/ml), the actual concentration of the protein of interest was low. A possible explanation can be found in the unique N-terminal extension of PDE3A1 that directs the protein to intracellular membranes in transfected HEK293 cells(2). This caused PDE3A1 to be recovered solely in microsomal fractions whereas PDE3A2 can be solely recovered in cytosolic fractions. Lysis of HEK293 cells with the current lysis buffer and technique

might favour cytosolic PDE3A2 over PDE3A1 in intracellular membranes resulting in a higher yield of PDE3A2 compared to PDE3A1.

Overlay of PKA-Cα with PDE3A1 WT

An overlay of the PKA-Cα with PDE3A1 WT was performed to find the binding sites of PDE3A1 WT to PKA-Cα. A visual representation of where the binding takes place according to this overlay was acquired. This type of figure solely gives an indication about where the binding of these proteins take place. However it does not give any implications about PKA's capacity to phosphorylate PDE3A1. In order to assess PKA for its activity and therefore phosphorylation capacity, a Peptag assay is required. This type of assay can essentially measure the capacity of a kinase to phosphorylate a peptide. Unfortenutaly, the scope of this project did not allow for such an assay.

Further research

To give a more conclusive answer to the question what way the mutations change hydrolytic activity of cAMP by PDE3A, a full hydrolysis experiment should be performed. a cAMP standard curve can be established to determine the sensitivity and dynamic range of the assay and eventually extrapolate the amount of cAMP in the experimental sample. Using Michealis-Menten parameters, qualification of enzyme kinetics of different mutations of PDE3A1 and PDE3A2, treatment with PMA and comparison to the Wildtype protein could possibly elucidate the mechanisms for increased hydrolytic activity of cAMP by these isoforms PDE3A1 and PDE3A2. PDE3A2 can be stimulated by PMA treatment as the phosphorylation of the 14-3-3 and Ser428 sites lead to increased activity of PDE3A2. Unlike PDE3A2, PDE3A1 activity is not affected by phosphorylation of either site(2). Vandeput et al. examined the effects of phosphorylation at S312 and S428 on PDE3A on cAMP hydrolytic activity in transfected HEK293 cells. They found that exposure of PDE3A2-transfected cells, to PMA led to an increase in cAMP hydrolysis that was blocked byt the introduction of an S428A substitution. When the cells were treated with the nonhydrolyzable analog dibutyryl cAMP (dBcAMP), which causes a receptor independent, non compartmentalized activation of PKA , this resulted in the phosphorylation at both s312 and S428 as well and the stimulation of catalytic activity was similar in magnitude to that seen in response to PMA (also similarly blocked by a mutation in S428A). This demonstrates that there is a role for phosphorylation at S428 in regulating the activity of PDE3A2.

In contrast to these findings regarding the PDE3A2 isoform, exposure of HEK293 cells transfected with PDE3A1 to isoprotenerol (non-selective β-adrenoreceptor agonist) did not stimulate cAMP hydrolytic activity, but exposure to dBcAMP led to a significant increase in the phosphorylation of the PDE3A1 isoform at both S312 and S428 and subsequently to a stimulation of cAMP hydrolytic activity. Interestingly, the same increase of catalytic activity in response to dBcAMP was observed when the cells were transfected with a PDE3A1 construct that had S312 and S428 substitutions. Their analysis with Western Blotting with anti-phospho PKA substrate atnibodies confirmed that PDE3A1-S312/S428A is phosphorylated at other sites in its sequence under these substitution condtions. This result indicates that neither s312A or s428a regulates the activity of PDE3A1. The stimulation of catalytic activity in response to dBcAMP is likely to results from the phosphorylatin of other PKA sites in the PDE3A1 sequence. The selective regulation of PDE3A isoforms may have therapeutic ramifications. As was shortly described earlier, PDE3 inhibitors, used to overcome a reduction in the receptor-mediated cMP generation in patients with heart failure, increase contractility by increase contractility by increasing the phosphorylation of PKA substrates involvend in the intracellular calcium cycling, but their long-term use is associated

with an increase in mortality that may result from PKA-mediated proapoptotic changes in gene expression. Vandeput et al. findings indicate that existing PDE3A, like they are used today, are actually targeting at least two functionally distinct isoforms of PDE3A that are likely to have different roles in regulating intracellular singaling in cardiac myocytes. PDE3A1 and PDE3A2 are equally sensitive to these PDE3 inhibitors, an agent that is capable of binding selectively to either one of the isofroms and disrupting its protein-protein interactions might be able to increase cAMP content in a particular intracellular microdomain, without it raising total intracellular cAMP content. By affecting the phosphorylation of a restricted set of PKA substrates in cardiac muscle cells, such an agent might have inotropic effects with fewer of the adverse effects observed in PDE3A inhibitors (catalytic site inhibitors) known today. Finding agents that can bind selectively to phosphorylated PDE3A1 or PDE3A2 would be an important step forward in this approach.(2)

Conclusion

Initially, the aim of this project was to transfect HEK293 cells with mutants of the PDEs and to compare cGMP and cAMP levels in these HEK293 cells as well as tissues of mutant rats with the immunoassay kits. When these kits did not have show sufficient stability to perform subsequent assays over the course of several weeks, the constructs were expressed in HEK293 cells, and the purified PDE3A was used in cAMP Alphascreen assays where fixed amounts of cAMP were incubated with this purified PDE3A to essentially investigate the kinetics of the hydrolysis by these phosphodiesterases. The mutants as well as the wildtype purified PDE3A showed t hydrolyzing properties of cAMP. However unfortunately, the limited time span of the project did not allow for a full hydrolysis experiment and the results were limited to hydrolysis pilots that do not allow for quantification of differences in mutant hydrolytic activity compared to the wildtype PDE3A. Further research should be conducted to quantify these findings and elucidate the genetic basis and the influence of selective phosphorylation and therefore regulation of the distict isoforms PDE3A1 and PDE3A2. As there are strong implications by Vandeput et al that this differential regulation of their catalytic activity and protein-interactosomes result form phosphorylation at alternative binding sites in response to activation of PKA and PKC respectively, it is plausible that the PDE3 inhibitors known today therefore target at least two functional distinct isoforms in the cardiac muscle cells that are likely to have different roles in intracellular signaling. This way, the possibility is raised that isoform-selective targeting may increase contractility in failing hearts without increasing mortality, providing a potential route for developing the therapeutics for heart failure of the future.

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Appendix

Appendix 1 Transfection of HEK 293 cells with PEI and Bradford total protein assay

Transfection of HEK 293 cells for the purification of PDE3A types

Monday

17:00 Splitting of HEK 293 cells for seeding

Take 15 cm plates (12 plates)
Or: 10 cm plates (33 plates)

In this case we used 10 cm plates:

Cell seeding example:

Confluent T75	9.40E+06 cells
Confluent 100 mm dish	7.70E+06 cells
Confluency	40%
Target cells	3.08E+06
Days to grow	1
Starting cells needed	1.54+E06 starting cells
Volume of T75 cells	10 ml
Volume needed	1.64 ml
Volume DMEM per T75	8.36 ml

Cell seeding example:

Confluent T75	9.40E+06 cells
Confluent 150 mm dish	2.02E+07 cells
Confluency	40%
Target cells	8.07E+06
Days to grow	1
Starting cells needed	4.04+E06 starting cells
Volume of T75 cells	10 ml
Volume needed	4.29 ml
Volume DMEM per T75	5.71 ml

Tuesday
<u>09:00 Transfection</u>
33 plates 10 cm

PEI	DNA
8530.50 µl OPTI MEM 544.50 µl PEI	9075.00 µl OPTI MEM 181.50 µg plasmid (= **363** µl of 500 ng/µl DNA)

510 µl per plate

13 plates 15 cm

PEI	DNA
10161.72 µl OPTI MEM 648.62 µl PEI	10810.34 µl OPTI MEM 216.21 µg plasmid (= **432.41** µl of 500 ng/µl DNA)

1310 µl per plate
(2 x 655 µl)

- Change cells into 6 ml P/S free medium (for 10 cm plates) and return them to the incubator. Take 18 ml for 15 cm plates
- Label 1 50 ml Falcon PEI and 1 50 ml Falcon DNA and add the corresponding amounts of OPTI MEM and PEI or plasmid
- Vortex PEI mix and incubate for 5 minutes at room temperature.
- Transfer the PEI substance into the DNA labeled Falcon and incubate for 25 minutes at room temperature
- Add PEI+Plasmid mix to dish (510 µl) and incubate at 37 degrees for 5 hours or O/N.
- Change cells into 10 (18 ml for 15 cm plates) ml DMEM with P/S (complete) per plate
- Incubate for 48 hours at 37 degrees

<u>Bradford total protein assay</u>
-Prepare 7 standards in 500 µl tubes from the albumin standards provided in the kit
* 1000 µg/ml
* 750 µg/ml
* 500 µg/ml
* 250 µg/ml
* 125 µg/ml
* 62,5 µg/ml
* 0 µg/ml
-Add 10 µl of the standards in quadruplicate to the 96 well plate
-Add 10 µl of the protein (4 samples) to the 96 well plate in quadruplicate
Add 10 µl of the flagpeptide to the 96 well plate in quadruplicate

-Add 300 µl of Bradford protein assay solution to all standards and samples

*Samples do not have to be diluted

Appendix 2 : Protocol Biovision cAMP/ cGMP direct immunoassay kit (Colorimetric)

Protocol Biovision cAMP direct immunoassay kit (Colorimetric)

Component	100 assays	Color Code Cap color	Storage Temperature
10X cAMP Assay Buffer	25 ml	WM	+4°C
Standard cAMP (10 nmol)	1 vial	Yellow	-20°C
Neutralizing Buffer	7,5 ml	NM	+4°C
Acetylating Reagent A	0,75 ml	Violet	+4°C
Acetylating Reagent B	1,5 ml	Black	+4°C
Anti-cGMP pAb/BSA	1 vial	Red	-20°C
cGMP-HRP/BSA	1 vial	Green	-20°C
HRP developer	10 ml	Amber	+4°C
Protein G coated plate	1 each	....	-20°C

Reagent preparations:
-Thaw the 10x cAMP buffer assay by putting it in hand warm water until fully liquid.
-Dilute the 10x cAMP buffer to 1 x Assay buffer with MiliQ water by adding 225 ml of Aquadest water to the 25 ml of 10x cAMP buffer to get 250 ml of 1x Assay buffer.
-Store in the fridge(4°C) or temporarily on ice.
-Put the vials on ice and thaw the HRP developer and neutralizing buffer by hand before putting them on ice.
-Add 1 ml of 0,1 M HCl to the Standard cAMP to reconstitute the Standard cAMP (pallet may not be visible).
-Vertex for 10 seconds to generate the 10 pmol/µl cAMP standard stock solution
-Dilute Rabbit anti-cAMP by adding 1,1 ml of 1x Assay buffer to the Rabbit anti-cAMP to as stock solution. Keep frozen.
-Dilute cAMP-HRP with 1,1 ml of dH_2O as stock solution. Keep frozen.

Note: the unused Protein G coated strips with descants and the kit should be stored at -20°C. The strips are stable for 1 month. The kit is stable for 1 tot 2 months after opening and reconstitution.

Sample preparation

Urine, plasma and culture medium samples:
-Dilute urine and plasma 1:20 tot 1.100 in 0,1 M HCl
-Dilute Culture Medium 1:10 to 1.200 in 0,1 M HCl

Cell samples:
-Add 1 ml of 0,1M HCl for every 35 cm^2 of surface area. For a 6 well-plate (surface area = 9,6 cm^2) this is 0,274 ml per well.

-Incubate at room temperature for 20 minutes
-Scrape cells of the surface with a cell scraper
-Dissociate sample by pipetting up and down until the suspension is homogeneous
-Transfer the suspension to a centrifuge tube and centrifuge at top speed for 10 minutes
-The supernatant may be assayed directly (protein concentrations ≥ 1mg/ml is recommended for reproducible results)

Tissue Samples:
-Freeze tissues directly after collection to avoid metabolisation of the cyclic nucleotides. E.g. with liquid nitrogen.
-Weigh the frozen tissue and add 5-10 times its volume in 0,1 M HCl.
-Homogenize the sample on ice using a Polytron-type homogenizer
-Spin at top speed for 5 min and collect the supernatant.
-The supernatant may be assayed directly.

Prepare cAMP standard curve and Sample
-Label 8 microcentrifuge tubes: 1.25, 0.625, 0.3125, 0.156, 0.078, 0.039, 0, 0_B pmol/50μl (final concentrations in the well) and one tube for the 1.25 pmol/μl cAMP working solution.
-Label tubes for test samples
-Dilute 25μl of the 10 pmol/μl standard cAMP stock with 975 μl 0,1 M HCL to generate 0,25 pmol/μl cAMP working solution.
Note: this solution should be fresh and used within an hour.
-Add 200 μl of the 0.25 pmol/μl cAMP standard into the tube labeled 1.25 pmol tube (enough for 20 assays)
-Add 100 μl of 0,1M HCl into the rest of the tubes
-Transfer 100 μl from the tube labeled 1.25 pmol tube to the labeled 0.625 pmol tube, mix by resuspending, then transfer 100 μl into the labeled 0.3125 pmol tube.
Continue the serial dilution by transferring 100 μl to 0.156, 0.078, 0.039 pmol tubes
-discard 100 μl from the 0.039 tube.

Time:

Note: the diluted cAMP should be used within 1 hour.
-Add 100 μl each test sample per labeled test sample tube. (use different dilutions for each sample and dilute with 0,1M HCl)
-Add 50 μl of Neutralizing Buffer to each tube (all standards cAMP and testing samples)
-Prepare Acetylating Reagent Mix: Mix 1 volume of Acetylating Reagent A (violet cap) with two volumes of Acetylating Agent B (Black cap) in a microtube.
-Prepare enough for the experiment: 5 μl each sample and standard tube.
For only standards: 8 standards x 5μl = 40μl. + extra 20 μl = 60 μl
20 μl of Acetylating agent A
40 μl of acetylating agent B

Time:

Note: Use within 1 hour

-Add 5 µl oft he Acetylating Mix directly into each test solution (all standards and samples) **IMMEDIATELY vortex 2-3 seconds following each addition without delay, one tube at a time.**
-Incubate at room temperature for 10 min to acetylate cAMP.
-Add 845 µl 1x Assay Buffer into each tube to dilute the acetylation reagents, mix well.
-The acetylated standard and samples are ready for quantification.

Quantification cAMP

-Add 50 µl oft the acetylated Standard cAMP and test samples from step 9 to the Protein G coated 96-well plate in duplicate.
-Add 10 µl of the reconstituted cAMP antibody per well to the standard cAMP and sample wells, except the well with 0_B pmol cAMP
Note: do not add cAMP antibody to the 0_B pmol cAMP well, instead add 10 µl of 1x Assay Buffer for background reading.
-Cover the plate with a sticky seal.
-Centrifuge briefly.
-Incubate for 1 hour at room temperature with gentle agitation (22°C, 300 rpm).

Start: End:

Note: use a repeating pipette to minimize pipette errors.
-Add 10 µl of cAMP-HRP to each well, incubate for 1 hour at room temperature with agitation (22°C, 300 rpm).

Start: End:

-Wash 5 times with 200 µl 1x Assay Buffer each wash.
-Completely empty the wells by tapping the plate on a new paper towel after each wash step.
-Add 100 µl of HRP developer and develop for 1 hour at room temperature with agitation (22°C, 300 rpm).

Start: End:

-Add 100 µl of 1 M HCl to each well. This will stop the reaction and the sample color should change from blue to yellow
-Read the plate at OD 450 nm.
Note: the OD450 nm reading may vary significantly among experiments depend on lot numbers, kit storage and experiment conditions. **Therefore, samples and standard curve must be performed at the same time and using the same kit reagents.**
-Substract OD 450 nm background reading (the well with 0_B pmol cAMP) from all samples and standards.

Instruction manual for AlphaScreen:

Start the machine and computer following the instructions on the AlphaScreen.
Choose programme.

Click load plate
Run programme
Export data onto stick (text file)

Protocol Biovision cGMP direct immunoassay kit (Colorimetric)

Component	100 assays	Color Code Cap color	Storage Temperature
10X cGMP Assay Buffer	25 ml	WM	+4°C
Standard cGMP (10 nmol)	1 vial	Yellow	-20°C
Neutralizing Buffer	7,5 ml	NM	+4°C
Acetylating Reagent A	0,75 ml	Violet	+4°C
Acetylating Reagent B	1,5 ml	Black	+4°C
Anti-cGMP pAb/BSA	1 vial	Red	-20°C
cGMP-HRP/BSA	1 vial	Green	-20°C
HRP developer	10 ml	Amber	+4°C
Protein G coated plate	1 each	….	-20°C

-

Reagent preparations:
-Thaw the 10x cGMP buffer assay by putting it in hand warm water until fully liquid.
-Dilute the 10x cGMP buffer to 1 x Assay buffer with MiliQ water by adding 225 ml of Aquadest water to the 25 ml of 10x cGMP buffer to get 250 ml of 1x Assay buffer.
-Store in the fridge(4°C) or temporarily on ice.
-Put the vials on ice and thaw the HRP developer and neutralizing buffer by hand before putting them on ice.
-Add 1 ml of 0,1 M HCl to the Standard cGMP to reconstitute the Standard cGMP (pallet may not be visible).
-Vertex for 10 seconds to generate the 10 pmol/µl cGMP standard stock solution
-Dilute Rabbit anti-cGMP by adding 1,1 ml of 1x Assay buffer to the Rabbit anti-cGMP to as stock solution. Keep frozen.
-Dilute cGMP-HRP with 1,1 ml of 1x assay buffer as stock solution. Keep frozen.

Note: the unused Protein G coated strips with descants and the kit should be stored at -20°C. The strips are stable for 1 month. The kit is stable for 1 tot 2 months after opening and reconstitution.

Sample preparation

Urine, plasma and culture medium samples:
-Dilute urine and plasma 1:20 tot 1.100 in 0,1 M HCl
-Dilute Culture Medium 1:10 to 1.200 in 0,1 M HCl

Cell samples:
-Add 1 ml of 0,1M HCl for every 35 cm^2 of surface area. For a 6 well-plate (surface area = 9,6 cm^2) this is 0,274 ml per well.
-Incubate at room temperature for 20 minutes
-Scrape cells of the surface with a cell scraper

-Dissociate sample by pipetting up and down until the suspension is homogeneous
-Transfer the suspension to a centrifuge tube and centrifuge at top speed for 10 minutes
-The supernatant may be assayed directly (protein concentrations ≥ 1mg/ml is recommended for reproducible results)
(dilute to get protein concentrations: 2, 4, 6 mg/ml etc depending on the Bradford assay results)

Tissue Samples:
-Freeze tissues directly after collection to avoid metabolisation of the cyclic nucleotides. E.g. with liquid nitrogen.
-Weigh the frozen tissue and add 5-10 times its volume in 0,1 M HCl.
-Homogenize the sample on ice using a Polytron-type homogenizer
-Spin at top speed for 5 min and collect the supernatant.
-The supernatant may be assayed directly.

Prepare cGMP standard curve and Sample
-Label 8 microcentrifuge tubes: 10, 5, 2.5, 1.25, 0.625, 0.3125, 0.156, 0.078, 0.039 0, 0_B pmol/50μl (final concentrations in the well) and one tube for the 2 pmol/μl cGMP working solution.
-Label tubes for test samples
-Dilute 200 μl of the 10 pmol/μl standard cGMP stock with 800 μl 0,1 M HCL to generate 2 pmol/μl cGMP working solution.
Note: this solution should be fresh and used within an hour.
-Add 200 μl of the 2 pmol/μl cGMP standard into the tube labeled 10 pmol tube (enough for 20 assays)
-Add 100 μl of 0,1M HCl into the rest of the tubes
-Transfer 100 μl from the tube labeled 10 pmol tube, mix by resuspending, then transfer 100 μl into the labeled 5 pmol tube.
Continue the serial dilution by transferring 100 μl to 2.5, 1.25, 0.625, 0.3125, 0.156, 0.078, 0.039 pmol tubes
-Discard 100 μl from the 0.039 tube.

Time:

Note: the diluted cGMP should be used within 1 hour.
-Add 100 μl each test sample per labeled test sample tube. (use different dilutions for each sample and dilute with 0,1M HCl)
-Add 50 μl of Neutralizing Buffer to each tube (all standards cGMP and testing samples)
-Prepare Acetylating Reagent Mix: Mix 1 volume of Acetylating Reagent A (violet cap) with two volumes of Acetylating Agent B (Black cap) in a microtube.
-Prepare enough for the experiment: 5 μl each sample and standard tube.
For only standards: 11 standards x 5μl = 55μl. + extra 20 μl = 75 μl
25 μl of Acetylating agent A
50 μl of acetylating agent B

Time:

Note: Use within 1 hour

-Add 5 µl oft he Acetylating Mix directly into each test solution (all standards and samples) **IMMEDIATELY vortex 2-3 seconds following each addition without delay, one tube at a time.**
-Incubate at room temperature for 10 min to acetylate cGMP.
-Add 845 µl 1x Assay Buffer into each tube to dilute the acetylation reagents, mix well.
-The acetylated standard and samples are ready for quantification.

Quantification cGMP

-Add 50 µl oft the acetylated Standard cGMP and test samples from step 9 to the Protein G coated 96-well plate in duplicate.
-Add 10 µl of the reconstituted cGMP antibody per well to the standard cGMP and sample wells, except the well with 0_B pmol cGMP
Note: do not add cGMP antibody to the 0_B pmol cGMP well, instead add 10 µl of 1x Assay Buffer for background reading.
-Cover the plate with a sticky seal.
-Centrifuge briefly.
-Incubate for 1 hour at room temperature with gentle agitation (22°C, 300 rpm).

Start: End:

Note: use a repeating pipette to minimize pipette errors.
-Add 10 µl of cGMP-HRP to each well, incubate for 1 hour at room temperature with agitation (22°C, 300 rpm).

Start: End:

-Wash 5 times with 200 µl 1x Assay Buffer each wash.
-Completely empty the wells by tapping the plate on a new paper towel after each wash step.
-Add 100 µl of HRP developer and develop for 1 hour at room temperature with agitation (22°C, 300 rpm).

Start: End:

-Add 100µl of 1 M HCl to each well. This will stop the reaction and the sample color should change from blue to yellow
-Read the plate at OD 450 nm.
Note: the OD450 nm reading may vary significantly among experiments depend on lot numbers, kit storage and experiment conditions. **Therefore, samples and standard curve must be performed at the same time and using the same kit reagents.**
-Substract OD 450 nm background reading (the well with 0_B pmol cGMP) from all samples and standards.

Instruction manual for AlphaScreen:

Start the machine and computer following the instructions on the AlphaScreen.
Choose programme.

Click load plate
Run programme
Export data onto stick (text file)

Appendix 3 Purification of PDE3A types

Thursday

Purification of PDE3A types

Lysis, binding and elution of transfected HEK cells with PDE3A expression

Lysis

PDE3A Lysis Buffer

500 ml					
20	mM HEPES 7.5	20	ml of	0.5	mM HEPES 7.5
150	mM NaCl	15	ml of	5	mM NaCl
1	mM EDTA	2	ml of	0.25	mM EDTA
1	% Triton	20	ml of	25	% Triton

Additions to 50 ml of this buffer:

Total volume	50 ml	
PMSF	0.0125 µl/µl	625 µl
Jenny's Mix	0.008 µl/µl	400 µl
Na3VO4	0.01 µl/µl	500 µl
NaF	0.05 µl/µl	2500 µl

-Add these additives (protease inhibitors) to 50 ml of buffer by pouring some buffer into a 50 ml measuring cylinder, then adding the additives, then bringing it up to 50 ml with buffer

-Transfer the buffer with additives to a 50 ml falcon and mark with a red piece of tape

-Take the plates out of the incubator and label 6 50 ml Falcons: 1,1, 2, 2, 3, 3.

-pipette the DMEM from the first plate and put it in a Falcon labeled 1
-Dislogde the cells from the plate mechanically by hitting the plate with the lid on it with a Eppendorf holder on all sides of the plate. Or use a cell scraper fort he 150 mm dishes
-Pipette the medium from the Falcon back into the plate and make sure all the cells are dislodged in the medium.
-Pipette the medium with the cells into the Falcon.
-Take another plate and pipette the medium off this plate, into the empty plate

-After dislodging the cells from the new plate, pipette the medium from the previous plate into the new plate.
-Pipette the medium with cells into the Falcon.
-Repeat until a total of 5 plates is dislogded (5 x 10 ml = 50 ml) (or 2 plates 2 x 24 ml = 48 ml)
-Repeat for the other 5 Falcons to dislogde a total of 30 plates (100 mm dish) or 12 plates (150 mm dish).

-After 10 plates (or 4 plates for 150 mm dish) are transferred into 2 Falcons, spin the tubes at 1200 rpm for 4 minutes.
-Pour off the DMEM from the cells, carefully, without losing the pallet, and resuspend in 20 ml of cold PBS
-Spin the cells again for 4 minutes at 1200 rpm.
-Pour off the old PBS, resuspend in another 20 ml of PBS and spin again.
-following this second wash, the PBS is removed and 4 ml of lysis buffer + protease inhibitors is added.
- Collect a 40 µl sample of the raw cell lysate
-The total volume of 24 µl is pooled and put on a roller plate for 5 minutes at room temperature.
-The cell lysate is transferred into 1,5 ml safelock eppendorf tubes (+/- 1 ml per tube)
-The eppendorfs are spun for 15 miutes at 14,000 rpm at 4 degrees to pellet the cell material
-Supernatant is removed and pooled into 2 15 ml Falcons. Pellets are discarded.
-Collect a 40 µl sample of supernatant

Binding
-Equilibrate 2 x 250 µl of M2 FLAG magnetic bead slurry with 3 washes in 800 µl Anti-Flag lysis buffer
-Add the equilibrated beads to the cell lysis supernatant in the 15 ml Falcon
-Incubate the beads and supernatant together for <u>3 to 4 hours</u> on a rolling mixer in a cold room

Elution
-Using a 15 ml Falcon magnet apparatus, precipitate the beads and remove most of the supernatant, leaving about 1 to 1,5 ml in the falcon.
-Collect a sample of 40 µl of supernatant (after beads)
-Tranfer the beads (and the remaining supernatant) into a 1,5 ml Eppendorf tube for a total of 2 Eppendorf tubes with beads.
-Using a 1,5 ml Eppendorf magnet apparatus, precipitate beads and remove all lysis buffer from the beads.
-Wash the beads (2x) with 800 µl of lysis buffer by taking the tube out of the magnet apparatus, adding buffer and removing the buffer after precipitation of the beads in the magnet apparatus
-Wash the beads (3x) with 20 mM HEPES pH 7.5, 200 mM NaCl buffer, for a total of 5 washes
-Following these washing steps, remove the 20/200 buffer from the beads and add 100 µl of 200 µg7ml FLAG peptide in 20/200 to the beads
    * 480 µl 20/200 buffer
    * 20 µl 5 mg/ ml FLAG peptide

-Incubate this elution mixture for 15 minutes at room temperature with approximately 1000 rpm in a thermomixer
-Remove the elution mixture (2 x 100µl) from the beads and pool it in a new Eppendorf tube and keep it on ice
-Add another 100 µl to the beads and repeat this process (incubate for another 15 min)
-Pool the elution mixture (4x 100 µl)
-Aliquot the maximum of a p10 pipette into 1,5 ml eppendorf tubes labeled with date, PDE3A2 and mutation type
-Freeze in liquid nitrogen and store at -80 degrees.

Bradford Total Protein assay (by Thermo Scientific)
-Prepare 7 standards in 500 µl tubes from the albumin standard (2000 µg/ml) provided in the Coomassie Plus (Bradford) Assay kit by Thermo Scientific.
-1000 µg/ml
-750 µg/ml
-500 µg/ml
-250 µg/ml
-125 µg/ml
-62,5 µg/ml
-0 µg/ml
-Add 10 µl of the standards in quadruplicate to a 96 well plate
-Add 10 µl of the protein (4 samples of 10 µl) to the 96 well plate in quadruplicate*
Add 10 µl of the flagpeptide to the 96 well plate in quadruplicate
*Samples do not have to be diluted

-Add 300 µl of Bradford protein assay solution to all standards and samples and develop for 10 minutes at room temperature
-Read the plate at 595 nm.

Appendix 3 Pouring gels, coommassie gel and immunoblotting

Pouring gels for coomassie gel and immunoblotting

-Assemble the gel holder and glass parts
-Check for leaks by filling the opening (chamber) up with water
-Meanwhile, prepare the gel mixture according to the protocols in the hood in a 50 ml Falcon
-Acrylamide and APS can be found in the fridge
Note: once the APS is added, the solidifying process will start
-Mix carefully by shaking it around for a second and fill up the chamber with a 5 ml pipette with the gel mixture up until the lower part of the green plastic
-Add isopropanol on top until it overflows a bit
-Close the hood and let this sit for about 30 minutes until the remains of the gel in the Falcon have solidified, don't screw the cap back on the Falcon
-When this is finished prepare the stacking gel in another 50 ml Falcon
- Pour off the isopropanol and let the remains flow into a filter paper while turning the chamber on it's side (see picture below)
- load the stacking gel on top of the running gel until it overflows a bit
-Insert a comb into the stacking gel (10 or 15 wells)

-Again, close the hood and let this sit for about 30 minutes until the remains of the gel in the Falcon have solidified, don't screw the cap back on the Falcon.
-After solidifying, take out the glass parts with the gel and wrap them in a wet paper towel.
-Put the gel (s) in a plastic bag and write name, date, percentage and amount of wells before putting it in the fridge.

Coomassie gel

- Assemble the buffer tank and the gel holder and put the (8%) acrylamide gel in the gel holder
- Prepare the samples
- Prepare all samples (supernatant before beads, supernatant after beads, cell lysate and the beads by adding **10 µl of the sample to 190 µl of the mastermix** except for the purified protein eluent sample.

Mastermix in 2 ml Eppendorf

Sample	-
DTT	100 µl
SDS (Lämli buffer)	500 µl
Buffer (water)	1300 µl
Total	**1900** µl

- Prepare the purified protein eluent sample:

Sample (purified protein)	15 µl
DTT	1 µl
SDS (Lämli buffer)	5 µl
Buffer (water)	-
Total	21 µl

- Heat the samples at 95°C for at least 10 minutes without any rotation
- Fill the tank with running buffer (tap) until the gel is completely covered

- Spin down the samples after heating
- Load the marker (3 µl) and probes (20 µl) with a pipette
- Attach the lid to the buffer tank and apply the electrodes and insert into the electronic machine.
- Turn it on, hit manual, constant mV and apply 25 mV. Hit run.
- Run the gel for about 1 hour and make sure the gel is covered all the time in running buffer. If the tank is leaking, add more buffer if necessary.
- Take out the gel in the gel holder and rinse it off.
- Take out the gel and place it in a plastic container with about 1-2 inches of coomassie staining and let this incubate for about 1 hour.
- After staining, remove the stain and rinse the gel with water.
- Apply 1-2 inches of destain and repeat after about 15-30 minutes until the gel is destained enough to read

Western blot (immunoblotting)

-Run the gel as described previously in Coomassie gel but apply 35 mA for about 40 min.

Preparing of the samples for Western blot

You want to load **100 µg of the protein** so for a 67,4 µg solution of PDE3A1 that is: 1,48 µl which is roughly 1,5 µl of the eluent. Prepare 10 times as much as you need. Bring up to volume with water.

Sample	15 µl (10 x 1,5)
Lämli buffer	50 µl
DTT	10 µl
water	75 µl
Total	**150 µl**

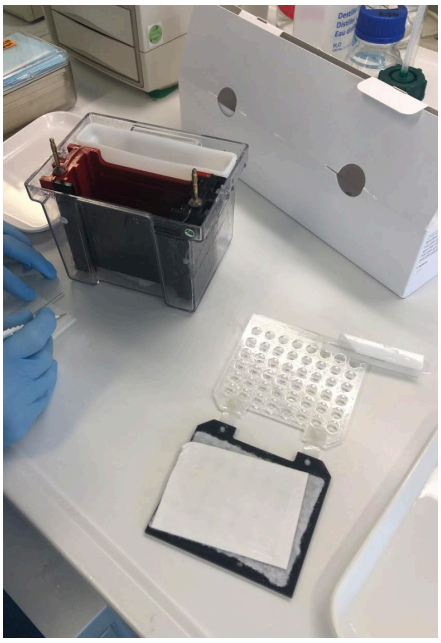
- Heat at 95°C for at least 10 minutes.
- Keep in the fridge O/N (heat again the next day) or use immediately
- Load 15 µl of the sample and 3 µl of the marker in the order: marker, protein 1, protein 2, marker, protein 1, protein 2 etc.

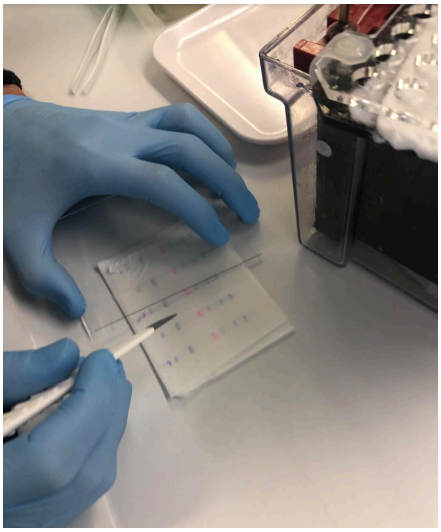
Transfer

-Take the gel out and cut off the top part were the comb used to be.
-place it in a container on a piece of fabric, followed by a filter paper and a membrane that was previously soaked in ethanol.
-put another filter paper and piece of fabric on top and make sure all the parts are aligned.

-Pour sufficient Tank buffer on.
-Take this construct out of the container and place it on the black part of the WB holder.
-press the transparent part to the construct and fix it with the white clip.
-Put the WB in the chamber and fill the chamber up with ice and tank buffer.

-Add a magnetic stirrer and take the chamber to the cold room
-Apply 110 mV for 2 hours to the WB chamber and make sure it is stirring at 850 rpm.
-After 2 hours, take out the membrane and cut it as wished using a sharp knife (razor) and putting the membrane on a filter paper and using a glass to cut precisely (See pictures below).





-block with 5%BSA or 5% skimmed milk in TBS-T.
Note: skimmed milk 5%: I.g. 1,2 g skimmed milk + 19 x 1,2 = 22,8 ml of TBS-T, prepare in 50 ml Falcon

-Put the seperate strips in 50 ml Falcons with 10 ml of the right blocking buffer and incubate at room temperature for 1 hour.
-Label the Falcons with the names oft he first antibody, that you will use and the date.

Western blot of PDE3A1,
8% AA gel
PDE3A1 reported: 996 aminoacids, MW: 109,980 kD(27)

5 different 1st antibodies: Flag-HRP, Bethyl PDE3A, Eurogentec Rat-PDE3A1 S292-293 Non-phospho (RRRRSSSVVAGE) (HM1) , Eurogentec Rat-PDE3A1 (CSGPAPPRERFGSQSS) (HM2), Eurogentec Rat-PDE3A1 (ARTKEEIPGWK) (HM3)

Blocking:
Flag-HRP, Bethyl : 10 ml 5% milk in TBS-T
(Homemade) HM 1, 2, 3: 10 ml 5% BSA in TBS-T

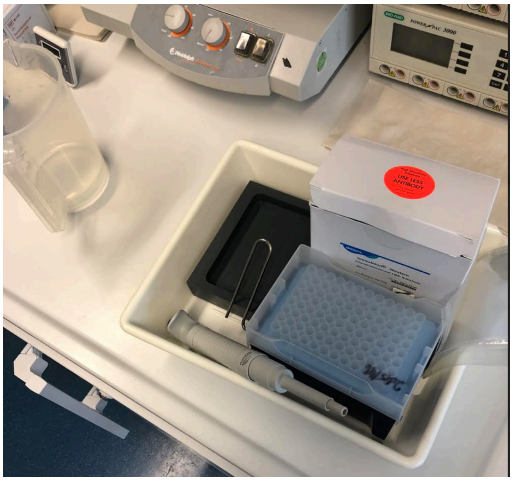
1st antibody: prepare in 50 ml falcons and transfer later to the right Falcons with blots after pouring off the blocking buffer.
Bethyl PDE3A: 5 µl in 5 ml 5% milk in TBS-T
Flag-HRP: 2 µl in 5 ml 5 % milk in TBS-T
HM 1, 2, 3: 2 µl in 5 ml 5% BSA in TBS-T

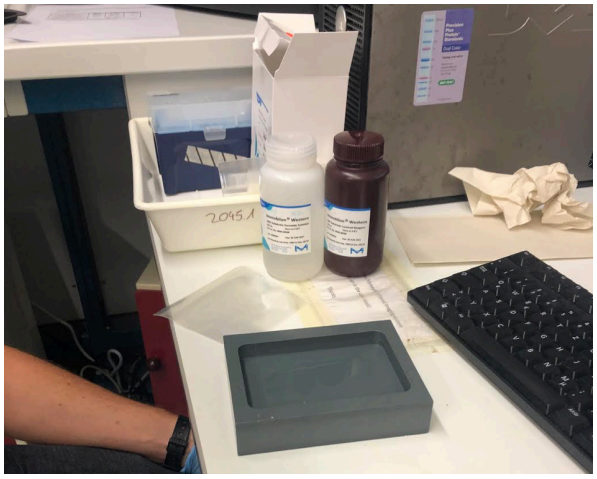
- Incubate O/N in the cold room

- Wash 3 times for 8-10 min with 10 ml of TBS-T, **except for** the Flag- HRP at room temperature

2nd antibody
Bethyl PDE3A: 2 µl anti rabbit in 10 ml 5% milk in TBS-T
Flag HRP: no 2nd antibody
HM 1, 2, 3: 2 µl anti rabbit in 10 ml 5% BSA in TBS-T

- Incubate the 2nd antibody for 2 hours
- Wash 3 times for 8-10 min with 10 ml of TBS-T, **including** the Flag- HRP at room temperature
- Read the IB (See pictures):





Overlay of the AKAP 2/PKA protein with PDE3A1 WT:

Control for interaction of the AKAP2/PKA with the Bethyl anti PDE3A antibody
-A membrane is prepared with the full sequence of the AKAP 2 protein in fractions of 25 aminoacids
-The membrane is activated in 100% ethanol
-Block the membrane in 5% milk in TBS-T for 3 hours at room temperature (0,75 g skimmed milk up to 15 ml TBS-T)
-Wash 3 x 5 min with TBS-T
-Peptide assay incubation buffer with 50 mM Tris pH 7.4 and 5 mM $MgCl_2$
-Incubate overnight at 4°C with incubation buffer.
-1st antibody: 10 µl of anti PDE3A in 15 ml 5% milk in TBS-T
-Incubate for 3 hours at room temperature
-2nd antibody: 2 µl of anti-rabbit in 15 ml 5% milk in TBS-T
-Incubate at room temperature for 1 hour.
-Wash 3 x 8 min with TBS-T
- Read image with the Odysseus
- Incubate overnight with 30 µl of PDE3A1 WT in 15 ml of incubation buffer
-Reincubate with the 1st antibody (Bethyl) for 3 hours
-Read the membrane

Appendix 4 Hydrolysis pilot

AlphaScreen Pilot for the assesment of the dynamics of PDE3A1 WT hydrolysis of cAMP

Buffer to make before experiment

cAMP AlphaScreen Buffer with 0,3% Tween (for 100 ml)		
20 mM Hepes pH 7,5	4 ml of	0,5 M stock
200 mM NaCl	4 ml of	5 M stock
5 mM MgCl2	0,5 ml of	1 M stock
0,1 % BSA	1 ml of	10
0,3% Tween-20	3 ml of	10% Tween

Protein concentrations	
Molecular weight	110958,29 amu
Protein concentration (ng/µl)	110 ng/µl
Protein concentration (nM)	991,36 nM
Target concentration	6,091 nM
Target volume	1000 µl
Volume protein needed	6,144 µl

Protein mix	1000 µl
AlphaScreen buffer	1 x 993.86 or 2x 496,93 µl
Volume protein needed	6,144 ml
	161,29 µl into each of 6 tubes

cAMP dilutions

cAMP mix 1 DON'T USE THIS 5000 nM, 500 µl	
50000 nM stock	50 µl
AlphaScreen Buffer	450 µl

1:10 dilution

cAMP mix 2 USE THIS ONE 500 nm, 500 µl	
5000 nM	50 µl
AlphaScreen Buffer	450 µl

1:100 dilution

Conditions
No cAMP
No PDE
0 min incubation
6 min incubation
30 min incubation

	No cAMP	No PDE	0 min	6 min	30 min
AlphaScreen Buffer (µl)	5	20	0	0	0
6,091 nM protein mix (µl)	20	0	20	20	20
500 nM cAMP mix (µl)	0	5	5	5	5
25 mM IBMX (µl)	2	2	2	2	2
Total (µl)	**27**	**27**	**27**	**27**	**27**

1. Set the thermomixer to 800 rpm, 37°C
2. Use a multichannel pipette to add 20 µl of PDE protein mix (or 20 µl AlphaScreen buffer for no PDE condition) to all 6 tubes per condition
3. Close the caps of the 6 and 30 minute conditions and leave them on ice
4. Add 2 (µl) of IBMX directly to the no cAMP, no PDE and 0 minute incubation conditions. Do not add IBMX to the other conditions.

* no cAMP
* no PDE
* 0 min

5. Add 5 µl of the Alphascreen buffer tot he no cAMP condition

* no cAMP

6. Add 5 µl of the 500 nM cAMP mix to the no PDE and 0 minute incubation conditions

* no PDE
* 0 min
7. The hydrolytic incubation of the PDE3A1 or PDE3A2 for the 30 minute:
Add 5 µl of cAMP mixture tot he first tube oft he 30 minute condition and put it in the thermomixer and continue this process at 20 second intervals.

After 30 minutes add 2 µl of IBMX to each of the 6 tubes, again at 20 second intervals.

1. 0:00 – 30:00
2. 0:20 – 30:20
3. 0:40 – 30:40
4. 1:00 – 31:00
5. 1:20 – 31:20
6. 1:40 – 31:40

8.During the 30 minute incubation, the 6 minute incubation can be performed
9. Add 5 µl of 500 nM cAMP mix to the first tube of the 6 minute condition, put the tube in the thermomixer and continue this process at 20 s intervals. After 30 minutes add 2 µl of IBMX to each of the 6 tubes, again at 20 second intervals.

1. 0:00 – 6:00
2. 0:20 – 6:20
3. 0:40 – 6:40
4. 1:00 – 7:00
5. 1:20 – 7:20
6. 1:40 – 7:40

10.Acceptor bead mix
If possible, durin the 30 minute incubation, also prepare the acceptor bead mix

Acceptor bead mix

75 Reactions	12,5 µl reaction volume	
6 X acceptor beads	3,75 µl of	250 X stock
1 x Alphascreen buffer	183,75 µl	1 X stock
	Load: 2,5 µl per well	

28,85 in each of 6 tubes

11. Add 2,5 µl of acceptor bead mix to each well (5 x 12 wells, duplo) of a 384 well plate (put 28,85 µl in 6 tubes first and transfer with the multichannel pipette)
12. Add 2,5 µl of the experimental conditions to each well
13. Cover te plate with a sticky seal and cover in Aluminium foil and attach tot he Thermomixer. Incubate at 23°C and 800 rpm for 30 minutes.
14. During this incubation, the Donor bead mix should be prepared:

Donor bead mix

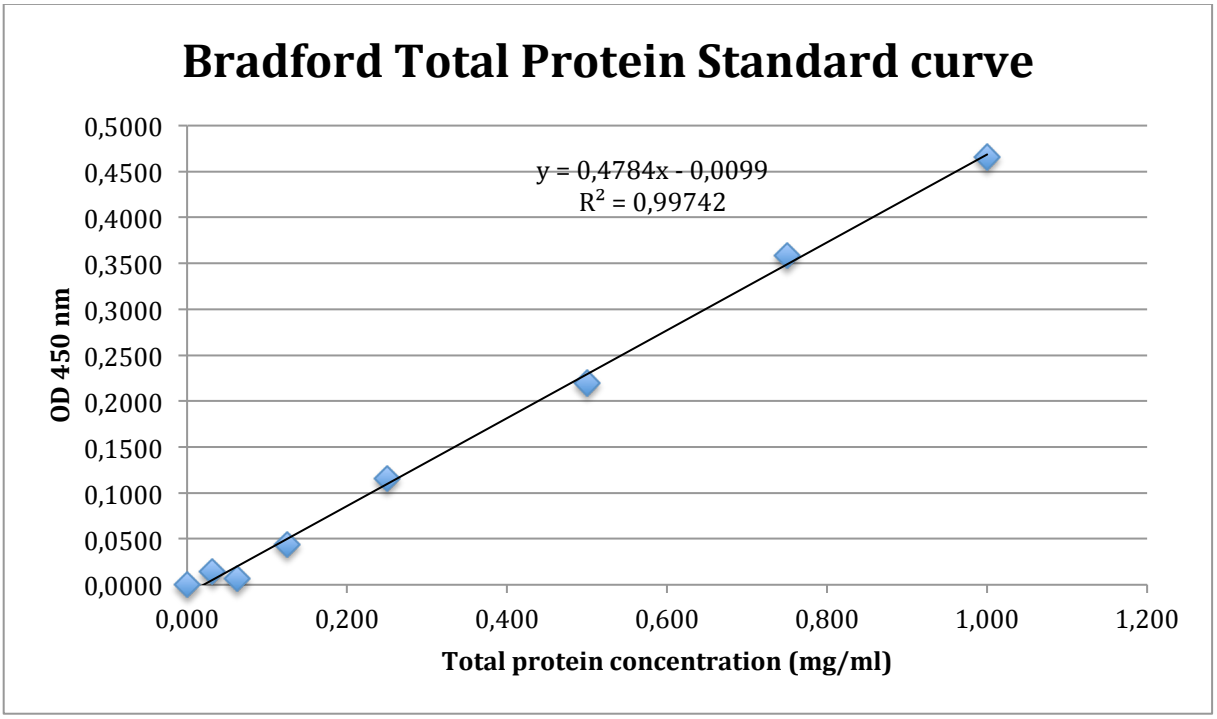
75 reactions	12.5 µl reaction volume	
25 nM Biotin cAMP	23,4375	1 µM stock
5 X Acceptor beads	3,75 µl of	250 X Stock
1 X Alphascreen	535,3125 µl of	1X Stock
Load	7,5 µl	Per well

86,54 in each of 6

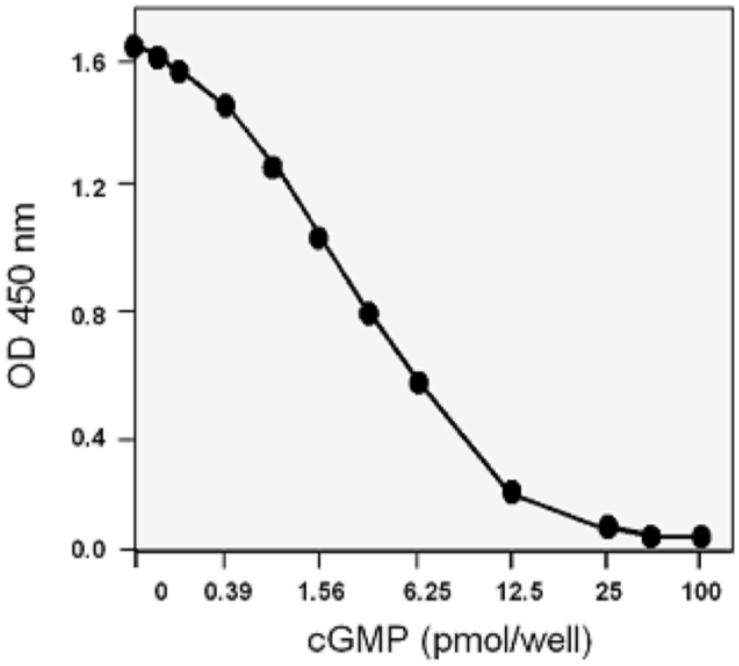
15. Attach the donor bead mix tot he thermomixer and incubate at 23°C for 30 minutes at 800 rpm.

16. Add 7,5 µl of the donor bead mix to each well.
17. Attach the plate to the thermomixer and incubate for 1 hour at 23°C at 800 rpm.
18. Read the plate using the Enspire plate reader using the programm called “2020 copy of Alphascreen“ program.

Appendix 5 Bradford total protein assay standard curve



Appendix 6 cGMP standard curve from the Biovision cGMP direct immunoassay (colorimentric) protocol



Appendix 7 cAMP standard curve from the Biovision cAMP direct immunoassay (colorimentric) protocol