Non-canonical Signaling of μ-opioid Receptors

Nicht kanonische Signaltransduktion von μ Opioidrezeptoren

Master Thesis / Master Arbeit

Study Program / Studienprogramm

FOKUS Life Sciences (Master of Science)

Faculty of Biology / Fakultät für Biologie

Julius-Maximilians-Universität Würzburg,

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Summary

According to the "canonical" paradigm of GPCR signaling, agonist-bound GPCRs only signal to the downstream adenylyl cyclase enzyme when they are seated at the plasma membrane. Upon prolonged binding of an agonist, receptor internalization usually takes place, leading to the termination of this downstream signaling pathway and activation of alternative ones. However, a set of recent studies have shown that at least some GPCRs (e.g. thyroid stimulating hormone receptor) continue signaling to adenylyl cyclase after internalization. In this study, I aimed to investigate canonical signaling by internalized μ opioid receptors (MORs), which are G_i-coupled receptors, using a fluorescence resonance energy transfer (FRET) sensor for cyclic AMP (cAMP) known as Epac1-camps. My results show that the cyclic AMP inhibition signal induced by the binding of DAMGO, a MOR agonist, persists after agonist washout. We hypothesized that this persistent signal might come from internalized DAMGO-bound receptors located in the endosomal compartment. To test this hypothesis, I used dynasore and Dyngo 4a, two dynamin inhibitors that are known to prevent clathrin-mediated endocytosis. Interestingly, dynasore but not Dyngo 4a pretreatment largely blunted the response to MOR activation as well as to adenylyl cyclase activation with Forskolin (FSK). In addition, DAMGO-induced cAMP signal remained persistent even in the presence of 30 μM Dyngo 4a. These results might point to a complex interplay between clathrin-mediated internalization and MOR signaling. Further experiments are required to elucidate the mechanisms underlying the persistent MOR signaling and to fully clarify whether MORs are capable of Gi signaling in the endosomal compartment.

Zusammenfassung

Nach dem „kanonischem“ Paradigma der Signaltransduktion akktivieren agonistbindende GPCR's nur dann die Adenylylcyclase, wenn sie sich in der Zellmembran befinden. Ist der Agonist länger gebunden führt dies meist zur Internalisierung des Rezeptors. Dies führt dazu, dass die Signaltransduktion beendet wird und andere Signalwege aktiviert werden. Jedoch haben einige neuere Studien gezeigt, dass zumindest einige GPCR's (z.B. der Thyreotropinrezeptor) auch nach Internalisierung weiter die Adenylylcyclase aktivieren. Das Ziel der vorliegenden Arbeit ist es kanonische Signaltransduktion von internalisierten μ Opioidrezeptoren (MORs) zu untersuchen, welche zu den G_i gekoppelten Rezeptoren gehören. Dazu wird ein Förster Resonanz Energie Transfer (FRET) Sensor für Cyclisches Adenosinmonophosphat (cAMP) benutzt, bekannt als Epac1-camps.

Meine Resultate zeigen, dass die Inhibierung des cAMP Signal durch das Binden von DAMGO, einem MOR Agonisten, bestehen bleibt auch nachdem der Agonist ausgewaschen wurde. Unsere Hypothese ist, dass internalisierte Rezeptoren im endosomalen Kompartment, die DAMGO gebunden haben, die Ursache für das fortbestehende Signal verantwortlich sind. Um dies zu überprüfen habe ich Dynasore und Dyngo 4a benutzt. Beides sind Dynamin Inhibitoren von welchen man weiß, dass sie die Clathrin gesteuerte Endocytose unterbinden. Interessanterweise hat nur die Vorbehandlung mit Dynasore die Reaktion auf die MOR und die Adenylylcyclase Aktivierung mit Forskolin (FSK) verringert, jedoch nicht Dyngo 4a. Desweiteren hielt das durch DAMGO induzierte cAMP Signal selbst nach Zugabe von 30 M Dyngo 4a an.

Diese Ergebnisse können ein Hinweis für einen komplexen Zusammenhang zwischen Clathrin gesteuerter Internalisierung und MOR Signaltransduktion sein. Jedoch braucht es weitere Experimente um den zugrundeliegenden Mechanismus der anhaltenden MOR Signaltransduktion zu beleuchten und um vollständig zu erklären ob MORs in der Lage für G_i Signaltransduktion im endosomalen Kompartment sind.

(Übersetzt von Kerstin Seier)

1 Introduction

1.1 “Canonical” and “Non-canonical” Signaling of G protein-coupled Receptors

With their very distinctive structure of seven-transmembrane domains, G protein-coupled receptors (GPCRs) make up one of the largest known families of receptors. This receptor family includes neurotransmitter receptors such as, dopamine receptors, and hormone receptors such as thyroid stimulating hormone receptors (TSHRs), as well as olfactory receptors. In addition, a very famous GPCR is the light receptor known as Rhodopsin which is activated by photons (Lefkowitz, 2000). A huge proportion of the medicinal drugs sold in the market target GPCRs which shows how they are deeply involved in the control and regulation of many biological processes. For example, some of the drugs that are used in hypertension treatment target α-1 adrenergic receptors which are GPCRs (Grimm, 1989). In addition, the treatment of Parkinson's disease requires drugs that activate Dopamine receptors (Silva et al., 1997) which also belong to the GPCRs family.

Although 3D structures for many GPCRs are unknown, scientists determined the primary amino-acid sequence of most of the known GPCRs and according to the degree of similarity of these primary sequences, they were able to classify GPCRs into three major families; Family A, B and C (Pierce, Premont, & Lefkowitz, 2002). Family A is the biggest family of the three and it hosts many receptors including adrenergic receptors and rhodopsin. Family B is a much smaller family consisting of only 25 receptors which are mainly peptide hormone receptors. Family C is also a small family whose members have a characteristic big N-terminus. In 2005, a group of scientists created the GRAFS classification system (Schiöth & Fredriksson, 2005) which specifically divided the human GPCRs into five subfamilies based on sequence homology of the Transmembrane regions. The five subfamilies are as follows: rhodopsin family (also known as class A), secretin receptor family (class B), glutamate receptor family (class C), adhesion receptor family and frizzled receptor family. the rhodopsin family is the biggest known subfamily hosting more than 700 receptors.

It is generally well-known that all GPCRs share the common structure consisting of the seven-transmembrane α-helical domains, and extracellular loops ending with the N-terminus, as well as intracellular loops ending with the C-terminus (Trzaskowski et al., 2012). The seven-transmembrane helices are believed to burrow a cylindrical hole into the membrane. Interestingly, the binding site of the agonist differs from one receptor type to the other; small organic agonists, such as acetylcholine, were found to bind to the transmembrane domain (Lu, Saldanha, & Hulme, 2001), while peptide ligands were often found to bind to the extracellular loops and the N-teminus of the receptor (Wheatley et al., 2012). Upon the binding of the ligand, several conformational changes take place leading to receptor activation which are known as "molecular switches". The most crucial conformational change known in GPCRs is the rotational movement of the intracellular part of the transmembrane region 6 (TM6) (Farrens, Altenbach, Yang, Hubbell, & Khorana, 1996; Ghanouni, Steenhuis, Farrens, & Kobilka, 2001; Kobilka, 2007). This TM6 movement is thought to play a key role in the movement of the intracellular loop which binds to and activates the G-proteins.

The name "G-protein-coupled" is based on the classical paradigm of the signaling pathway belonging to this receptor family. This paradigm states that when GPCRs are activated, they undergo a conformational change that leads to the coupling of the receptor to guanine-nucleotide regulatory proteins known as the trimeric G-proteins. As the word "trimeric" indicates, the structure of the G-protein is comprised of 3 subunits; α, ß and γ subunits (also recognized as G_α, G_β, G_γ respectively) and each subunit serves a different function. Once the G-protein is coupled to the active receptor, the active receptor acts as a guanine exchange factor (GEF) replacing guanosine di-phosphate (GDP) with guanosine tri-phosphate (GTP) on the α subunit (Digby, Lober, Sethi, & Lambert, 2006). This GTP binding event leads to the activation and the dissociation of the α subunit from the ß and γ subunits (which remain attached as a single ßγ entity).

These different subunits mediate different downstream effects by binding to distinct effectors. However, the type of activity that an individual receptor mediates is thought to be heavily determined by the type of α subunit in the G-protein that couples to it. Depending on which effector they bind to, G_α subunits are classified to 3 different types; Firstly, G_s which activates the enzyme adenylyl cyclase (AC) which, in turn, synthesizes the second messenger known as cyclic adenosine monophosophate (cAMP), secondly,

G_i which inhibits adenylyl cyclase thus reducing the amount of cAMP, and finally G_q which activates the enzyme phospholipase C (PLC) (Gilman, 1987). Interestingly, the G_α subunit is capable of terminating its own activity by intrinsically hydrolyzing GTP to GDP and Phosphate (P_i). This process spontaneously occurs in the matter of minutes after which the deactivated G_α subunit returns back to ßγ subunits and re-unites with them. Another way the G-protein activity is terminated is when an enzyme called Regulator of G-protein signaling (RGS) hydrolyzes the GTP to GDP (De Vries, Zheng, Fischer, Elenko, & Farquhar, 2000) deactivating the G-protein and reuniting the dissociated G_α and βγ subunits.

Just like many biological processes, GPCR activity is heavily regulated by a variety of mechanisms. This phenomenon known as "desensitization" is observed when the agonist binds to the receptor for a prolonged period of time and is characterized by reduced responsiveness of the receptor to the binding agonist and by gradual "dampening" of downstream signals. The mechanism of this desensitization process begins usually with the phosphorylation of the receptor either by kinases that are activated by second messengers such as protein kinase C (PKCs) or by specific kinases known as G protein-coupled receptor kinases (GRKs) that specifically phosphorylate GPCRs (Ferguson, 2001). GRKs do not phosphorylate any GPCR, they only phosphorylate GPCRs which are agonist-bound in an active conformation. The phosphorylated receptor recruits β-arrestin, which binds to the receptor and prevents its coupling to the G-protein, thus terminating the signal.

The regulatory process does not just stop at the signal termination step, it goes further when the β-arrestin mediates the internalization of the bound receptor through a complex mechanism involving clathrin-coated pits (Zhang, Ferguson, Barak, Ménard, & Caron, 1996). Receptor internalization via clathrin-coated pits was first described by Kobilka and Von Zastrow in a study they performed on ß2-adrenergic receptor (von Zastrow & Kobilka, 1994). Clathrin is a protein composed of 3 heavy chains and 3 light chains and it is known to play a main role in internalization by coating plasma membrane pits (Huang et al., 1997). β-arrestin, while interacting on one side with the phosphorylated receptor, on the other side, it starts interacting with one of clathrin's heavy chains as well as clathrin's adaptor protein known as AP-2. The bringing of all these components with the help of β-arrestin stimulates the formation of the pit

containing the receptor and ligand to be internalized. This pit then detaches from the plasma membrane into the cytoplasm with the help of dynamin. Dynamin is a GTPase which dissociates the budding vesicle from the membrane by forming a coil around the neck of the vesicle. There have been a lot of debate about what happens after the coil formation, which resulted in two main competing hypotheses; the "poppase" mechanism and the "pinchase" mechanism. The poppase mechanism suggests that the dynamin coil extends (by GTP hydrolysis) and stretches the membrane neck trapped between its coils until the neck is broken, and the vesicle "pops" off (Stowell, Marks, Wigge, & McMahon, 1999) (Wiejak & Wyroba, 2002). On the other hand, the pinchase mechanism proposes that the dynamin coil constricts and tightens (by GTP hydrolysis) around the neck of the vesicle until the vesicle neck breaks (Sweitzer & Hinshaw, 1998). To this date, it is not known which vesiculation mechanism is more accurate but there are review articles discussing these hypotheses and which mechanism is more biologically likely to occur (Praefcke & McMahon, 2004). Interestingly, experiments done with dynamin inhibitors have shown that the blockage of dynamin function inhibits internalization of the receptors (Macia et al., 2006), a property that would be exploited in this study.

The processes of desensitization and internalization happen only after few minutes of interaction with the agonist. However, the next regulatory steps take longer time (up to several hours). Following receptor internalization, there is more than one possible fate that the receptor may undergo (Drake, Shenoy, & Lefkowitz, 2006); it can either be recycled back to the cell surface (slow or fast) or it can undergo lysosomal degradation.

As the previous paragraphs have discussed, the classical theory of GPCR signaling shows that the signaling stops upon the binding of β-arrestin and subsequent internalization events. However, it was discovered that β-arrestin can activate a different signaling pathway known as mitogen-activated protein kinase (MAPK) pathway by bringing 3 of the signaling members of this pathway close to the receptor (P. H. McDonald et al., 2000). Normally, MAPK pathway is translocated to the nucleus, where it performs its downstream effects, but the binding of β-arrestin keeps MAPK retained in the cytosol. This cytoplasmic retention of MAPK was shown to have a variety of downstream signaling outcomes.

A more recent set of studies have shown that even after internalization, GPCRs can continue signaling to the Adenylyl cyclase enzyme. Among the G_s-coupled receptors subfamily, a study done by Calebiro et al (Davide Calebiro et al., 2009) was able to show, using fluorescence resonance energy transfer (FRET) Microscopy, that cAMP production continued steadily after the internalization of thyroid stimulating hormone receptor (TSHR) in isolated murine primary thyroid follicles. Interestingly, they were able to show that TSH receptor internalizes together with its α_s subunit and the adenylyl cyclase (which it continues to activate) and moves to the trans-golgi network where it produces physiological effects that are quite different from those produced at the cell surface. Calebiro et al employed transgenic mice expressing ubiquitously the very versatile Epac1 FRET sensor, which will be introduced later in this study. The same endosomal signaling phenomenon was shown in parathyroid hormone receptor (PTHR) (Ferrandon et al., 2009), as well as D1 dopamine receptors (D1R) (Kotowski, Hopf, Seif, Bonci, & von Zastrow, 2011), which are also G_s-coupled.

However, this is not a phenomenon restricted to G_s-coupled receptors; as another study performed by Mullershausen et al discovered that when the G_i-coupled, Sphingosine-1-phosphate (S1P1) receptor is stimulated by the agonist FTY720-phosphate, it internalizes together with the agonist and continues to inhibit cAMP production for "hours" (Mullershausen et al., 2009). Interestingly enough, the study showed that the sustained cAMP inhibition mediated by S1P1 receptors leads to the inhibition of lymphocyte migration, which explains why the FTY-720P agonist has such powerful clinical impact in the treatment of multiple sclerosis. So far, this has been the only G_i coupled-receptor for which persistent signaling was shown. All the previous studies have employed FRET-based cAMP sensor to investigate this phenomenon except the study done on sphingosine-1-P receptor which used the traditional cAMP assay, which has a very low temporal resolution compared with the FRET-based methods. However, the one common thing between all the studies is the fact that they observed mainly cAMP levels to detect persistent signaling.

All the aforementioned findings have led to serious considerations on the development of a new "non-canonical" model for the signaling of GPCRs. However, the exact mechanism through which sustained signaling takes place is still under investigation, and it is still unknown if all GPCRs undergo persistent signaling as only a very few

number of receptors have been reported to show this kind of signaling behaviour. More studies need to be done in this direction. A schematic diagram representing the persistent endosomal GPCR signaling is shown in Figure 1.

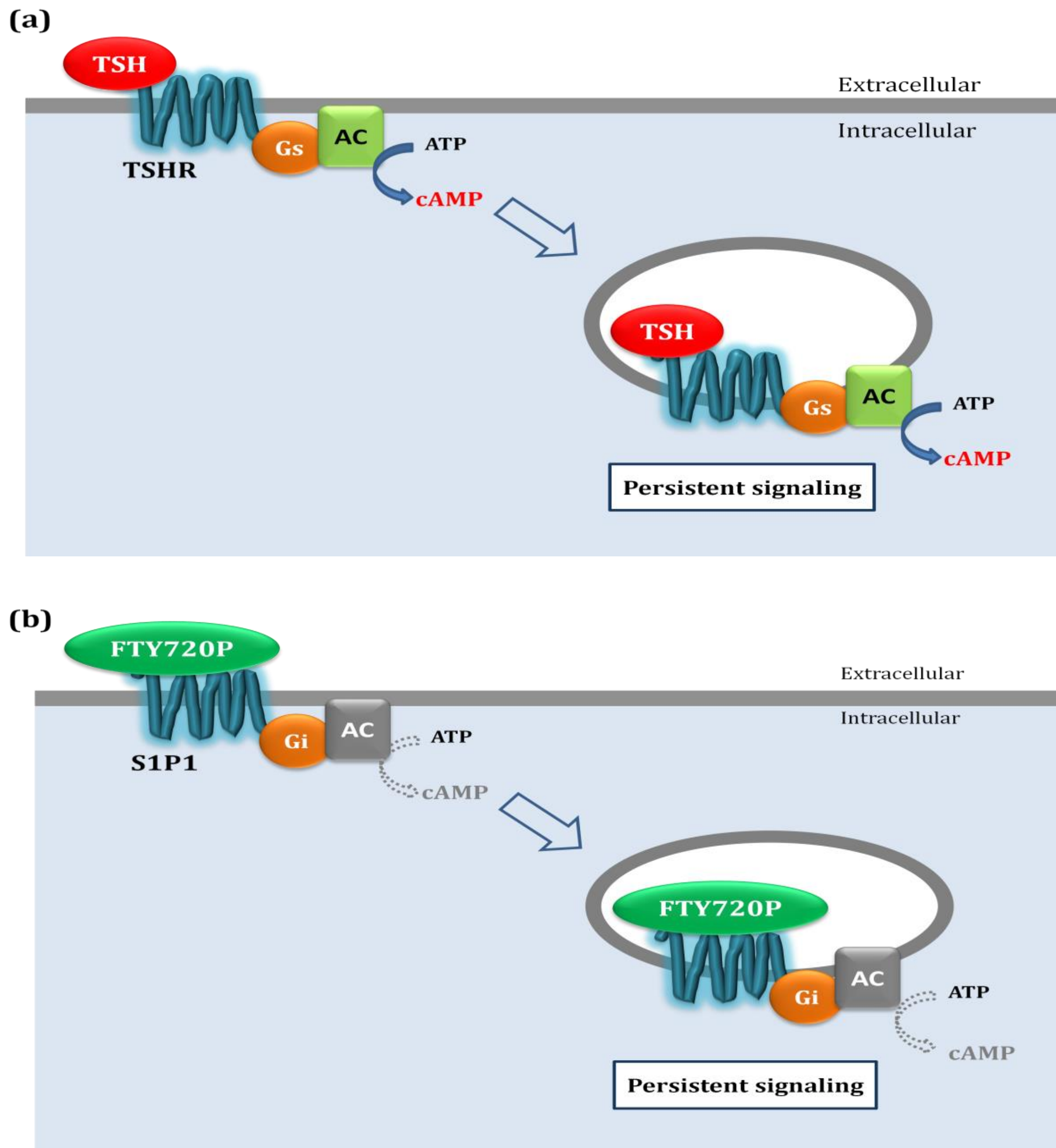


Figure 1: Schematic Diagram of persistent endosomal signaling by GPCRs. (a) TSHR which is cAMP stimulatory gets coupled to G_s subunit which in turn stimulates adenylyl cyclase and the production of cAMP. This process continues after internalization as the receptor, the ligand, the G_s and the adenylyl cyclase enzyme are grouped together inside the endosomes, and the stimulation of cAMP production continues. (b) The same is shown for S1P1 receptors attached to FTY720P agonist where the S1P1 inhibits the production of cAMP at the cell surface and continues to inhibit cAMP production in the endosomal compartment. The figure was adapted from (D. Calebiro, Nikolaev, & Lohse, 2010).

1.2 μ-opioid receptors

μ-Opioid receptors (MORs) are a class of neuromodulatory receptors that play a main role in the reduction of pain (also known medically as anti-nociception) and the development of addiction by acting on the reward pathways in the brain. These receptors are expressed in the central nervous system in many loci along the pain modulation pathway such as the periaqueductal gray in the brain stem and the dorsal horn in the spinal cord (Basbaum, Clanton, & Fields, 1976), and in the reward and addiction centers like nucleus accumbens (Trezza, Damsteegt, Achterberg, & Vanderschuren, 2011). They belong to the family of opioid receptors including also κ and δ opioid receptors which are two classes that only exert a minimal pharmacological influence compared with the μ class (J. McDonald & Lambert, 2005). Within the μ class, there are different isoforms known as μ_1, μ_2 and μ_3 with μ_1 (or MOR-1) being the most extensively researched variant. As MORs are G_i-coupled receptors, their main molecular action is the inhibition of adenylyl cyclase activity but, they were found to activate G protein-coupled inwardly rectified potassium channels (GIRK) and to inhibit calcium channels as well (Al-Hasani & Bruchas, 2011). Given the huge clinical involvement of MORs, a lot of research has been dedicated to understand the molecular mechanisms through which they produce their therapeutic action, addiction and tolerance.

The oldest known agonist that binds to MOR is morphine, which is obtained from opium plant and is famous for its wide clinical use and non-clinical abuse. However, there are endogenous peptide ligands secreted inside the brain known as endorphins and enkephalins. MORs are known to have the highest affinity for endorphins (Pasternak & Pan, 2013). Enkephalins are composed of 5 amino acids which bind with very high affinity to δ opioid receptors but can also bind to a lesser extend to MORs (Cotton, Kosterlitz, Paterson, Rance, & Traynor, 1985; Knapp & Yamamura, 1990). The use of peptide ligands for research is a challenge because of their rapid hydrolysis by peptidases. This rapid degradation encouraged the development of a synthetic enkephalin known as "DAMGO" ([D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin) which is resistant to the action of peptidases. DAMGO was developed initially for δ opioid receptors but it was found instead to be only specific for MORs (Handa Bk Fau - Land et al.). Both morphine and DAMGO are full MOR agonists, but interestingly enough, it was

shown that DAMGO induces receptor endocytosis upon prolonged binding to the receptor as opposed to morphine, which does not induce internalization of the receptor (Keith et al., 1996). This points to a significant difference in the downstream signaling outcomes that these ligands produce. As a matter of fact, it has already been shown that morphine causes tolerance with prolonged use (Saeki & Yaksh, 1993), DAMGO is able to alleviate the tolerance that is caused by Morphine. This tolerance alleviation has been traced back to the ability of DAMGO to promote receptor internalization (He, Fong, von Zastrow, & Whistler, 2002). This study also suggested that the withdrawal-reducing effect of DAMGO may be due to the absence of the adenylyl cyclase upregulation after the end of DAMGO administration. DAMGO is not the only MOR agonist that induces internalization and causes interesting effects after internalization. Actually, another interesting study by Macey et al has demonstrated that the internalization induced by dermorphin is a contributing factor to its anti-nociceptive effect, and that blocking internalization subsequently reduces the anti-nociception (Macey et al., 2010). Another noteworthy study (Lin, Higgins, Loh, Law, & Liao, 2009) showed that high dose fentanyl was able to induce internalization of MORs, which lead to the increase of the number of new dendritic spines as well as AMPA receptors. Intriguingly enough, the blockage of fentanyl-induced internalization produced the opposite effect. The explanation for these physiological observations is not known. However, the explanation may lie in the dynamics of the downstream signaling of MORs. Carefully studying the signaling to adenylyl cyclase after the binding and internalization of agonists -that induce robust internalization- such as DAMGO can provide us with an explanation of how internalization influences these aforementioned physiological outcomes and give us more insight into how MORs signal in general.

1.3 Fluorescence/Förster Resonance Energy Transfer (FRET)

As we have mentioned previously, most of the studies investigating the persistent signaling phenomenon have used FRET-based measurements. This is mainly due to the fact that FRET has a very high temporal resolution compared with older biochemical pharmacological methods such enzyme immunoassays. Thus, it allows real-time detection and accurate measurement of a persistent GPCR signal. This is why FRET will

be exploited in this study as it will be able to resolve this signaling mode as it happens. However, as much promise as FRET can hold as a tool for biological kinetic measurements, it also has its own set of limitations which will be discussed later in this study.

FRET is a physical optical phenomenon, historically discovered by Theodor Förster, in which one excited fluorophore transfers its emission energy to another fluorophore or using an electronic long-range dipole-dipole coupling mechanism (Andrews, 1989). The former is known as "FRET donor" and the latter is known as "FRET acceptor" and it is not always fluorescent. The occurrence of FRET and the FRET rate of transfer rely heavily on several conditions. First, the distance between the donor and the acceptor should be within 1-10 nm separation range; bigger separation translates into lower FRET and vice versa (Jares-Erijman & Jovin, 2003). In addition, the donor emission spectrum and the acceptor absorption spectrum should overlap sufficiently; the greater the overlap, the faster is the FRET transfer rate. Last but not least, the donor and the acceptor should be in an appropriate orientation to each other.

The rate of FRET transfer is inversely related to the sixth power of the distance between the donor and the acceptor meaning it is very sensitive to any minimal change in distance in the nanometer range (Andrews, 1989). This breaks the traditional diffraction barrier of the optical methods. It is this particular sensitivity that made it very tempting to transform this phenomenon into a tool to answer interesting biological questions such as measurement of protein-protein interaction, detection of changes in conformations of different molecules or even the measurement of relative changes in levels of a second messenger (as will be shown later) . Fortunately, it was a successful pursuit as the last 20 years have revealed that FRET can indeed be used for such biological experiments (Clegg, 1995).

In order to detect conformational changes in a protein, usually the two fluorophores have to be placed on the same protein, and in this case, “intramolecular FRET” is detected. While if interactions between two proteins are to be measured, each fluorophore is placed on a separate protein and “intermolecular FRET” is then measured. For the detection of cAMP levels, the principle of intramolecular FRET is used as will be explained in the following chapter.

1.4 FRET-based Measurement of cAMP using Epac1-camps

As was mentioned previously, FRET is a method having a high temporal and spatial resolution, which can provide answers to biochemical and physiological questions that cannot be addressed by the classical biochemical methods. Detection of changes in cAMP levels and distribution using FRET is of major biological significance, because cAMP is a universal second messenger. In order to track cAMP dynamics and localization in living cells, FRET-based fluorescent probe known as "Epac1-camps" was developed that is sensitive to changes in cAMP levels (Nikolaev, Bünemann, Hein, Hannawacker, & Lohse, 2004). This sensor consists of the human variant of cAMP-binding domain (amino acids from E157 to E316) of Epac1. Epac1 is a guanine nucleotide exchange protein which is activated by cAMP and targets Ras-like GTPases (Schmidt, Dekker, & Maarsingh, 2013). In the Epac1-camps sensor, the truncated Epac1 protein is flanked from both sides by the variants of the green fluorescent protein: Cyan Fluorescent Protein (CFP) and Yellow Fluorescent Protein (YFP) where CFP acts as the FRET donor and YFP as the FRET acceptor. As shown in Figure 2, when CFP is excited by a wavelength of 430 nm, it emits light with a wavelength of 480 nm, which in turn excites the neighbouring YFP into emitting yellow light with a wavelength of 535 nm. This leads to a decrease of emission of CFP and increase of the emission of YFP which translates into baseline FRET (Nikolaev et al., 2004). Upon the binding of cAMP to Epac1, conformational changes take place which lead to the movement of CFP and YFP away from each other and consequently, a proportional reduction in FRET.

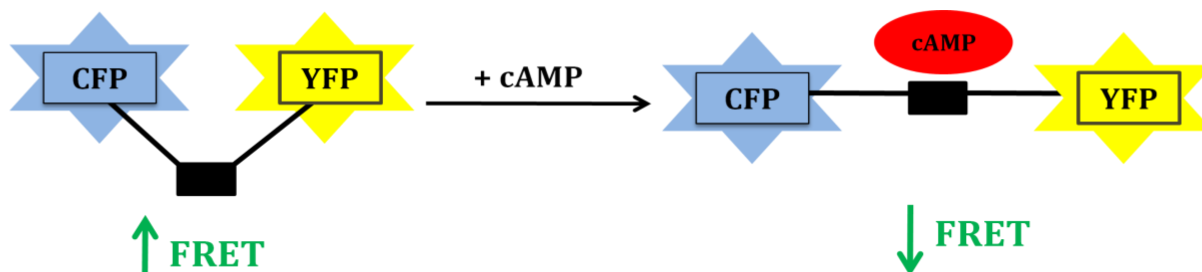


Figure 2: Epac1-camps sensor FRET-based mechanism

Epac1 with CFP as the FRET donor and YFP as the FRET acceptor. When there is no cAMP bound to the sensor, there is high FRET between CFP and YFP. Upon the binding of cAMP, YFP and CFP move away from each other and this results in a reduction of FRET. The figure was adapted from (Borner et al., 2011)

There are other FRET-based sensors for measurement of cAMP levels such as the one based on Protein Kinase A (PKA) which is also a direct effector of cAMP (Zaccolo et al., 2000). However, Epac1-camps proved to have much higher speed compared with the PKA-based sensor, because having four different cAMP binding sites, the PKA-based sensor has to undergo a complex slow mechanism of activation (Lohse, Bünemann, Hoffmann, Vilardaga, & Nikolaev, 2007). While on the other hand, Epac1-camps sensor contains only a single binding site and faster activation. Therefore, to this date, Epac1-camps is the most sensitive and fastest sensor available for cAMP measurement in intact cells.

1.5 Analysis of sustained cAMP signaling using FRET

As a versatile sensor of cAMP, Epac1-camps can be used to detect changes in the levels of cAMP over a prolonged period of time in intact cells. This can allow us to elucidate the presence or absence of sustained GPCR signaling. This technique has been employed by the studies that discovered the persistent signaling in TSHR, PTHR and dopamine receptors (Davide Calebiro et al., 2009; Kotowski et al., 2011). The approach followed by the TSHR study was to stimulate the receptor with the agonist for a prolonged period which would allow enough time for internalization to take place (≃10 min.). This is

followed by prolonged ligand washout by applying buffer solution using a perfusion system (≃ 20 min.). This is to ensure that all ligand-receptor complexes on the plasma membrane would effectively dissociate. Since the receptor under investigation in our current study is MOR which is a G_i coupled-receptor, the agonist stimulation step is preceded by the stimulation of cAMP production by a permeable direct adenylyl cyclase activator known as Forskolin (FSK). The reason for this is to ensure that any reduction in cAMP caused by the addition of the MOR agonist would be detectable in the recorded FRET ratio. With this method, any sustained inhibition of cAMP would be seen as a stable increase in FRET that would remain after prolonged ligand washout.

1.6 The use of dynamin Inhibitors in studying sustained signaling

In case of the presence of a sustained signal, it is necessary to determine if the sustained signal is caused by the internalized receptor signaling from the endosomal compartment. As a means to that end, the same set of experiments as the one mentioned in the previous chapter have to be done in the presence of an internalization inhibitor. The main class of internalization inhibitors used in the previously mentioned studies were dynamin inhibitors. It was mentioned in the first chapter that dynamin is an important GTPase that cleaves the budding vesicle from the plasma membrane (Wiejak & Wyroba, 2002), and that the inhibition of the activity of dynamin by inhibition of the GTPase activity is enough to stop internalization of the GPCRs altogether (Macia et al., 2006). Therefore, dynamin inhibitors can be useful for the detection of endosomal sustained signaling, since the inhibition of dynamin would lead to inhibition of internalization, thus resulting in complete abolishment of the endosomal signal.

The most widely used non-competitive dynamin inhibitor is dynasore which has been shown to block more than all endocytosis at a concentration of 80 µM. Dynasore inhibits the GTPase activity of both Dynamin 1 and Dynamin 2 (Macia et al., 2006). Another inhibitor which is a trihydroxyl analog to dynasore is Dyngo 4a which has been shown to be about 6 times more potent endocytosis blocker than dynasore blocking

endocytosis effectively at a concentration of only 30 μM, which is much less than the concentration used with dynasore.

1.7 The limitations of using FRET for analysis of sustained cAMP signaling

As established as the aforementioned FRET-based technique is, it still suffers from its hindrances. First of all using the donor-acceptor pair, CFP and YFP, present several limitations in themselves, because the CFP and YFP emission spectrum overlap considerably. This results in a large bleed-through (more than 50%) of the CFP emission into the YFP channel. In addition, YFP can be directly excited by the 436 nm wavelength. Also, if the experiment lasts for a long duration, there is a risk of photobleaching for one or the two fluorophores used. The obtained FRET data need to be corrected for such factors in order to calculate accurate FRET ratios.

For the detection of sustained cAMP signaling, a perfusion system is used in order to allow for proper ligand washout after stimulation of the receptor. This system consists of reservoirs that are connected with a tube to the custom chamber containing the cells. The chamber has an outlet that aspirates fluids from the chamber with the influence of gravity. With this setup, diffusion of ligands is a rate-limiting factor to the observed response.

1.8 Aim of this study

The aim of this study is to determine whether MORs can exhibit non-canonical endosomal sustained signaling upon binding to DAMGO which is a μ opioid agonist known for its ability to internalize the receptor. For this purpose, the extent and duration of cAMP inhibition was measured following ligand stimulation and washout using FRET-based cAMP sensor, Epac1-camps. In order to detect whether cAMP signals come from the endosomal compartment, FRET signals measured in the presence and absence of dynamin inhibitors, dynasore and Dyngo 4a.

2 Materials and Methods

2.1 Materials

2.1.1 Agonists and Blockers

[D-Ala2, N-MePhe4, Gly-ol]-enkephalin (DAMGO), Tocris (Cat No. 1171)

Forskolin, Tocris (Cat. No. 1099)

3-Isobutyl-1-methylxanthin (IBMX), Sigma-Aldrich (I5879)

Dynasore Hydrate, Sigma-Aldrich Co. (D7693)

Dyngo 4a, Abcam (ab120689)

All compounds except DAMGO were dissolved in DMSO and then subsequently diluted in FRET buffer to the final concentrations needed to carry out the experiments. DAMGO stock solution was prepared by dissolving the powder in FRET buffer and then diluted with same vehicle for the experiments.

2.1.2 Chemicals, Enzymes and Cell culture materials

Calcium Chloride ($CaCl_2$), AppliChem GmbH, Darmstadt

Dimethyl Sulfoxide (DMSO), cell culture grade, AppliChem GmbH, Darmstadt

Dulbecco's modified eagle medium (DMEM), without sodium pyruvate, with

4.5 g/l glucose, Life Technologies GmbH, Darmstadt

Dulbecco's phosphate-buffered saline (DPBS), Life Technologies GmbH, Darmstadt

Ethanol, Sigma-Aldrich Co., Steinheim

Fetal Calf Serum (FCS), Biochrom AG, Berlin

HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), AppliChem GmbH, Darmstadt

L-glutamine, PAN Biotech GmbH, Aidenbach

Magnesium Chloride ($MgCl_2$), AppliChem GmbH, Darmstadt

Paraformaldehyde, Applichem GmbH, Darmstadt

Penicillin Streptomycin, PAN Biotech GmbH, Aidenbach

Poly-L-Lysine hydrobromide, Sigma-Aldrich Co., Steinheim

Potassium Chloride (KCl), AppliChem GmbH, Darmstadt

Sodium Chloride (NaCl), AppliChem GmbH, Darmstadt

Sodium Hydroxide (NaOH), AppliChem GmbH, Darmstadt

Sodium Pyruvate mM solution, Sigma-Aldrich Co., Steinheim

Trypsin EDTA 1x solution, PAN Biotech GmbH, Aidenbach

2.1.3 Buffers and Solutions

FRET buffer: 5 mM KCl, 137 mM NaCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, pH 7.3

LB medium: 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl

LB agar: made by dissolving 1.2 g agar in every 100 ml LB medium

P1 buffer: 50 mM Tris-HCl, 10mM EDTA, 100 µg/ml RNase A, pH 8.0

P2 buffer: 200 mM NaOH, 1% SDS (w/v)

S3 buffer: 3.0 M potassium acetate, pH 5.5

Binding buffer (BB): one of the buffers provided in the Midi kit for plasmid isolation and purification. Composition is kept confidential by QIAGEN.

Endotoxin removal buffer (ETR): one of the buffers provided in the Midi kit for plasmid isolation and purification. Composition is kept confidential by QIAGEN.

2.1.4 Plasmid Constructs

pcDNA3_OPRM_wildtype	C. Hoffmann, Würzburg
pc-Flag-SNAP-mMOR	U. Zabel, Würzburg
pcDNA3-EYFP-Epac1-ECFP	Nikolaev, Buneman Würzburg

2.2 Biochemical Methods

2.2.1 Transformation of competent E. coli

1 µg of the plasmid of interest was added to 100 µl of competent E. coli and 10 µl KCM solution and diluted with 80 µl sterile water. The mixture was left in ice for about 20 min. then transferred to room temperature where it stood for 10 min. After that, 1 ml of LB medium was added without any antibiotics and then was placed in the Thermomix shaker at 37°C for 50 min. On an agar gel coated with the selection antibiotic, Ampicillin (100 ng/ml), some of the transformed bacteria were plated using an inoculation loop then the plates were incubated at 37°C for 16 hrs overnight. This was followed by plasmid purification.

2.2.2 Large-scale Plasmid Preparation (Midi)

First of all, one of the transformed bacterial colonies was inoculated into a 50 ml LB medium containing 100 ng/ml ampicillin. This culture was incubated at 37 °C for 16 hrs with constant shaking. Afterwards, the medium was transferred to 50-ml Falcon tube and the bacterial pellet was obtained by

centrifugation at 4000 rpm for 15 min. at 4 °C. The pellet was then resuspended in 4 ml of buffer P1 (composition of all buffers used is listed in 2.1.3 Buffers and Solutions section of this study) by pipetting up and down. An equal volume of buffer P2 was added and then the reagents were mixed by gently inverting the Falcon tube 4-6 times. The mixture was then allowed to incubate at room temperature for 3-5 min., after which buffer S3 was added and also mixed by inverting the tube 4-6 times until the solution turned colourless. The mixture was centrifugated at 4000 rpm for 5 min. at room temperature. A QIAfilter cartridge was placed on top of a 15 ml falcon tube and closed with a stopper. The lysate was decanted into the QIAfilter cartridge and it was left to stand for about 5 min. After that, the stopper was removed from the QIAfilter cartridge and the plunger was inserted and pushed very slowly until a clear filtrate was collected in the 15- ml Falcon tube. 2 ml of buffer BB was added to the collected filtrate and mixed by inverting 4-6 times. The mixture was transferred to a vacuum pump assembly. The vacuum pump was started and the mixture was allowed to filter through the provided column. The columns were removed from the vacuum pump assembly and placed in a 2 ml collection tube. 700 µl buffer ETR was then added and the column was centrifuged in a tabletop centrifuge at 10,000 rpm for about 1 min. The flowthrough in the collection tube was discarded and 700 µl buffer PE was added to the column. The previous centrifugation step was repeated. The flowthrough was discarded and the column was centrifuged one more time to ensure that all the buffers had been efficiently removed. Finally, 100 µl autoclaved PCR grade water was added to the column and the 2 ml collection vessel was replaced by a 1.5 ml Eppendorf tube. After letting the assembly stand for about 1-2 min, the DNA was eluted by centrifugation at 10,000 rpm for 1 min.

2.2.3 Cell culture

HEK 293 cells were cultured in DMEM supplemented with 10% FCS, 5 mM sodium pyruvate, 5 mM L-glutamine, 100 µg/ml Streptomycin and 100 U/ml penicillin. The cells were kept in a humidified incubator at 37 °C and 5% CO_2.

When the cells were 80%-100% confluent, they were split either 1:5, 1:10 or 1:20 in 10-cm cell Petri dishes.

In order to prepare the cells for the experiments, they were trypsinized from a 80%-100% confluent 10-cm plate and re-seeded in 6-well plates where each well contained a 24-mm glass coverslip coated with poly-L-lysine. The cell density for seeding was 250,000-300,000 cells/well.

2.2.4 Transient transfection

The next day after seeding the HEK 293 cells in the 6-well plates, they were transfected with MOR cDNA and Epac1-camps sensor for FRET experiments. Transfection was done using Effectene transfection reagent following the manufacturer's protocol. Before transfection, the medium of the cells had been replaced by fresh DMEM. The mixture of reagents used for transfecting each well was prepared as follows:

1 µg total plasmid DNA was diluted in 100 µl EC buffer. Then 4 µl Enhancer was added and mixed by gentle pipetting. The mixture was left to incubate at room temperature for 3 min. after which 12 µl Effectene (ratio of effectene to DNA used was 12:1) was added and mixed by gentle pipetting. In order to allow the DNA to be integrated with Effectene, the mixture was incubated at room temperature for 10 min. After that, the mixture was diluted in 400 µl DMEM. A total of 500 µl transfection mixture was added dropwise per well. The FRET experiments were carried out 48 hrs after transfection.

2.3 Optical methods

2.3.1 FRET measurements

Transfected HEK 293 cells on poly-L-lysine coated coverslips were used to carry out the FRET experiments 48 hrs after transfection. The cells were mounted in a perfusion-customized FRET chamber and FRET buffer (see section 2.1.3 for the exact composition of the buffer) was added in order to keep the cells in a viable condition while performing the experiment. FRET measurements were carried out on an Axiovert 200 inverted microscope (Zeiss, Jena) equipped with a 63x oil immersion objective, a Visichrome High Speed Polychromator System (Visitron Systems, Puchheim) and an EMCCD camera. The light exposure time was adjusted to about 5-20 ms. Images were acquired every 5 s. Since the FRET donor is CFP, the excitation wavelength was configured to be 436 nm ±10 nm (dichroic long-pass beam splitter, 455 nm). The emission of both CFP and YFP were recorded simultaneously at 480nm ± 20nm (F_{480}) and 535 nm ± 15nm (F_{535}) respectively (dichroic long–pass beam splitter, 505 nm).

Due to the spectral overlap between the CFP and YFP emission, the emission intensities were corrected for the bleed-through of the CFP into the YFP channel (which was 57% of the total emission of CFP (F_{480})) as well as the direct YFP excitation by the 436 nm laser (The YFP emission at 436 nm divided by the YFP emission at 490 nm excitation was only 7%). These corrections resulted in the corrected emission intensities of F_{CFP} and F_{YFP}. FRET was measured as the ratio between the F_{YFP} and F_{CFP}.

The cells were superfused with FRET buffer throughout the experiments. Agonists, FSK and IBMX stock solutions were diluted in FRET buffer and were applied to the cells using a perfusion system that functions by gravity flow. The fluorescence intensities of the cells were recorded throughout the experiment using Metaflour software (Molecular Devices, Sunnyvale, CA) which also performed ratiometric imaging. After carrying out necessary corrections to the emission intensities, the data were analyzed using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA).

2.3.2 Confocal microscopy

Confocal images were acquired using Leica TSC SP5 setup. HEK 293 cells which were transiently transfected with Flag-SNAP-tagged-μOR were first stained using SNAP 549 (red) dye for 20 min. at 37°C after which the dye was washed 3 times with DMEM (with FCS). In between washing steps, the cells were incubated for 5-10 min. at 37°C to make sure all extra unbound dye is removed. Afterwards, the cells were incubated with DMEM (without FCS) + Hepes 15 mM for 20 min. Then 10 μM DAMGO was added and cells were allowed to incubate for 20 more min. after which they were washed with DPBS and fixed with 4% PFA in DPBS.

The SNAP 549 dye was excited using 561 nm Diode-pumped solid-state (DPSS) yellow-green laser. The Ultra Violet (UV) 405 nm laser diode was used to detect background fluorescence. The images were acquired using a 63x oil immersion objective. The configuration settings for acquiring the images (pinhole size, laser power, detector gain, etc.) were kept the same for all the experiments.

3 Results

3.1 MORs show persistent cAMP signaling with DAMGO at 37° C

In order to investigate whether MORs show persistent signaling after prolonged binding with agonist, HEK 293 cells were seeded on poly-L-lysine coated 24-mm glass coverslips then they were transfected with MOR plasmid as well as Epac1-camps sensor plasmid previously described. 48 hours after transfection, the coverslips were mounted on a custom perfusion chamber where they were superfused in FRET buffer (Figure 3 A). By the adjustment of a built-in thermostat, the cells were kept at approximately 37°C throughout the experiment in an attempt to bring the experimental conditions closer to physiological conditions.

First cAMP production was stimulated using a cell permeable direct adenylyl cyclase activator known as Forskolin (FSK) (Seamon Kb Fau - Daly & Daly, 1981) which was applied at a 10 µM concentration using perfusion setup. After that, MOR was stimulated using DAMGO 10 µM for 10 min. The DAMGO concentration used was decided based on the EC50 of the Dose Response Curve (DRC) constructed by Benedikt Schmidt (AG Hoffmann, Institute of Pharmacology and Toxicology, University of Würzburg) measuring the extent of MOR-ß-arrestin interactions in response to different doses of DAMGO by FRET. The application of DAMGO 10 µM resulted in an effective inhibition of cAMP production. Reduction in cAMP is seen as an increase in FRET because the distance between CFP and YFP in the sensor is reduced after the dissociation of cAMP from Epac1 (Nikolaev et al., 2004).

Following the 10-minute DAMGO stimulation, the agonist was washed out for 20 min. using perfusion. Despite extensive washout of the ligand, the DAMGO-induced cAMP inhibition was only partially reversible in the cells as shown in figure 3 (C) (on the following page).

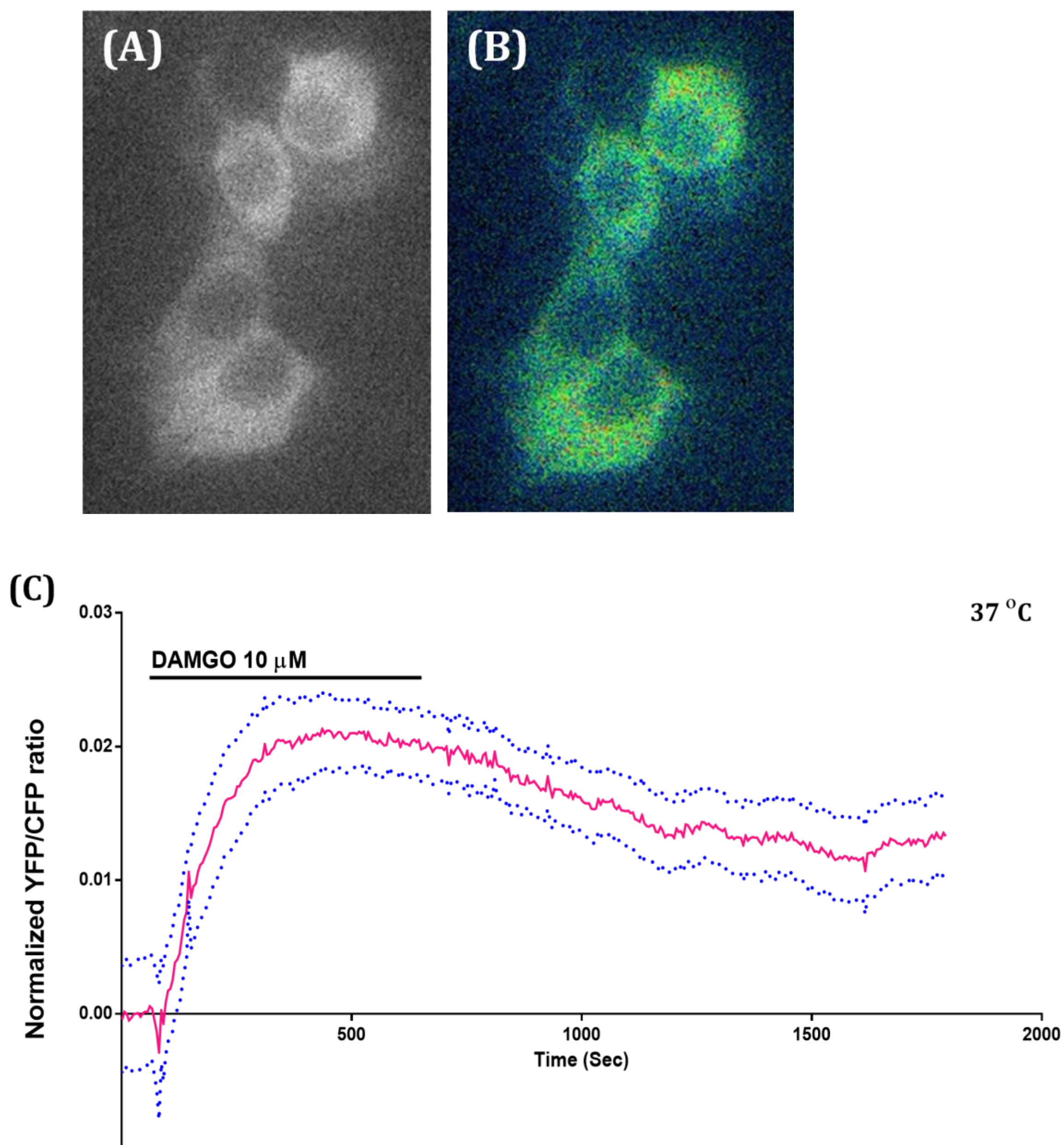


Figure 3: MORs exhibit persistent signaling upon prolonged binding to DAMGO at 37 °C

(A) HEK293 cells transfected with MOR cDNA and Epac1-camps cDNA for 48 hrs viewed in the FRET channel. (B) shows the cells as they are viewed in the ratiometric pseudocolour channel which represents the YFP/CFP ratio through a range of colours. (C) Stimulation of MOR using 10 μM DAMGO resulted in an increase in FRET corresponding to a reduction in cAMP. This inhibition of cAMP production is only partially reversible even after the washout of DAMGO for 20 min. The data represents the mean ratios obtained from 41 cells and the blue-dotted line represents ±SEM.

3.2 Does the persistent cAMP signal come from the endosomes?

Part I: FRET experiments in the presence of dynasore at 37 °C

Previous studies have shown that the persistent cAMP signaling for different receptors (e.g. TSH, PTH, S1P1) come from the endosomal compartment (Davide Calebiro et al., 2009; Ferrandon et al., 2009; Mullershausen et al., 2009). Based on these studies, we also constructed a similar hypothesis for the persistent MOR signal triggered by DAMGO. The most common method to test this hypothesis was the incubation of the cells with an endocytosis inhibitor to prevent receptor internalization, thus inhibiting endosomal signaling if present. The experiments presented in the previous part were repeated the same way at the same temperature (37 °C) but after incubation for 20 minutes with 80 μM dynasore, which is the dynamin inhibitor described in the introduction section (Macia et al., 2006). According to literature, 80 μM dynasore is sufficient for nearly complete blockage of endocytosis (Davide Calebiro et al., 2009; Kirchhausen, Macia, & Pelish, 2008; Kotowski et al., 2011).

Unexpectedly, there was no FRET cAMP response to the initial pre-stimulation with FSK in the presence of 80 μM dynasore (as shown in Figure 4). Therefore, the experiments could not be completed as the first cAMP stimulation step is crucial for the completion of the experiment. The reason for this observation is not known. However, in an attempt to optimize the technical conditions for the experiment, the experiments were repeated at room temperature (RT).

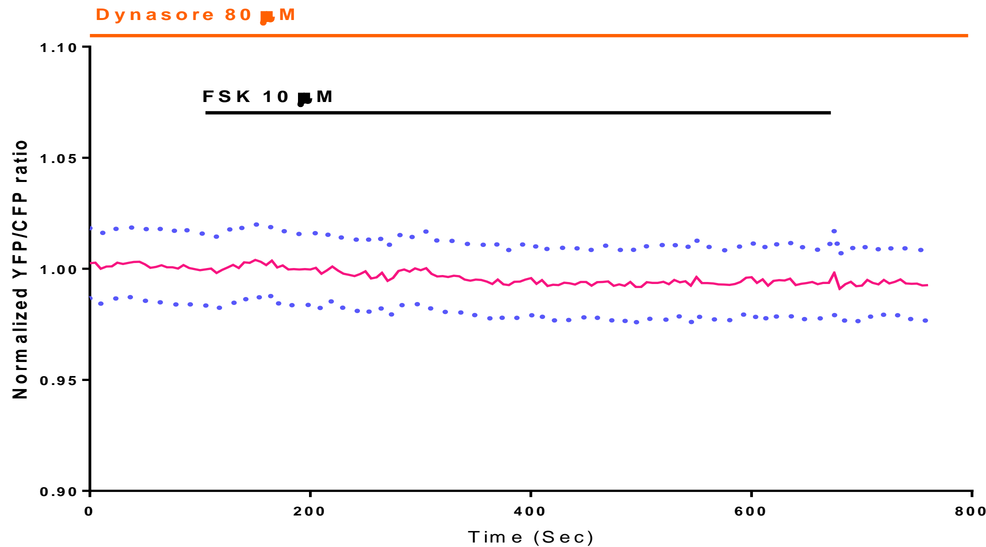


Figure 4: No FRET Epac1-camps response observed to addition of FSK in presence of 80 µM dynasore

The graph represents the normalized YFP/CFP FRET ratios before and after the addition of 10 µM FSK. There is no change in the FRET ratio after addition of FSK. The data in this graph represents the mean of 13 cells (red line) ±SEM (blue-dotted line).

Part II: FRET Experiments in the presence of dynasore at room temperature

Prior to repeating experiments with 80 µM dynasore at room temperature, first a set of room control experiments were done. This is to validate that the signal is still persistent at room temperature in the absence of internalization inhibitor. Results of the control experiments show that the signal is still persistent at room temperature. Thus, it can be inferred that this phenomenon can also be seen at room temperature.

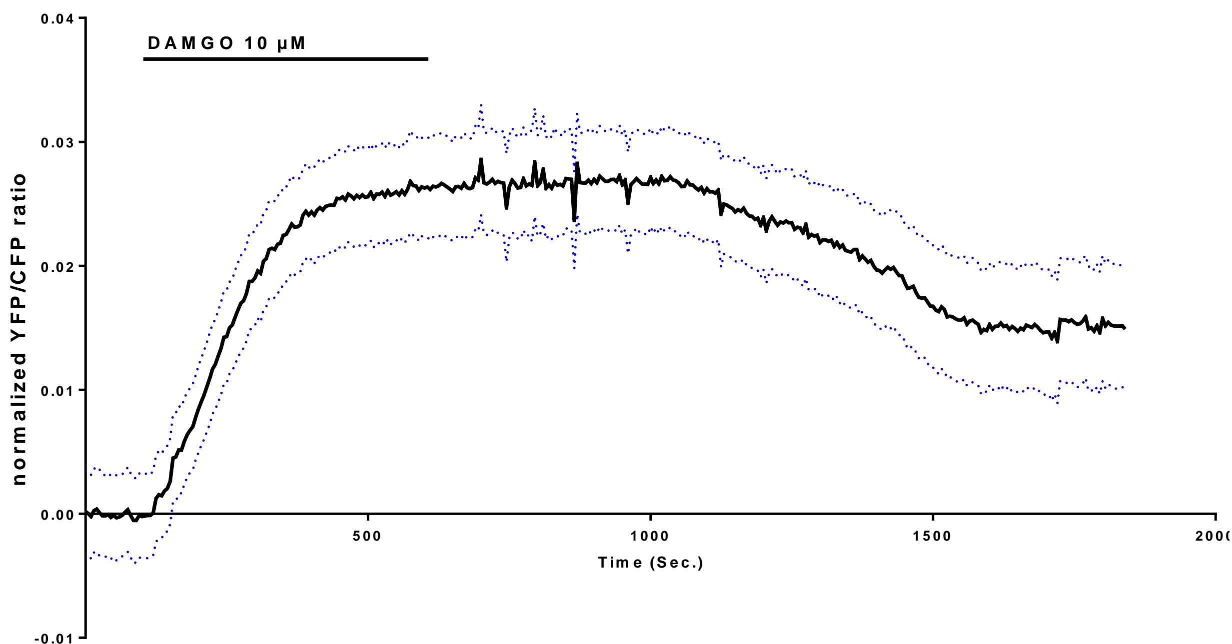


Figure 5: MORs still exhibit persistent signaling with DAMGO at Room temperature

Similar to what was seen at 37 °C, the addition of 10 µM DAMGO resulted in a subsequent increase in FRET ratio which corresponds to a reduction of cAMP. This signal remained partially persistent even after 20 min. of agonist wash out. This graph represents the mean of 34 cells (black line) ±SEM (blue-dotted line).

The experiments were then repeated in the presence of 80 µM dynasore at room temperature. As shown in figure 6 A, a cAMP response (reduction in FRET ratio) was observed upon perfusing the cells with 10 µM FSK. However, the cells had no response to subsequent addition of 10 µM DAMGO (Figure 6, B). Therefore, once again, the experiments could not be completed. Since all the trials to do FRET measurements in the presence of dynasore have not been successful both at 37 °C and at room temperature. Therefore, a trihydoxyl analogue of dynasore, Dyngo 4a was used instead as the endocytosis inhibitor in the FRET experiment. Dyngo 4a is mentioned in the literature to be at least 6 times as potent as dynasore (McCluskey et al., 2013). In addition, it is also mentioned to have less off-target effects than dynasore. Thus, the same set of experiments were then done at 37 °C in the presence of Dyngo 4a.

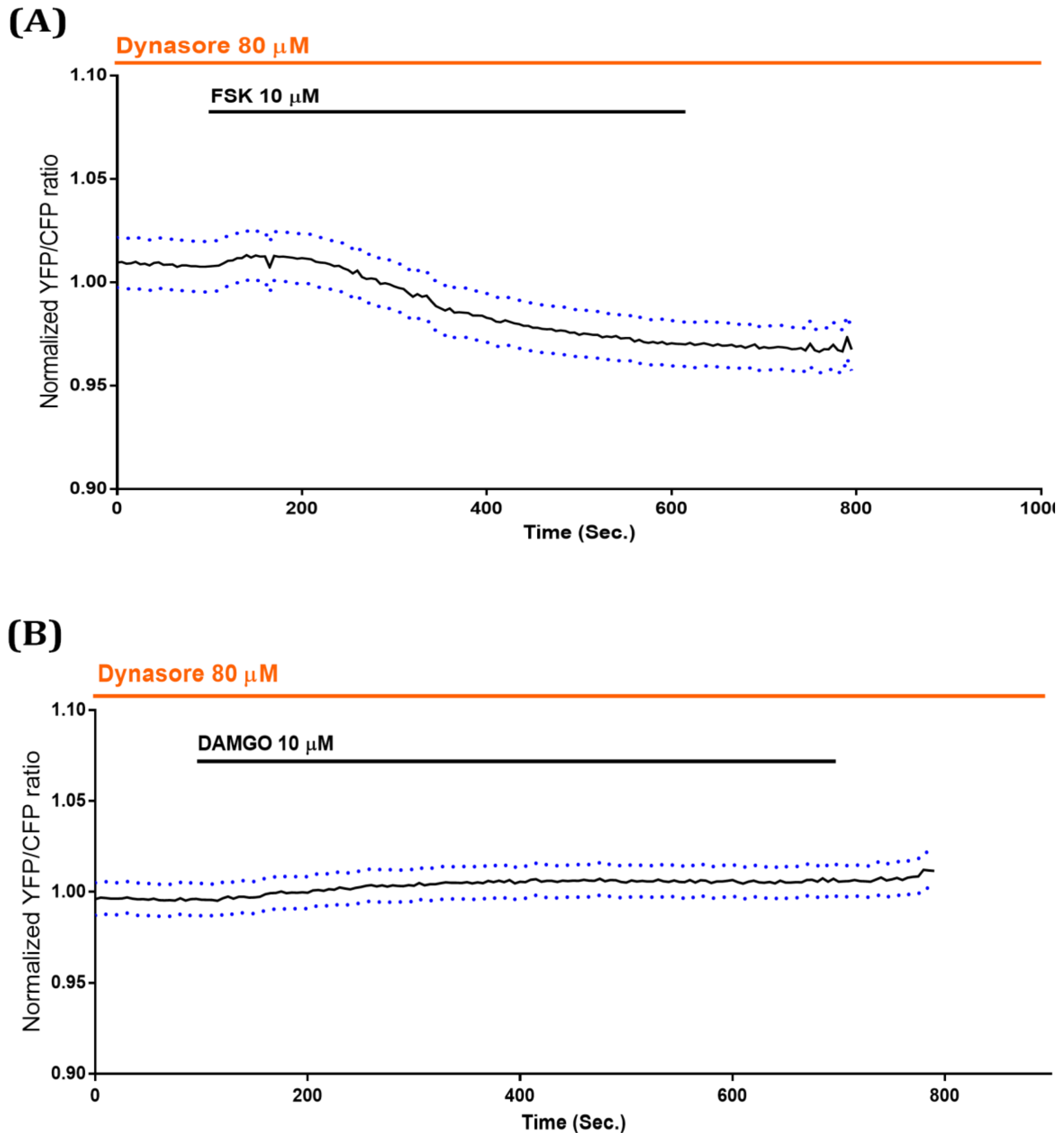


Figure 6: At room temperature, HEK293 cells show a small FRET cAMP response to FSK 10 µM stimulation but no FRET cAMP response to 10 µM DAMGO in the presence of 80 µM dynasore.

When cells are preincubated with 80 µM dynasore, (A) There was small change in corrected YFP/CFP FRET ratio upon 10 µM FSK stimulation, and (B) no change in FRET ratio took place upon addition of 10 µM DAMGO for 10 min. Each of the two graphs represents an average of 12 experiments (black line) ± SEM (blue-dotted line).

Part III: Preliminary FRET experiments in the presence of Dyngo 4a at 37 °C

Following the same protocol used for the previous experiments, we performed FRET experiments with DAMGO in the presence Dyngo 4a as the replacement dynamin inhibitor for dynasore. However, the concentration of Dyngo 4a used for pre-incubation is lower since the IC_{50} of Dyngo 4a (IC_{50} = 5.7 μM) is much lower than that of dynasore (IC_{50} =15 μM). Therefore, based on literature, the Dyngo 4a concentration employed for the following FRET experiments was 30 μM (McCluskey et al., 2013; Tsvetanova & von Zastrow, 2014).

First, preliminary FRET measurements were attempted at 37 °C**,** but this time preceded with the incubation with 30 μM Dyngo 4a. Yet the observation seen was similar to that seen with dynasore; a FRET cAMP response to FSK was not observed (Figure 7). Therefore, similarly to that done with dynasore, we repeated these experiments also at room temperature.

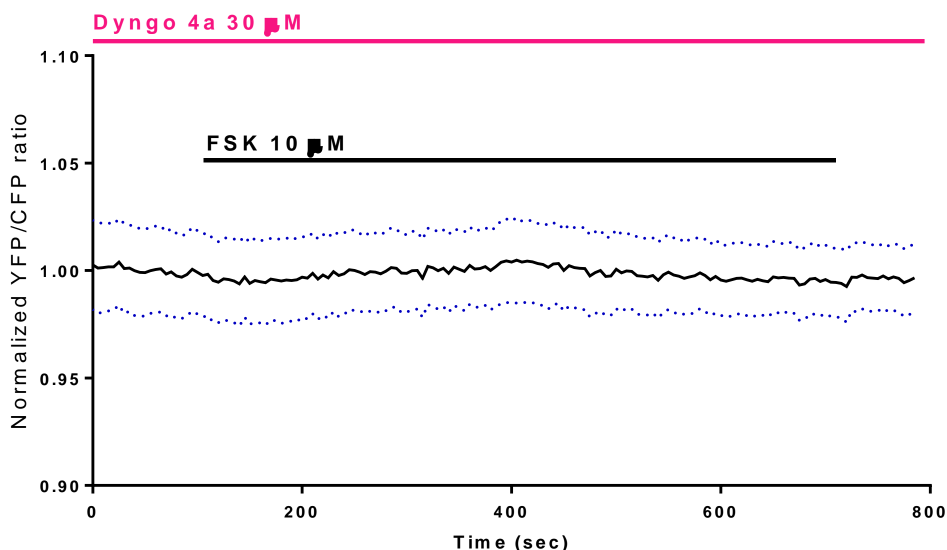


Figure 7: No FRET response to 10 μM FSK stimulation in the presence of 30 μM Dyngo 4a

The data presented in this graph is the mean data obtained from 11 cells (black line) ± SEM (blue-dotted line)

Part IV: FRET experiments in the presence of Dyngo 4a at Room Temperature.

At room temperature in the presence of Dyngo 4a, the cells responded to 10 µM FSK and 10 µM DAMGO. However, the DAMGO-induced signal remained persistent nevertheless (Figure 8). This observation indicates one of two things: either that Dyngo 4a does not block internalization of the receptor, or it blocks internalization of the receptor, but the signal persists through a different mechanism.

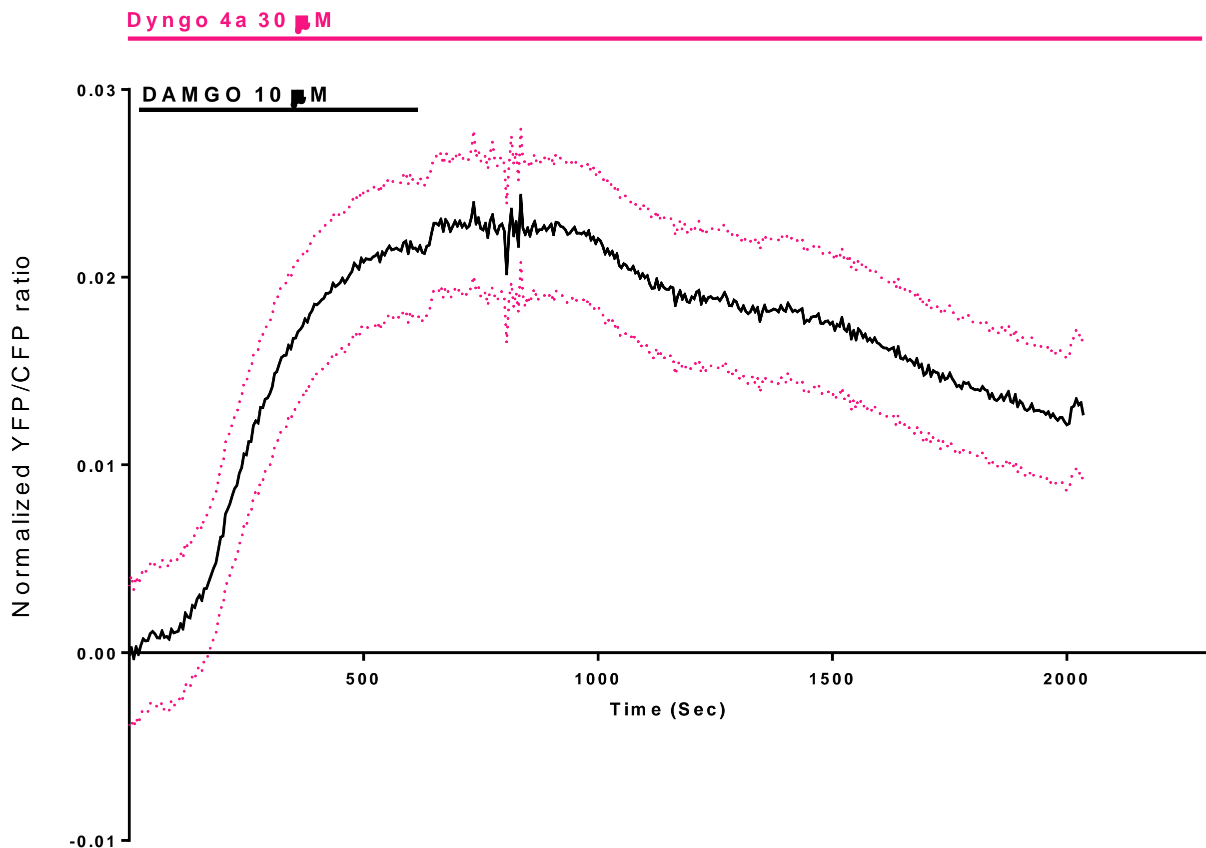


Figure 8: cAMP inhibition signal is persistent even in the presence of 30 µM Dyngo 4a

After 10-minute stimulation with 10 µM DAMGO, cAMP inhibition takes place leading to an increase in FRET ratio which remains persistent even after agonist washout for 20 min. The data here is the mean obtained from 33 cells (black line) ± SEM (red-dotted line).

3.3 Confocal Microscopy Experiments

As previously mentioned, the FRET experiments carried out at room temperature with 30 μM Dyngo 4a pre-incubation showed that the signal remained partially irreversible even in the presence of this highly potent internalization blocker. Therefore, in order to see whether that Dyngo 4a is blocking internalization, confocal microscopy experiments were necessary. The first step before testing Dyngo 4a is to validate whether internalization takes place in the control condition (i.e. the absence of dynamin inhibitor). The rationale behind these confocal microscopy experiments relies on using HEK 293 cells that transiently express a fluorescently-tagged receptor. Before adding DAMGO, it is expected that the receptor would be observed sitting on the plasma membrane and would then be re-located into the cell after the addition of DAMGO.

In this set of experiments, we employed SNAP-tagged MOR. SNAP-tag is a protein sequence that can fused with any protein under investigation. SNAP-tag is capable of performing enzymatic binding with certain ligands such as fluorescent dyes (Keppler, Pick, Arrivoli, Vogel, & Johnsson, 2004). The fluorescent dye we chose for the confocal experiments was SNAP-surface 549 dye which cannot penetrate the plasma membrane is excited by 560 nm laser and has an emission maximum of 575 nm. (New England Biolabs, 2015)

The exact labeling process is described in the materials and methods section of this study. In the next steps after labeling, the DMEM used for incubation contained no serum. After labeling with the dye, the cells were incubated for 20 min. either with DMEM + HEPES 15 mM. After the first incubation, 10 μM of DAMGO was added to some of the coverslips to activate the receptor and stimulate internalization, and the other coverslips were kept without agonist. The cells were allowed to further incubate with/without the agonist for 20 min. then they were fixed using 4% Paraformaldehyde (PFA) and viewed under the confocal microscope.

DAMGO-induced internalization of MORs in HEK 293 cells

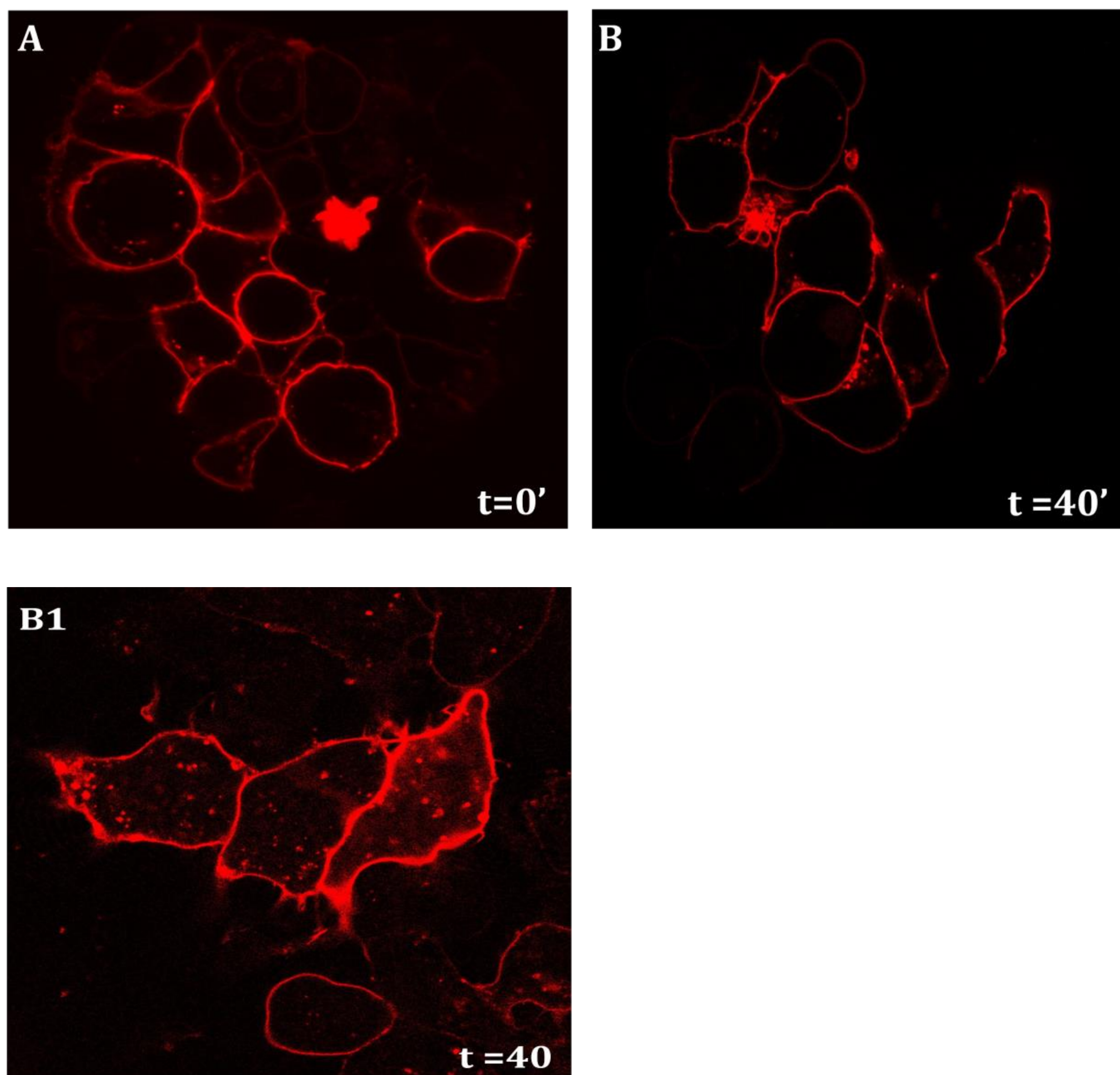


Figure 9: Basal images of MORs before stimulation with DAMGO. Null control.

All images show cells with SNAP-tagged MOR and labelled with SNAP surface 549 dye. The dye was excited with DPSS 561 laser in the confocal microscope setup. Image (A) shows cells were fixed directly after labeling using 4% PFA. (B) shows cells that were incubated after labeling for 40 min. with only DMEM and 15 mM HEPES and then fixed using 4% PFA. The MORs are only seen on the cell membrane. (B1) shows cells that underwent the same treatment as the cells in B but, a small degree of constitutive internalization is seen in B1.

Coverslips that were fixed at time zero all show cells that have receptors sitting on the plasma membrane and have no internalized receptors. After 40 minutes (of incubation with DMEM and HEPES 15 mM), images show that 68% of cells that have receptors exclusively sitting at the plasma membrane. However, the other 32% of the cells show a small degree of constitutive internalization (Figure 9, B1).

After stimulation with DAMGO 10 μM, internalization was seen in 44.6% of the cells (Figure 10 A, A1). On the other hand, a bigger proportion of the cells showed no internalization. This means that stimulated coverslips have 12.6% more internalized cells than the unstimulated coverslips (Figure 9).

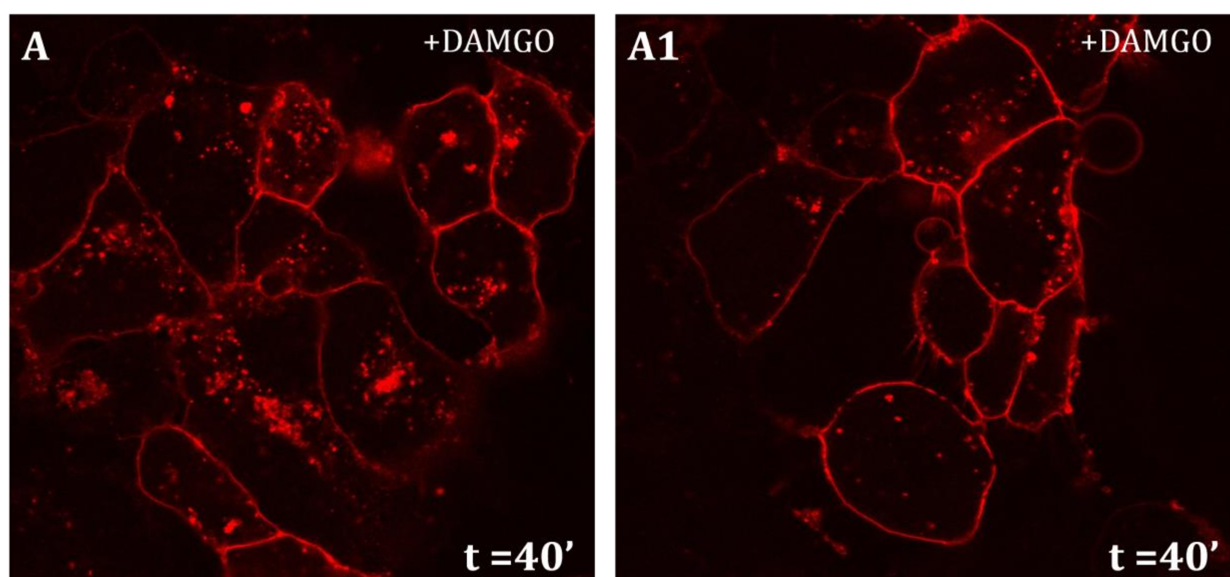


Figure 10: MOR internalization after stimulation with 10 μM DAMGO

After labeling, cells were incubated for 20 min. with DMEM + HEPES 15 mM then 10 μM DAMGO was added and cells were allowed to incubate for 20 more min. before they were fixed using 4% PFA.

(A) 44.6% cells show internalization with 10 μM DAMGO. (A1) the rest of the cells show only a small degree of internalization or no internalization. [For images of unstimulated please refer to Figure 9]

4 Discussion

4.1 Persistent signaling of MORs

This study aimed to identify whether the μ-opioid receptors (MORs) exhibit non-canonical endosomal persistent signaling. As mentioned previously, it has been shown in few GPCRs that the signaling to the second messenger cAMP continues even after internalization of the receptor which contradicts the classical signaling model for GPCRs (Davide Calebiro et al., 2009; Ferrandon et al., 2009; Jalink & Moolenaar, 2010; Kotowski et al., 2011). Only one of the previously-studied GPCRs is a Gi-coupled receptor which is S1P1 receptor that has demonstrated persistent inhibition of cAMP production even hours after FTY720P washout (Mullershausen et al., 2009) . However, to this date, no published studies have shown similar behaviour in other Gi-coupled receptors.

Here in our study, using FRET-based method, we were able to demonstrate that another Gi-coupled receptor, MOR transfected in HEK 293 cells indeed exhibits persistent signaling with DAMGO even after prolonged 20-minute agonist washout. However, we could not identify whether the source of these signals is cell surface or the endosomal compartment, because of technical issues that will be mentioned later in the discussion.

These findings are actually consistent with the previous studies done on the other GPCRs like TSHR, PTHR, dopamine receptors (D1R) and S1P1 receptors which demonstrated that the signal does remain persistent even after long agonist washout (Davide Calebiro et al., 2009; Ferrandon et al., 2009; Jalink & Moolenaar, 2010; Kotowski et al., 2011). Not all of the aforementioned studies used the physiological model for the receptors: the PTHR study used HEK 293 cells stably transfected with PTHR (Ferrandon et al., 2009) and the D1R study used neurons as well HEK 293 cells for their investigations (Kotowski et al., 2011). Regardless of the model used, all the previous studies were able to show such sustained signaling phenomenon in both the endogenous models and HEK 293 cells.

However, one noteworthy study compared the signaling of endogenous TSHR in thyroid cells and transfected TSHR in HEK 293 cell line. Interestingly, this study observed that

the cAMP signals, although quite persistent in the thyroid follicles, they were reversible in HEK 293 cells (Werthmann, Volpe, Lohse, & Calebiro, 2012). It was mentioned in that study that the HEK 293 cell line probably lacked the machinery required for sustained signaling of TSHR. On the other hand, the studies done on PTHR, D1R and our study have successfully demonstrated persistent signaling of these different GPCRs in HEK 293 cells. This indicates that TSHR may have very distinct requirements for persistent signaling that are otherwise not available in HEK 293 cells.

We have been able to demonstrate only 20 minutes of persistent signal due to the nature of the method we employed. 20 minutes is the time period which can be determined in our investigation, but that does not mean that the sustained cAMP signal only lasts 20 minutes. This is consistent with the previous studies that observed the same phenomenon in other receptors. However, the single exception is the study done on S1P1 receptor for reasons that will be mentioned later in the discussion.

4.2 Finding the source of persistent signaling with dynamin inhibitors

Based on previous studies done on this type of signaling, it is thought that the signaling comes from the endosomal compartment (Davide Calebiro et al., 2009; Ferrandon et al., 2009; Jalink & Moolenaar, 2010; Kotowski et al., 2011). As mentioned before, GPCRs rely mainly on clathrin-mediated endocytosis (Claing, Laporte, Caron, & Lefkowitz, 2002; von Zastrow & Kobilka, 1994). For this reason, we employed the use of internalization blockers that mainly block dynamin function: dynasore or Dyngo 4a with concentrations of 80 μM and 30 μM respectively. These concentrations were chosen based on the ones employed in the literature as other studies have shown them to be effective and of no visible toxicity (Kotowski et al., 2011; Macia et al., 2006; McCluskey et al., 2013). However, with dynasore we have seen two unusual observations as follows. In the presence of dynasore, on one hand, the cells did not give a cAMP response to FSK pre-stimulation at 37 °C which completely hindered the continuation of the experiment. On the other hand, at room temperature, a normal FSK response was observed, but the DAMGO response was blunted. The exact reason for this observation

is not understood, but we have hypotheses that might explain this aforementioned observation. Dynasore has been shown in several previous studies to be a very potent inhibitor for clathrin-mediated internalization of GPCRs (Davide Calebiro et al., 2009; Kotowski et al., 2011). Also, current ongoing studies in our lab on have employed dynasore successfully in FRET experiments, and it proved to be a very efficient internalization inhibitor without any observed effect on the response to the agonist addition. This abolishment of the agonist-mediated response -in the presence of dynasore- was only seen in our study on MORs. On the other hand, the use of Dyngo 4a in our study did not abolish the DAMGO response, but it did not reverse the persistent signal caused by our receptor. These negative results seen with Dyngo 4a might indicate that Dyngo 4a does not inhibit MOR endocytosis. Therefore, based on these observations, we hypothesized that MOR internalization may be an important requirement for agonist-mediated cAMP response. In other words, we assume there might be a complex interplay between internalization and MOR signaling. Interestingly, one study done on ß2-adrenergic receptors (ß2-AR) showed that there was an overall reduction in agonist-mediated cAMP response when the internalization of ß2-AR was inhibited (Tsvetanova & von Zastrow, 2014). This indicated that ß2-AR is required for full cAMP response. Aside from our study, no one has seen before a complete abolishment of agonist-mediated cAMP response in the presence of internalization inhibitor. The limitation to this assumption is that we have not personally validated the efficacy of dynasore and Dyngo 4a for the specific inhibition of MOR internalization. However, this would leave room for future experiments to check if our assumption is valid, and to see whether there is a connection between internalization and signaling of MORs.

It might be also be assumed that dynasore has caused some unspecific effects that lead to general reduction in cAMP response. The problem with this assumption is that no previous studies have reported any adverse effect of transient incubation of HEK 293 cells with dynasore. One study has shown that the viability of HeLa cells was not affected by transient exposure to dynasore (McCluskey et al., 2013). However, the same study reported cell growth inhibition across different human cancer cell lines after a long non-transient 72 hours of incubation with dynasore. It is worth mentioning that the study done on dopaminergic neurons has not reported any problems after using

similar concentration of dynasore with HEK 293 cells while measuring FRET using Epac1-camps (Kotowski et al., 2011). In addition, the aforementioned studies and ongoing research in our lab have shown that 80 µM is the concentration effective for the blockade of internalization in 293 cells as well as other models. (Davide Calebiro et al., 2009; Kirchhausen et al., 2008; Kotowski et al., 2011). Therefore, we have reasons to assume that the lack of DAMGO response might not be due to unspecific dynasore effects. However, only future experiments would be able to reveal the mechanism through which dynasore abolished the agonist-mediated response.

Although dynasore is known to be a very potent endocytosis blocker, it has the limitation of being fluorescent. Dynasore was reported to have wide fluorescence range, which allows it to interfere with GFP and RFP fluorescence as was seen in a study done by Matthews et al (Matthews et al., 2012). However, dynasore fluorescence did not interfere with our FRET experiments. In addition, previous and ongoing studies using Epac1-camps had no issues at all with FRET measurements in presence of dynasore (Davide Calebiro et al., 2009; Kotowski et al., 2011).

Since the experimental attempts with dynasore were not successful, we switched to the trihydoxyl analogue of dynasore, Dyngo 4a. Dyngo 4a is claimed to be of 6-times higher potency towards endocytosis inhibition than dynasore . Also, Dyngo 4a was reported to have less unspecific effects than dynasore (Harper et al., 2011; Harper, Popoff, McCluskey, Robinson, & Meunier, 2013; McCluskey et al., 2013). This information made Dyngo 4a a rather promising candidate for our experiments. In our FRET measurements at room temperature, the cells showed normal cAMP FRET response to FSK and DAMGO in the presence of 30 µM Dyngo 4a. However, the presence of 30 µM Dyngo 4a strangely did not reverse the persistent cAMP signal. We hypothesized thus that this concentration of Dyngo 4a did not block internalization of the receptor. Interestingly enough, this concentration has been shown in literature to be highly effective for dynamin-mediated endocytosis inhibition in neurons and reduction of transferrin uptake remarkably (McCluskey et al., 2013). One study demonstrated that beta-2 adrenergic receptors (ß2-AR) internalization was strongly inhibited in the presence of 30 µM of Dyngo 4a (Irannejad et al., 2013). Another study done by Henry et al showed that 30 µM Dyngo 4a does indeed inhibit internalization of MOR in HEK 293 cells (Henry et al., 2012). In the case of our results, Dyngo 4a blocking internalization at this

concentration was highly questionable. In addition, no higher concentration of Dyngo 4a was previously characterized for potency and toxicity so, it would be of potential risk to resort to a higher concentration of Dyngo 4a without properly characterizing the current Dyngo 4a concentration and to validate the potential use of higher concentrations. Therefore, future experiments should perform confocal experiments in order to see whether or not the current concentration of Dyngo 4a blocks the internalization of our receptor.

4.3 Validating MOR internalization in HEK 293 cells

In order to resolve the question whether dynasore and Dyngo 4a are inhibiting MOR internalization, we have started a set of confocal experiments that employed a MOR tagged with SNAP-549 dye. However, it was necessary to validate first whether MOR internalization takes place in the control condition (in the absence of internalization inhibitor). Unstimulated cells showed a degree of internalization that was not due to agonist stimulation (36% of total unstimulated cells). After DAMGO stimulation, we saw that 44.6% of the HEK 293 cells showed internalization. This means that there is only 12.6% increase in internalization when compared to unstimulated cells. The degree of internalization induced by DAMGO is lower than previous reports. In a previous study done by Anselmi et al (Anselmi, Jaramillo, Palacios, Huynh, & Sternini, 2013), the degree of MOR internalization was 70% in neurons which is several folds higher than what we saw in HEK 293 cells. In addition, another study showed that 10 μM DAMGO induced about 30% internalization in HEK 293 cells (Bailey et al., 2003) which is also much higher than the agonist-induced internalization in our study. The reason for such low observed internalization in our study is to be investigated. However, the temperature influence could be excluded since the coverslips were kept incubated at 37 °C. This calls for future optimization for this experimental setting so that DAMGO-induced internalization could be more efficient.

4.4 Limitations of technical setup and potential improvements

We have employed a FRET-based method for investigating the cAMP signaling of MORs. FRET has a variety of advantages such as the fact that it is a real-time method as opposed to other older biochemical methods such as the enzymatic cAMP assay which could not record such fast fluctuations in cAMP and requires a lot of sample preparation involving lysis of the cells. FRET is capable of measuring relative changes in intact cells and without complicated sample preparatory procedure. In addition, our studies have employed cAMP FRET sensor known as Epac1-camps which has been shown in previous studies to have a high binding affinity of 2.35 μM to cAMP. The fact that it is constructed from a single cAMP-binding site allows it to exhibit faster activation in response to cAMP compared to full length Epac1 sensor as well as Epac2 sensor (Nikolaev et al., 2004). This indicates that Epac1-camps has a very high sensitivity for cAMP and was proven to be far more sensitive than other cAMP FRET sensors such as those based on PKA (Nikolaev et al., 2004; Nikolaev & Lohse, 2006). Although the technique has a great set of advantages, but it also enjoys a share of disadvantages. For example, like any fluorescent microscopy method, although temporal resolution is high, the experiment has a limited total time because of the photobleaching of fluorophores throughout the experiment. This is particularly evident when our results are compared with those of the study done on S1P1 receptor has applied a wash out time of 5 hours which is much longer than the one used in our study or the other previous studies. The reason we have not applied such long washout periods is due to the nature of our optical method that allows only limited experiment time (1-2 hours). On the other hand, the S1P1 study used traditional biochemical cAMP assays which are more open to interval measurements over a huge period of time.

Another limitation is that our FRET method cannot resolve the absolute amounts of cAMP which makes it very hard to determine if there is any change in the basal "starting" amounts of cAMP between different conditions (e.g., with or without Dyngo 4a). Therefore, we suggest that in the future for persistent signaling studies, the two methods should be used. We believe this would give more insight to the quantitative nature of the persistent cAMP signals.

4.5 Outlook

In our study, we have been able to show that MORs indeed undergo persistent signaling with DAMGO. However, much remains to be unraveled as to "where" this signaling takes place. To answer this question, we have already attempted the use of two dynamin inhibitors: dynasore and Dyngo 4a, both of which are structural analogues to each other having similar dynamin inhibition mechanism of action. Unfortunately, both have not provided us with answers to our question (for different reasons that are previously discussed in extensive detail). However, their capability of inhibiting MOR internalization has not been assessed. Therefore, the first step should be proper characterization of these inhibitors. This can be done using confocal microscopy experiments using a fluorescently-tagged receptor. We have done preliminary control experiments in (the absence of these internalization inhibitors) in order to evaluate the internalization of the MORs under normal conditions. However, future experiments should focus on performing these experiments in the presence of 80 μM dynasore and 30 μM Dyngo 4a. This can provide insight into the underlying reasons for the observations seen in our FRET experiments.

Based on our FRET experiments, we have assumed that 30 μM Dyngo 4a did not block internalization. Therefore, future confocal microscopy experiments can also be done to characterize higher concentrations of Dyngo 4a for potency and toxicity. Toxicity can be assessed by measuring several parameters such as cell viability and cell proliferation. For the measurement of cell viability, lactate dehydrogenase assay and Tetrazolium dye MTT assay are generally very commonly used and can be informative about the viability state of the cells (Chan, Moriwaki, & De Rosa, 2013; Fotakis & Timbrell, 2006). For the measurement of cell proliferation, 5-bromo-2'-deoxyuridine (BrdU) assay is used because, this synthetic nucleoside binds to the newly synthesized DNA and can then be labeled using fluorescent anti-BrdU antibody and subsequently quantified (Gratzner, 1982). Once the higher concentrations are properly characterized, the FRET experiments can be re-attempted at the higher effective Dyngo 4a non-toxic concentration(s).

The second rationale is to attempt the use of the bigger variety of internalization blockers on the market. The internalization blockers available are not necessarily

limited to dynamin inhibitors such as the ones used in our study. One way to block endocytosis is to use inhibitors of clathrin-mediated internalization particularly those reported to inhibit GPCR internalization such as Concanavalin A. Concanavalin A is a plant-derived lectin, which can adhere to glycosyl groups on GPCRs thus, inhibiting their internalization upon binding of the agonist (Kim, Kim, Lee, Ahn, & Baik, 2004; Pippig, Andexinger, & Lohse, 1995; Xiang, Devic, & Kobilka, 2002). Concanavilin A was shown before to inhibit MOR internalization (Arttamangkul, Torrecilla M Fau - Kobayashi, Kobayashi K Fau - Okano, Okano H Fau - Williams, & Williams, 2006). However, the problem with Concanavilin A is that it was shown in our institute to interfere with FSK-mediated cAMP accumulation (unpublished data). Therefore, we do not suggest the use of Concanavilin A in future studies.

It is worth mentioning that dynasore and Dyngo 4a used in our experiments are not the only available dynamin inhibitors out there. There are many different classes of dynamin inhibitors each acting through a different mechanism. For example, there are the long-chain ammonium salts such as myristyl trimethyl ammonium bromide also known as MiTMAB, and octadecyltrimethyl ammonium bromide (OcTMAB). These ammonium salts stop the recruitment of Dynamin to the membrane (Nimitvilai, McElvain, & Brodie, 2013; Quan et al., 2007). Another class, Dynoles, are known to have potent action against dynamin. In particular, dynole 34-2 was demonstrated in literature to be 15-fold more potent than dynasore (Hill et al., 2009). There are many other classes of internalization inhibitors such as iminodyn, pthaladyn and pyrimidyn which were shown also to block the process of clathrin-mediated endocytosis by blocking dynamin GTPase function after the recruitment of dynamin to the membrane (Hill et al., 2010; McGeachie et al., 2013; Odell et al., 2010). Many of these inhibitors have proven versatile in several experimental settings and effective for endocytosis blockage. We believe that definitely one or several of these blockers can finally provide the answer to the missing part in our puzzle.

The third rationale to follow is to transfect the cells with a dominant-negative form of dynamin. Several studies claim that the chemical dynamin inhibitors are generally advantageous to genetic approaches since they are more applicable and their effects are easily reversed upon washing. However, if the chemical inhibitors are toxic to the cells or are fluorescent, they can be problematic as well. In fact, in two previous studies done

on MORs, the expression of dominant-negative forms of dynamin-1, was shown to effectively impair endocytosis of MORs in neurons (Lin et al., 2009; Macey et al., 2010; Whistler & von Zastrow, 1999). Other forms of dominant-negative dynamin were used with other receptors like PTHR and were also shown to be potent internalization blockers and to reverse the persistent signal induced by internalized PTHR. Therefore, we believe these dynamin mutants can be suitable alternatives for the chemical internalization blockers.

The spectrum of approaches to block clathrin-mediated receptor internalization is not limited at all to the aforementioned methods. Several studies have shown that hypertonic sucrose (0.43 M) was able to block G-protein coupled receptor (GPCR) internalization such as metabotropic glutamate receptor (mGluR), TSHR and D1R (Davide Calebiro et al., 2009; Dale, Bhattacharya, Seachrist, Anborgh, & Ferguson, 2001; Heuser & Anderson, 1989; Kotowski et al., 2011). The mechanism through which hypertonic sucrose acts is thought to be through the interference with the clathrin lattice structure, and the inhibition of the initial formation of the coated pits. We also suggest that hypertonic sucrose could be a cheap effective alternative to chemical and genetic-based approaches for internalization inhibition. It is worth mentioning that the study done on D1R have separately and successfully applied all the aforementioned methods to block internalization of D1R (Kotowski et al., 2011).

Whether the persistent cAMP signal comes from the cell surface or from the internalized receptors is a crucial question to answer, and it will provide great insight into understanding how MORs mediate their signaling and what functional outcomes this can have on the cellular and even medical level. This was already seen with another Gi-coupled receptor, S1P1 in which endocytosis was induced by FTY720-P-. Subsequent endocytic signaling of this S1P1 receptor was found to lead to an increase in the migration of primary human umbilical vein endothelial cells and cause certain immunological therapeutic outcomes (Mullershausen et al., 2009). As for MORs, as we mentioned in the introduction chapter, one study has demonstrated that internalization contributes to the anti-nociception effect mediated by dermorphin, a MOR agonist (Macey et al., 2010). Another study done by Lin et al has shown that fentanyl (another potent MOR agonist) increased the number of neuronal dendritic spines, an effect that was only visible upon MOR internalization (Lin et al., 2009). If the persistent signaling

observed in our study proved to originate from internalized MORs, it will provide a very interesting explanation for the findings of the aforementioned MOR studies and a platform for understanding a lot of medically-significant phenomena such as addiction, tolerance and withdrawal. Interestingly enough, it is already thought that opioid drugs or peptides that mediate strong internalization produce less addiction than those that do not produce internalization (e.g. Morphine)(Keith et al., 1996). Therefore, the last missing piece of our puzzle can be the very platform that would help understand many MOR-related medical observations at the cellular level.

The scope of studying this phenomenon does not end after answering this question. Previous studies done on persistent signaling of GPCRs have also studied the trafficking of the receptor after internalization. Studying trafficking is important to elucidate the mechanism through which persistent signaling takes place, and to determine which compartment each receptor relocates to after internalization. In the study done on PTHR signaling, the PTHR receptor was found to co-localize together with the $G_{\alpha s}$ subunit as well as the adenylyl cyclase enzyme in the early endosomes but not in the late endosomes (Ferrandon et al., 2009). The same observation was made for the D1R (Kotowski et al., 2011). Another study done on the Gi-coupled receptor, S1P1 receptor has shown that the receptor is translocated to the Golgi compartment after internalization (Mullershausen et al., 2009). This somehow implies that the internalized receptor targets its signals to a specific location in order to mediate certain specific outcomes that are different from those mediated by the receptors signaling at the cell surface. This has already been shown in the PTHR study that demonstrated that PTHRs signaling at the cell surface and those signaling from the endosomes explain the difference between paracrine and endocrine signaling of PTHR. Therefore, we would like to suggest that, later on, trafficking studies should also be done on MORs to understand how the signaling takes place and reveal how this leads to certain intriguing biochemical and physiological results.

Future investigations will be able to tell us the full story of the persistent cAMP signaling of MORs. Our finding is just the foot threshold to a very vast world of GPCR signaling phenomena that we are just beginning to explore.

5 List of Abbreviations

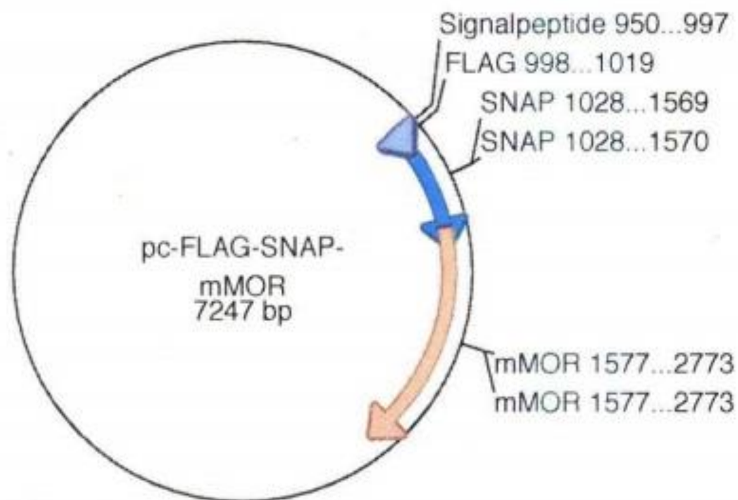
µg	Microgram
µl	Microliter
µM	Micromolar
AC	Adenylyl Cyclase
$CaCl_2$	Calcium Chloride
cAMP	Cyclic Adenosine Monophosphate
CFP	Cyan Fluorescent Protein
D1R	Dopamine 1 Receptors
DAMGO	(D-Ala^2, N-MePhe^4, Gly-ol)-enkephalin
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
DPSS	Diode-pumped solid-state
FCS	Fetal Calf Serum
FRET	Fluoescence Resonance Energy Transfer
FSK	Forskolin
g	Gram
GEF	Guanine Exchange Factor
GIRK	G protein-coupled inwardly-rectifying potassium channel
GPCRs	G protein-coupled Receptors
GRK	G protein coupled Receptor Kinase
GTPase	Guanosine Monophosphate
HEK	Human Embryonic Kidney
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
IBMX	3-Isobutyl-1-methylxanthin
KCl	Potassium Chloride
LB	Lysogeny Broth
MAPK	Mitogen activated protein kinase pathway
$MgCl_2$	Magnesium Chloride

mGluR	Metabotropic Glutamate Receptor
ml	milliliter
MORs	μ Opioid Receptors
NaCl	Sodium Chloride
ng	nanogram
nm	nanometer
PBS	Phosphate-buffered Saline
PFA	Paraformaldehyde
PKC	Protein Kinase C
PLC	Phospholipase C
PTHR	Parathyroid Hormone Recepor
RGS	Regulator of G-protein signaling
RT	Room Temperature
S1P1	Sphingosine-1-phosphate 1
TSHRs	Thyroid Stimulating Hormone Receptors
UV	Ultraviolet
YFP	Yellow Fluorescent Protein

6 Appendix

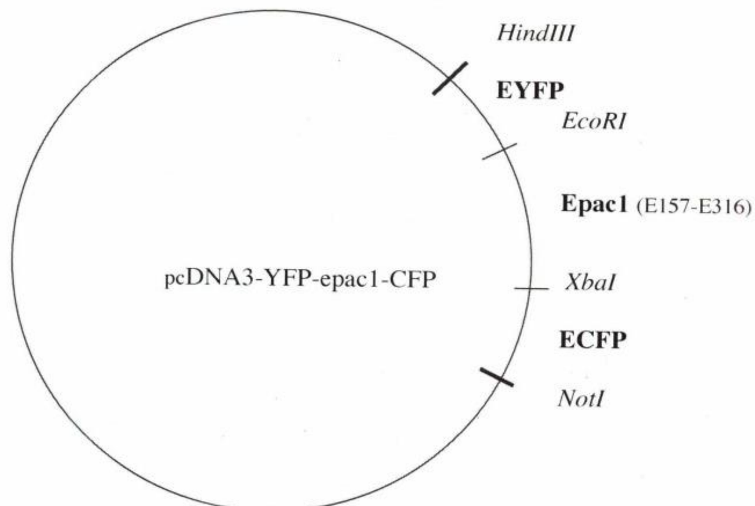
6.1 Plasmid map of FLAG-SNAP-mMOR construct

(pc-Flag-SNAP-mMOR)



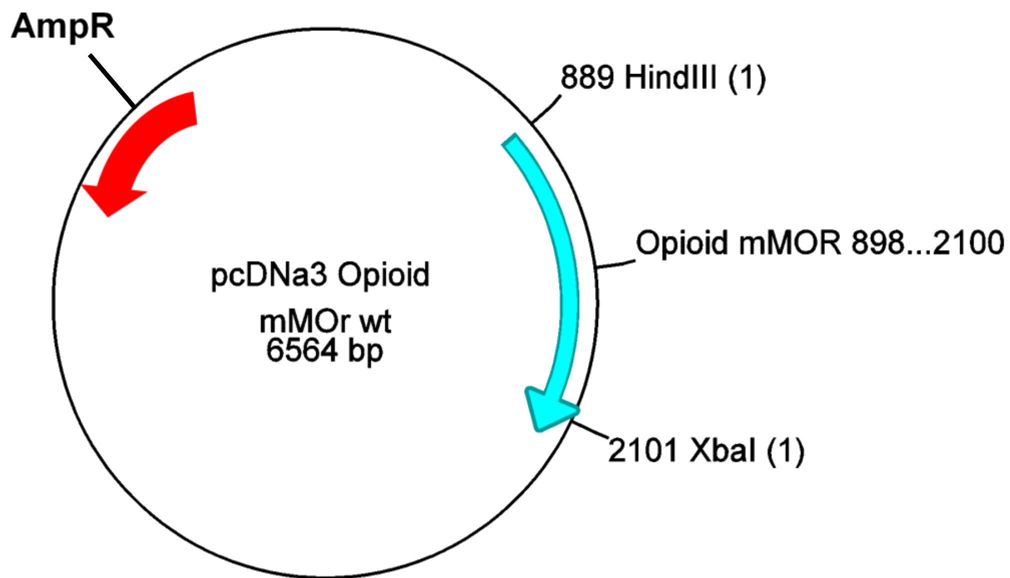
6.2 Plasmid map of Epac1-camps cAMP FRET-based sensor construct

(pcDNA3-EYFP-Epac1-ECFP)



6.3 Plasmid map of wild type mMOR construct

(pcDNA3_OPRM_wildtype)



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8 Acknowledgments

I would like to thank my supervisor **Dr. Davide Calebiro,** for giving me the opportunity to work on this very interesting project, and for being my direct mentor throughout my thesis. I am grateful that he gave me the opportunity to be a student at the Pharmacology and Toxicology institute where I learned about the latest developments in the fields of G protein-coupled receptors and imaging.

I would also like to thank **Dr. Isabella Maiellaro,** for teaching me the very basics, for helping me start this project, for thoroughly and patiently training me during my internship, and for her motivation. Without what she taught me, I would not have been able to work on my thesis.

Thanks to **Sandra Lyga,** for teaching me how to do statistical analysis for FRET graphs, and for teaching me the basics of confocal microscopy.

Very sincere and special thanks to **Sana Mohamedgamil,** for constantly cheering me on and encouraging me and for being my older sister, to **Kerstin Seier** for everything, but most of all, for your moral support during the thesis-writing stage (and of course for translating my summary to German!). Many thanks to **Kerstin Bathon** for reading my thesis and giving me insightful suggestions on how to improve it.

I would also like to thank the rest of **the members of AG Calebiro** group for being friendly and welcoming, and for being there to help when needed.

Thank you very much, my closest friends, **Norhan Issa**, **Alaa Leila**, **Nourhan El-Sonbaty** and **Nourhan Ehab** for always making me feel that I am not far away from home, and that home is just a message or a call away. Thank you for being there for me through the ups and downs.

Last but not least, I would like to send my deepest and warmest gratitude to **my parents** for believing in me and for encouraging me to pursue my dreams. Without their support, starting my scientific career abroad would not have been possible. I owe them my life. My dear parents, this is for you.