Agonist-dependent Mechanism of Mu-opioid Receptor

Desensitization

A DISSERTATION
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF THE UNIVERSITY OF MINNESOTA
BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

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DECEMBER, 2009
Acknowledgements

I need to extend my gratitude and appreciation to many people who made this doctoral thesis possible.

I would like to express my sincere appreciation to my advisor Dr. Ping-Yee Law, who provided me the opportunity to be one of his students. I want to thank all his guidance, encouragement and continuous supports. His keen scientific insights, enthusiasm for research benefited me throughout my Ph. D study, and will continue to benefit me.

I would like to thank my great committee: Dr. Timothy F. Walseth (chair), Dr. Stanley Thayer, Dr. Sabita Roy and Dr. Esam El-Fakahany for their inspiring discussions and help. I want to thank Dr. Horace H. Loh for all his advices and guidance. I also want to thank my DGS: Dr. Jonathan Marchant, Dr. Sabita Roy and Dr. Colin Campbell for their helping and encouragement.

Many thanks go to my colleagues in Dr. Horace H. Loh and Dr. Ping-Yee Law’s laboratory. In particular, I thank Hui Zheng, Yuhan Zhang, Lei Zhang, Xiaohong Guo, Wei Wang, Guilin Wang. I want to thank them for all their interesting and valuable discussion and technique support.
In the end, I want to thank my parents, Chongxin Chu and Yuwu Fan, my husband Xianping Wu, without your numerous supports, love and fully understanding, I would never accomplish all this things.
Dedication

This dissertation is dedicated to my grandparents, I love you.
Desensitization of the µ-opioid receptor (MOR) has been implicated as an important regulatory process in the development of tolerance to opiates. Desensitization of G-protein coupled receptor (GPCR) is thought to involve receptor phosphorylation and subsequent recruitment of β Arrestins (βArrs). However, the roles of receptor phosphorylation and βArr in morphine-induced MOR desensitization remain to be demonstrated; this may result from the insensitivity of the methods used to study receptor function. Using MOR-induced intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) release to monitor receptor activation, [D-Ala\(^2\), N-Me-Phe\(^4\), Gly\(^5\)-ol]-enkephalin (DAMGO) induced MOR desensitization in a receptor phosphorylation- and βArr-dependent manner. DAMGO-induced desensitization was blunted in HEK293 cells expressing the MORS375A mutant and was eliminated in MEF cells isolated from βArr2 knockout mice expressing the wild type MOR. However, although morphine induced a more rapid desensitization of [Ca\(^{2+}\)]\(_i\) release than DAMGO did and could induce the phosphorylation of the Ser\(^{375}\) residue of MOR, morphine-induced desensitization was not influenced by mutating MOR phosphorylation sites or in MEF cells lacking βArr1 and 2. In contrast, morphine induced MOR desensitization via protein kinase C (PKC). By using subtype-specific inhibitors, PKC\(\varepsilon\) was shown to be the PKC subtype activated by morphine and the subtype responsible for morphine-induced desensitization. Meanwhile, DAMGO did not increase PKC\(\varepsilon\) activity and DAMGO-induced MOR desensitization was not affected by a PKC\(\varepsilon\) inhibitor. Among the various proteins within the receptor signaling complex, G\(\alpha_i2\) was phosphorylated by morphine-activated PKC\(\varepsilon\). Moreover, mutating
three putative PKC phosphorylation sites, Ser^{44}, Ser^{144} and Ser^{302} on Gαi2 to Ala attenuated morphine-induced, but not DAMGO-induced desensitization. In addition, pretreatment with morphine desensitized cannabinoid receptor CB1 agonist WIN 55212-2-induced [Ca^{2+}]_{i} release, and this desensitization could be reversed by pretreating with a PKCε inhibitor or overexpressing of Gαi2 with the putative PKC phosphorylation sites mutated. Thus, depending on the agonist, activation of MOR could lead to heterologous desensitization and probable crosstalk between MOR and other Gαi-coupled receptors such as the CB1 receptor.
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Part II: MOR agonists pretreatment rapidly desensitize MOR without affecting [Ca^{2+}]_{i} store or P2Y receptor activity.

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Chapter I

Introduction
In ancient Egypt, opium, which is the morphine-contained latex extract from poppy seeds, was used and recorded for its analgesic effect. In the 19th century, morphine was firstly isolated from opium, and identified as the effective component in the opium mixture. The made it possible for morphine, as a purified agent, to be a predictable and reliable compound and can be precisely used in modern medical care. Because morphine’s outstanding analgesic clinical usefulness is compromised by its serious unwanted side effects, the pharmacological characteristics of morphine have begun to be extensively studied. Meanwhile, more opioid agents, which are referred to the opium-like substances, have been synthesized.

The opioids include alkaloids, peptides, and endogenous opioids. Usually, the opioid alkaloids are natural or derivations of natural opioids, such as morphine, heroin or etorphine. The opioid peptides are the fully synthetic opioids, such as [D-Ala2, N-Me-Phe4, Gly5-ol]-enkephalin (DAMGO), fentanyl. The endogenous opioid peptides were not be identified (Hughes, Smith et al. 1975) until the 1970s, when opioid receptors were first characterized by radioligands binding assay from both human and guinea pig(Pert and Snyder 1973; Simon, Hiller et al. 1973; Terenius 1973; Kosterlitz and Waterfield 1975). These include enkephalins, β-endorphin and dynorphin. Although opioid structures vary from alkaloids to peptides, all of them can bind to opioid receptors and execute their function through opioid receptor-activated G-proteins (Table 1.1).
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1.  Opioid receptors and signaling transduction

1.1 Opioid receptor structures

After the successful cloning of delta (δ)-opioid receptor (DOR) in 1992, three types of opioid receptors [mu (µ)- (MOR), delta (δ)- (DOR), kappa (κ)-] has been well-defined and cloned from central and peripheral nervous system (Evans, Keith et al. 1992; Kieffer, Befort et al. 1992; Chen, Mestek et al. 1993; Chen, Mestek et al. 1993; Fukuda, Kato et al. 1993; Li, Zhu et al. 1993; Meng, Xie et al. 1993; Yasuda, Raynor et al. 1993; Knapp, Malatynska et al. 1995). These three opioid receptors belong to the class A (Rhodopsin) family of Gi/Go-coupled receptor family (Pierce, Premont et al. 2002; Waldhoer, Bartlett et al. 2004). Like the rhodopsin, the opioid receptor is formed by an extracellular N-terminal and intracellular C-terminal; in between, there are seven α-helices crossed the cell bi-layer membrane and connected with hydrophilic loops (Pogozheva, Lomize et al. 1998). Based on their sequence analysis, all three opioid receptors were revealed to have ~60% identical sequence, with the greatest identity found in the transmembrane domains (73–76%) and intracellular loops (86–100%). The most sequence divergency was found within the receptors’ N-terminals (9–10%); and C-terminal (14–20%) (Chen, Mestek et al. 1993) (Fig. 1.1).
Fig. 1.1 Serpentine model of the opioid receptor
Each transmembrane helix is labeled with a roman number. The white empty circles represent nonconserved amino acids among the MOR, DOR, KOR, and ORL1 receptors. White circles with a letter represent identical amino acids among all four opioid receptors. Violet circles represent further identity between the MOR, DOR, and KOR. Green circles highlight the highly conserved fingerprint residues of family A receptors, Asn I:18 in TM1, AspII:10 in TM2, CysIII:01 in TM3, TrpIV:10 in TM4, ProV:16 in TM5, ProVI:15 in TM6, and ProVII:17 TM7. Yellow circles depict the two conserved cystines in EL loops 1 and 2, likely forming a disulfide-bridge. IL = intracellular loop, and EL = extracellular loop. (Waldhoer, Bartlett et al. 2004)
1.2 opioid receptor functions

The three major opioid receptor subtypes, MOR DOR and KOR, are primarily expressed and execute their analgesia function in the central nervous system (CNS) (Dubner and Bennett 1983; Basbaum and Fields 1984; Mansour, Fox et al. 1995; Mansour, Fox et al. 1995; Henriksen and Willoch 2008); however, the mRNA and protein of these three opioid receptors were also found at peripheral nervous system (Stein, Millan et al. 1988; Stein 1993; Wittert, Hope et al. 1996; Stein 2003; Truong, Cheng et al. 2003; Puehler, Rittner et al. 2006) as well as in the immune system (Lawrence, el-Hamouly et al. 1995; Smith 2003).

Using selective opioid receptor agonists and opioid receptor deficient mouse reveal that opioids mediate in vivo analgesia primarily through MOR (Matthes, Maldonado et al. 1996; Sora, Funada et al. 1997; Sora, Takahashi et al. 1997; Loh, Liu et al. 1998). In addition to analgesia, MOR also mediates the most important opioid functions, including hyperlocomotion, respiratory depression, constipation and immunosuppression as well as opioid reward and withdrawal symptoms (Matthes, Maldonado et al. 1996; Sora, Funada et al. 1997; Sora, Takahashi et al. 1997; Loh, Liu et al. 1998). Therefore, MOR cellular signaling transduction and regulation are most intensively studied among three receptor subtypes. DOR is suggested to functionally relate to MOR in analgesia effect and opioid tolerance development (Abdelhamid, Sultana et al. 1991; Fundytus, Schiller et al. 1995; Zhu, King et al. 1999). This opioid receptor subtype is also suggested to contribute to emotional and rewarding responses.
In addition to these three types of opioid receptors, there are two other receptors that have been identified and were suggested to relate to opioid receptors. One of them is sigma (σ)-receptor. σ-receptor originally was classified as one of the opioid receptor subtypes, because some opioids executed their cough relieving effect through it (Martin, Eades et al. 1976; Walker, Bowen et al. 1990). However, since σ-receptor has been cloned, it was found that σ-receptor has no structure similarity to opioid receptor and it was not able to be activated by endogenous opioid peptides (Walker, Bowen et al. 1990). Currently, σ-receptor is not classified as opioid receptor.

By using opioid receptor cDNA as probes, several groups isolated a cDNA that encode opioid receptor-like protein (Bunzow, Saez et al. 1994; Chen, Fan et al. 1994; Fukuda, Kato et al. 1994; Mollereau, Parmentier et al. 1994; Wang, Johnson et al. 1994; Wick, Minnerath et al. 1994; Lachowicz, Shen et al. 1995; Wick, Minnerath et al. 1995). This GPCR protein was named as opioid receptor-like 1 (ORL1) (Fig. 1.1). When it was first identified, this ORL1 was shown to have no affinity for endogenous opioid
ligands; however, in 1995, two groups isolated ORL1 endogenous ligands which were different from the MOR, DOR and KOR’s endogenous ligands (Meunier, Mollereau et al. 1995; Reinscheid, Nothacker et al. 1995). Moreover, this newly discovered opioid receptor subtype has shown an anti-analgesic action which is significantly different from the major three opioid receptor subtypes (Meunier 1997; Taylor and Dickenson 1998; Calo, Guerrini et al. 2000; Mogil and Pasternak 2001; New and Wong 2002; Meis 2003; Farooqui, Geng et al. 2006). The functional role of ORL1 is under active investigation; thus far, the system’s involvement in opioid analgesic and dependence or tolerance developments are not yet clear (Meunier 1997; Taylor and Dickenson 1998; Calo, Guerrini et al. 2000; Mogil and Pasternak 2001; New and Wong 2002; Meis 2003; Farooqui, Geng et al. 2006).

1.3 opioid receptor signal transduction

Like GPCRs, opioid receptors activate G proteins after binding to agonists. Opioid receptor mediated signaling can be abolished by pertussis toxin (PTX) pretreatment, indicating that opioid receptor couples to Gi/Go α subunits, which are PTX sensitive (Hsia, Moss et al. 1984; Wong, Demoliou-Mason et al. 1989). In addition, MOR was reported to associate with a PTX-insensitive G protein Gz to execute its analgesic function both in vivo and in vitro (Sanchez-Blazquez, Juarros et al. 1993; Sanchez-Blazquez, Garcia-Espana et al. 1995; Standifer, Rossi et al. 1996; Garzon, Martinez-Pena et al. 1997). Despite the different in vivo functions of these three opioid
receptor subtypes, they all belong to the Gi/Go-coupled receptor family, and regulate the same spectrum of effectors.

As the prototypic Gi/Go-coupled receptors, opioid receptors regulate the signals including: Gαi-mediated inhibition of adenylyl cyclase (Sharma, Klee et al. 1977; Fedynyshyn and Lee 1989; Childers, Fleming et al. 1992; Nijssen, Sexton et al. 1992), Gβγ-mediated inhibition of N- and L-type voltage-dependent Ca²⁺ channels (VDCC) (Xiao, Spurgeon et al. 1993; Kaneko, Fukuda et al. 1994; Wilding, Womack et al. 1995; Kaneko, Yada et al. 1997; Fukuda, Shoda et al. 1998; Morikawa, Mima et al. 1999; Gullapalli and Ramarao 2002; Rola, Jarkiewicz et al. 2008) and activation of inward rectifying K⁺ channel (GIRK) (Twitchell and Rane 1994; Ikeda, Kobayashi et al. 1995; Ma, Miller et al. 1995; Ikeda, Kobayashi et al. 1996). Extracellular Ca²⁺ influx is critical for neuron transmitter release (Wu, Westenbroek et al. 1999). Therefore the inhibition of MOR for Ca²⁺ channels is suggested to result in diminishing sensory input to the central nervous system (Omote, Sonoda et al. 1993; Soldo and Moises 1998; Johnson, Oldfield et al. 2006). Activation of GIRK induces postsynaptic hyperpolarization, thus decreases neuronal excitability (Emmerson and Miller 1999; Ikeda, Kobayashi et al. 2002) (Fig. 1.2).

Besides these most well-known opioid receptor signal transduction pathways, opioid receptor-mediated activation of mitogen-activated protein kinases ERK1 and ERK2 has also been reported (Fukuda, Kato et al. 1996; Li and Chang 1996; Kramer and Simon 2000). This function was suggested to relate to opioid receptor
phosphorylation, desensitization (Polakiewicz, Schieferl et al. 1998; Kramer, Andria et al. 2000; Schmidt, Schulz et al. 2000); and moreover it was indicated to involve in Fig. 1.2 μ-opioid receptor signaling.

Abbreviation: AC, adenylate cyclase; Ag, agonist; ER, endoplasmic reticulum; ERK, extra-cellular signal regulated kinase; DAG, diacyl glycerol; GIRK, G-protein-activated inwardly rectifying potassium channel; IP3, inositol 1,4,5-triphosphate; MEK, Mitogen-activated protein kinase kinase; PIP2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; VDCC, voltage-dependent calcium channel.
opioid-mediated gene expression and cellular adaptation response, thus may result in
opioid-induced tolerance and physical dependence (Kim, Clark et al. 2006; Chen, Geis
et al. 2008; Zheng, Loh et al. 2008). Recently, Src family nonreceptor tyrosine kinases
were implicated in GPCR functions (van Biesen, Hawes et al. 1995; Hawes, Luttrell et
Luttrell and Luttrell 2004); and three opioid major subtypes have all been reported to
activate Src kinase (Kramer, Andria et al. 2000; Okamoto, Tsuneto et al. 2007; Zhang,
Zhao et al. 2009). Although the exactly activation mechanism of Src by opioid receptors
was still under-investigation, it was strongly suggested to contribute to mu-opioid
receptor-mediated adenylyl cyclase super-activation in vitro (Zhang, Zhao et al. 2009);
which is the molecular model for morphine withdrawal in vivo (Avidor-Reiss, Nevo et
al. 1996; Williams, Christie et al. 2001; Bie and Pan 2005). Observations of opioid
receptor-regulated phospholipase Cβ (PLCβ) and intracellular Ca2+ [Ca2+]i mobilization
were controversial. Direct activation of PLCβ and release [Ca2+]i by opioid receptors
were observed in a number of cell lines (Jin, Lee et al. 1992; Smart, Smith et al. 1994;
Smart and Lambert 1996; Yoon, Lo et al. 1999); however, more reports indicated
activating Gq protein was required for opioid receptor-mediated [Ca2+]i release (Yeo,
Samways et al. 2001; Samways, Li et al. 2003). In other word, opioid receptor itself was
not able to stimulate [Ca2+]i release alone, but it potentiated Gq-coupled receptor-
mediated [Ca2+]i release. This phenomenon has been observed and estimated in
numerous Gi-coupled receptors (for review) (Werry, Wilkinson et al. 2003).
There are four identified PLCβ isoforms which could be activated by Gβγ subunits of Gi/Go-coupled receptor. However they have markedly un-identical activity level (Rhee 2001; Wing, Houston et al. 2001); the different expression profile of PLCβ isoforms within cells explain could explain the differences in observations on the opioid receptor-mediated [Ca^{2+}]_i release between different cell lines.

On the other hand, the mechanism of this cross-talk between Gi/Go-coupled receptor and Gq-coupled receptor was also unclear. There are several possible hypotheses, which include the direct modulation of PLC activity by Gβγ subunits of Gi/Go-coupled receptor (Wu, Katz et al. 1993; Jiang, Kuang et al. 1996; Murthy, Coy et al. 1996; Biber, Klotz et al. 1997; Katz and Rothenberg 2005). PLCβ can be directly activated by either Gβγ subunits or Gαq subunits, the binding sites within PLCβ are distinct (Lee, Shin et al. 1993; Xie, Samoriski et al. 1999); and simultaneous occupation to these sites by Gβγ and Gαq could produce synergistic effect (Zhu and Birnbaumer 1996). Participation of PKA or PKC should also be considered; PKA- or PKC-mediated phosphorylation of PLCβ inhibits its function (Yuen, So et al. 2004), however, this inhibition could be reversed by Gβγ subunits (Litosch 1997). Thus the activities of protein kinases could provide another potential mechanism through which activation of a Gai-coupled receptor enhance PLCβ signaling with Gαq-coupled receptor activation (Fig. 1.3).
In the unstimulated PLCβ enzyme (A), the Gβγ-subunit-binding region is inaccessible to its “ligand”. However, in (B), binding of Gaq causes both PLCβ activation and alteration of protein conformation, such that the Gβγ-binding site becomes accessible, allowing Gβγ also to stimulate PLCβ activity. It is not clear whether “unfolding” is required for PLCβ activation by Gaq. (Werry, Wilkinson et al. 2003).
2 Regulation of opioid receptor activity

One important feature of biological system is that they tend to eliminate their responsiveness to continuous outside stimulus to prevent overstimulation and thereby maintain biological equilibria. Desensitization describes this phenomenon and is observed widespread in diverse organisms and biological regulation processes (Lohse 1993; Lachowicz, Shen et al. 1995). At cellular level, multiple receptors including GPCRs (Gainetdinov, Premont et al. 2004; Kelly, Bailey et al. 2008; Klaasse, Ijzerman et al. 2008), receptor tyrosine kinases (Cochet, Gill et al. 1984; Blake, Hayes et al. 1987; Pillay and Makgoba 1991; Countaway, Nairn et al. 1992; Penengo, Rubin et al. 2003) as well as ion channel receptors (Betz 1990; Changeux 1990; Cooper, Arnot et al. 2000), gradually lose their activity after agonist activations, which are called receptor desensitization.

GPCRs family is the largest and most diverse receptor family in nature, it participates in various biological and pathological processes and accounts for about 50% of all prescription medicines on the market (Davies, Secker et al. 2008). Therefore, the regulation of GPCRs signaling is under intensive studies. Based on the causation, receptor desensitization is subjected into homologous or heterologous desensitization (Pei, Kieffer et al. 1995; Chuang, Iacovelli et al. 1996). Homologous desensitization is an activation-dependent regulation of receptor, what means only agonist-activated receptors lose their response. In contrast, heterologous desensitization does not necessarily result from its activation; it could results from the other receptors activation.
Usually GPCR homologous desensitization is resulted from G-protein-coupled receptor kinases (GRKs)-mediated receptor phosphorylation and subsequent β-arrestins (βArRs) recruitment; and heterologous desensitization is mediated by the second-messenger-regulated kinases they activate. Commonly, homologous desensitization is thought to be the adaptive response at the level of the GPCR itself, whereas heterologous desensitization refers to a more profound cellular adaptive response after GPCR activation (Gainetdinov, Premont et al. 2004; Gabra, Bailey et al. 2007).

2.1 GRKs and βArRs dependent desensitization

The serine/threonine protein kinase GRK family includes seven GRK subtypes. Four of them express throughout the body and are suggested to account for most GPCRs regulation (Bohn, Gainetdinov et al. 2000). GRKs are able to distinguish the agonist-activated receptor from inactive one, and then phosphorylated receptor (Lefkowitz 1998; Claing, Perry et al. 2000); it might because GRKs are recruited by activated Gβγ subunits (Kozasa and Gilman 1996; Li, Xiang et al. 2003; Metaye, Gibelin et al. 2005). The ability of receptor coupling to G-protein is decreased by GRK-mediated phosphorylation; by that, GPCR signaling is decreased (Lefkowitz 1998). After the firstly study showing phosphorylation of DOR by GRKs in 1995 (Pei, Kieffer et al. 1995; Kovoor, Nappey et al. 1997), GRKs-mediated three types of opioid receptor phosphorylation were identified in both native and heterologous expression systems (Appleyard, Patterson et al. 1997; Kovoor, Nappey et al. 1997; Zhang, Ferguson et al.
1998; Appleyard, Celver et al. 1999; Deng, Yu et al. 2001; McLaughlin, Myers et al. 2004).

Usually, the GRK-phosphorylated sites locate at GPCR third intracellular loop and C-terminal. From the deletion or truncation mutation studies, agonist-induced phosphorylation of the opioid receptors occurs mainly in receptor C-terminal. DOR phosphorylation sites were first investigated by deletion the last 31 amino acids or after Thr^{344} in C-terminal. Without affecting receptor responsiveness after agonist application, these deletion mutant DOR which excluded all C-terminal phosphorylation sites completely abolished agonist-induced DOR phosphorylation (Zhao, Pei et al. 1997; Murray, Evans et al. 1998). Truncation or deletion different part of MOR also revealed the importance of C-terminal in agonist-induced MOR phosphorylation (Deng, Yu et al. 2000; El Kouhen, Burd et al. 2001).

Site-directed mutagenesis experiments provided more details about the specific agonist-induced receptor phosphorylation sites. A series Ser/Thr sites in MOR were suggested to contribute to either receptor basal or agonist-induced phosphorylation (Chavkin, McLaughlin et al. 2001; Johnson, Christie et al. 2005). Among them, Ser^{375} was shown to be consistently phosphorylated in both native and heterologous expression systems (Schulz, Mayer et al. 2004). Besides Ser^{375}, other phosphorylation sites also contribute to DAMGO-induced MOR phosphorylation(El Kouhen, Burd et al. 2001; Schulz, Mayer et al. 2004). However, Ser^{375} seems account for over 90% of morphine-induced MOR phosphorylation in HEK293 cells (Schulz, Mayer et al. 2004).
Meanwhile, several Ser/Thr sites has also been identified to responsible for DOR and KOR phosphorylation (Guo, Wu et al. 2000; McLaughlin, Xu et al. 2003; McLaughlin, Myers et al. 2004).

Phosphorylation of opioid receptor by GRKs serves two basic functions: (1) decreased affinity of receptor to G-proteins. (2) increased affinity of agonist-receptor complex to cytosolic protein arrestins (Arrs), thus further terminates receptor signaling (Lefkowitz 1998).

The arrestin family includes four members, all of which interact with GPCRs after receptor activation. Arr1 and 4 are visual arrestins, and are found exclusively in retinal rods and cones. In contrast, Arr2 and 3, which are also called βarrestin1(βArr1) and 2, are expressed in all tissues and predominantly expressed in the CNS (Lefkowitz and Whalen 2004). These two types of βArrs are responsible for regulating most GPCRs signaling. The visual arrestins were originally identified in the context of desensitization of the photoreceptor rhodopsin (Pfister, Chabre et al. 1985); then βArr1 and 2 was found as protein potentiated the GRK2-induced β2-adrenergic receptor (Lohse, Benovic et al. 1990; Attramadal, Arriza et al. 1992). Using β2-adrenergic receptor as the model, Lefkowitz’s lab proposed that once phosphorylated by GRKs, the activated receptor recruits βArrs. βArrs recognize both GRK phosphorylation sites on the receptor as well as the active conformation of the receptor; both of the facts together drive robust βArrs association (Luttrell and Lefkowitz 2002; Perry and Lefkowitz 2002). Thus, by physically disrupting receptor–G-protein coupling, phosphorylation and Arrs
Evidence for the involvement of the proteins illustrated is discussed in text. While the phenomena of opioid receptor activation, uncoupling and internalization are well described, the precise mechanisms underlying the experimental observations are largely undefined. Serine/threonine protein kinases that might phosphorylate the μ-opioid receptor include PKA, calmodulin-dependent protein kinase, and protein kinase C; specific tyrosine kinases that might phosphorylate the μ-opioid receptor have not been identified. Abbreviations are AP-2, adaptor protein 2; Arr3, arrestin 3; GRK, G protein-coupled receptor kinase; P, phosphate. (Connor, Osborne et al. 2004)
interdict G protein activation and eventually dampen signal transduction processes despite the continued activation of the receptor by agonists (Fig. 1.4).

Currently, site-directed mutagenesis, over-expression wild type or dominant negative mutants and specific knocking-down GRKs and βArrs are the most commonly used methods to study their effects in agonist-induced opioid receptor desensitization. By using those methods, the involvement of GRKs and βArrs in opioid receptor regulation was demonstrated in various models.

Numerous studies demonstrate phosphorylation minus mutant receptor attenuated agonist-induced DOR or KOR desensitization; at the same time, over-expression of GRKs increases DOR or KOR phosphorylation level, and accelerates receptor desensitization rate (Pei, Kieffer et al. 1995; Kovoor, Nappey et al. 1997; Hasbi, Polastron et al. 1998; Zhu, Luo et al. 1998; Appleyard, Celver et al. 1999; Guo, Wu et al. 2000; Kouhen, Wang et al. 2000; McLaughlin, Xu et al. 2003; McLaughlin, Myers et al. 2004; Qiu, Loh et al. 2007). In contrast to DOR and KOR, regulation of MOR by GRKs and βArrs is more obscure. In HEK293 cells, activation of DOR results in significant accumulation of GRKs at the plasma membrane; however, MOR activation does not trigger translocation of GRK2 and 3 toward the cell membrane (Schulz, Wehmeyer et al. 2002). Instead of rapidly desensitizing in min as DOR, desensitization of MOR after agonist treatment usually is much slower and incomplete (Kovoor, Nappey et al. 1997; El Kouhen, Kouhen et al. 1999; Law, Erickson et al. 2000; Lowe, Celver et al. 2002), and does not co-relate to receptor phosphorylation timeline. Over-expression of GRK3 in the *Xenopus* oocytes which co-express MOR and DOR
together significantly decreases DOR initial response, but not MOR’s (Lowe, Celver et al. 2002). In addition, altering opioid receptor phosphorylation level by using Calyculin A or over-expression of GRK3 in HEK293 cells, does not affect desensitization of MOR, but does affect desensitization of DOR (El Kouhen, Kouhen et al. 1999). Available data suggest a relatively weak effect of receptor phosphorylation on MOR desensitization although observed agonist-induced MOR phosphorylation has been observed.

In the live cell, by using real-time microscopy, GFP-fused βArr1 and 2 are able to recruit to etorphine-activated MOR (Whistler and von Zastrow 1998; Bohn, Dykstra et al. 2004); and truncating MOR after Ser\(^{363}\) attenuated βArrs-mediated internalization (Qiu, Law et al. 2003). When GIRK is measured, three opioid receptor subtypes desensitizations in \textit{Xenopus} oocytes are accelerated with GRKs and βArr2 co-expression (Kovoor, Nappey et al. 1997; Appleyard, Celver et al. 1999); however, MOR still desensitizes in a much slower manner (El Kouhen, Kouhen et al. 1999; Lowe, Celver et al. 2002). By using surface plasmon resonance measurement with synthetic opioid receptor domain peptides, both the third intracellular loop and the C-terminal of DOR can interact with βArr1 and 2, and only C-terminal of KOR can bind to βArrs. In contrast, under the same conditions, MOR cannot interact with either βArrs (Cen, Xiong et al. 2001). The different efficiencies of opioid receptor subtypes on recruiting βArrs are further illustrated by over-expression of βArr1 in HEK293 cells; it uncouples DOR and KOR but not MOR from G-protein by using \(^{[35\text{S}]\text{GTP}\gamma\text{S}}\) binding assay
(Cheng, Yu et al. 1998). This observation corresponds to reports that MOR is less efficient on recruiting βArrs (Lowe, Celver et al. 2002).

Phosphorylation of MOR by different agonists has also been studied intensively in trying to correlate their physiological activity \textit{in vivo} to the pharmacological activity \textit{in vitro} (Yu, Zhang et al. 1997; Deng, Yu et al. 2001; Schulz, Mayer et al. 2004). Apparently, phosphorylation of MOR is agonist-dependent which suggests not all MOR agonists equally induce GRK-mediated MOR phosphorylation; in another word, agonist-induced MOR phosphorylation does not always follow the agonist intrinsic efficacy (Yu, Zhang et al. 1997; Deng, Yu et al. 2001; Schulz, Mayer et al. 2004). The best-studied MOR agonist morphine is no more or much less capable of inducing MOR phosphorylation than the other MOR agonists (Yu, Zhang et al. 1997; Zhang, Ferguson et al. 1998; Koch, Schulz et al. 2001); while it still efficiently activates receptor and induces robust MOR signals \textit{in vitro} (Emmerson, Clark et al. 1996; Selley, Sim et al. 1997) as well as it is a potent analgesic functioning through MOR \textit{in vivo} (Matthes, Maldonado et al. 1996; Sora, Takahashi et al. 1997). On the other hand, without overexpression GRKs, morphine-induced MOR phosphorylation seems to be dependent on PKC activation (Johnson, Oldfield et al. 2006); however if GRKs or βArrs were over-expressed in systems, morphine-induced MOR phosphorylation level would be increased (Zhang, Ferguson et al. 1998). In addition, recruitment of βArrs by MOR is also agonist-dependent. Etorphine or DAMGO pretreatment is able to promote robust βArr2 translocation while morphine fails to do so (Whistler and von Zastrow 1998). However, over-expression of βArrs by more than 30-fold than the endogenous level
would force the binding of βArrs to morphine-activated receptor (Whistler and von Zastrow 1998). In another hand, although morphine is much less efficient on inducing MOR phosphorylation, morphine-induced MOR desensitization was reported (Borgland, Connor et al. 2003; Dang and Williams 2005; Walwyn, Wei et al. 2006). Therefore, morphine-induced receptor desensitization should involve mechanisms other than the GRK/βArr pathway.

2.2 Receptor trafficking

Removing active receptor from cell surface intro intracellular compartments is another common regulatory mechanism of GPCR. βArrs not only physically uncouple receptor from cognate G proteins, but also serve as adaptor proteins for eliciting GPCR internalization. This βArrs- and subsequent dynamin-dependent GPCR internalization has been proposed as the paradigmatic model (Claing, Perry et al. 2000). Once recruited by opioid receptor, βArrs-bound receptors accumulate into clathrin-coated pits at the plasma membrane with another adaptor protein AP2. Then the dynamin, a large GTPase protein, moves receptors into clathrin-coated pits from cell surface into intracellular compartments (Chuang, Killam et al. 1997; Murray, Evans et al. 1998).

In early studies, using labeled ligand binding assay suggested agonist-induced internalization of MOR and DOR (Gaudriault, Nouel et al. 1997). However, this method can only observe the receptors which bind to ligands. When using this method, Lee et al. observed in primary cultured cortical neurons, fluo-labeled ligands accumulated in the
soma after internalizing with receptors. Meanwhile, immunohistochemistry assay indicated both MOR and DOR dissociated from ligands and recycled back to the plasma membrane (Kim, Cheong et al. 2002). Therefore, many studies used tagged opioid receptor heterologous expressed cells or immunohistochemistry assay with antibody to the C-terminal of receptors to follow receptor trafficking. In HEK293 expressing epitope-tagged-MOR cells, observation of opioid receptor internalization was firstly reported when DAMGO was used (Arden, Segredo et al. 1995). Soon, DOR and KOR’s internalization was also reported (Trapaidze, Keith et al. 1996; Li, Luo et al. 1999). In addition to the works in heterologous expressed cell lines, the internalization of opioid receptor in vivo was observed (Sternini, Spann et al. 1996; Keith, Anton et al. 1998; Marvizon, Grady et al. 1999).

Evidences has been accumulated for demonstrating the role of receptor phosphorylation and βArrs in opioid receptor internalization. Through over-expression GRKs, agonist-induced opioid receptor internalization is accelerated, and this effect is correlated to the increased receptor phosphorylation by GRKs (Whistler and von Zastrow 1998; Zhang, Ferguson et al. 1998; Schulz, Wehmeyer et al. 2002). And using GRKs inhibitor, internalization of DOR is attenuated (Hasbi, Allouche et al. 2000). Site-directed mutagenesis studies indicated the Ser\textsuperscript{375} in MOR is a positive regulatory phosphorylation site for internalization in MOR, while Ser\textsuperscript{363} and Thr\textsuperscript{370} and Thr\textsuperscript{394} play a negative role (Wolf, Koch et al. 1999; El Kouhen, Burd et al. 2001). The same method demonstrated that Ser\textsuperscript{344} and Ser\textsuperscript{363} in DOR and Ser\textsuperscript{369} in KOR are also involved in receptor internalization (Law, Kouhen et al. 2000; McLaughlin, Xu et al. 2003;
McLaughlin, Myers et al. 2004). Overexpression of βArr1 significantly accelerates etorphine-induced MOR internalization in HEK293 cells, while dominant negative mutant βArr1 impaired it (Zhang, Ferguson et al. 1998; Claing, Perry et al. 2000). In addition, an increased internalization rate, which induced by overexpression of GRK2, is prevented by dominant negative mutant βArr1 (Claing, Perry et al. 2000).

Since opioid receptor internalization is initiated by receptor phosphorylation and βArrs recruitment, it is reasonable to conclude that like those two processes, opioid receptor internalization is also agonist-dependent. Distinct agonists differ in their ability to induce internalization of both MOR and DOR, as been demonstrated in early study (Gaudriault, Nouel et al. 1997). After that, a large number of reports indicate that some opioid agonists such as etorphine or DAMGO cause a major fraction of opioid receptors to internalize after short period agonists’ exposure, while morphine is unable to internalize receptors in heterologous cell models (Keith, Murray et al. 1996; Whistler and von Zastrow 1998; Zhang, Ferguson et al. 1998; Whistler, Chuang et al. 1999; Kieffer and Evans 2002; Cichewicz, Cox et al. 2004). Although in rat striatal neurons, rapid internalization after morphine treatment is observed (Haberstock-Debic, Kim et al. 2005), agonist-selective internalization processes occur in various neurons (Sternini, Spann et al. 1996; Keith, Anton et al. 1998; Trafton, Abbadié et al. 2000; Borgland 2001; Alvarez, Arttamangkul et al. 2002; Bushell, Endoh et al. 2002). On the other hand, studies indicated morphine-induced receptor internalization can be greatly enhanced when GRKs or βArrs are over-expressed (Whistler and von Zastrow 1998; Zhang, Ferguson et al. 1998).
After internalization, opioid receptors face two destinations. They can be dephosphorylated and recycled back to cell surface. After dephosphorylation, it is believed that receptors have regained their signal transduction ability. This process is called resensitization (Zhang, Barak et al. 1997; Zhang, Ferguson et al. 1998). They also can be sorted into lysosomes for degradation (Whistler, Enquist et al. 2002); in this process, receptors lose their functions permanently. Depending on the interaction between GPCRs and βArrs, two types of GPCRs (A and B) has been classified (Oakley, Laporte et al. 2000). After receptor internalization, class A GPCRs can dissociate themselves from βArrs, and then recycle to plasma membrane. However, the strong interaction between class B and βArrs prevent further dissociation, receptor dephosphorylation and recycling. It was reported that after internalization, DOR prefers to traffic into lysosomes and degrade finally (Tsao and von Zastrow 2000). In contrast, MOR prefers to recycle and resensitize (Koch, Schulz et al. 1998; Wolf, Koch et al. 1999; Law, Erickson et al. 2000). Therefore, the C-terminals of MOR and DOR, which is the major site bind to βArrs, play a significant role in opioid receptor sorting after internalization.

2.3 GRKs and βArrs independent desensitization

2.3.1 Second messenger-dependent kinases
During agonists binding to opioid receptors, not only GRKs are recruited and phosphorylate receptors, but some other second messenger-dependent kinases are also activated and participate in opioid receptor desensitization. Moreover, second messenger-dependent kinases involve in downstream effectors regulation (Sheng, Thompson et al. 1991; Lane-Ladd, Pineda et al. 1997; Trujillo 2000; Cen, Xiong et al. 2001); thus produce profound cellular adaptation change and might relate to opioids functions \textit{in vivo} (Nestler 2001; Wang and Wang 2006).

**PKA** cAMP-dependent protein kinase (PKA) is one of the first protein kinases to be discovered (Walsh, Perkins et al. 1968); and is also the most studied and well-understood protein kinases (Shoji, Parmelee et al. 1981; Uhler, Carmichael et al. 1986). Inhibition of cAMP synthesis is a major and landmark signaling pathway of Gi/Go-coupled opioid receptor. After chronic morphine treatment, MOR phosphorylation in mouse brain was found temporarily decreased, however moderately (Bernstein and Welch 1998). This observation suggested it may result from the MOR-mediated inhibition of adenylyl cyclases activity, and MOR has basal PKA phosphorylation level. \textit{In vitro} experiments confirmed MOR can be PKA substrate when MOR contained Neuro2A cell membrane was incubated together with PKA catalytic subunit and MOR agonists (Chakrabarti, Law et al. 1998). Interestingly, the ability of PKA phosphorylates MOR is agonist-dependent; MOR can be phosphorylated when morphine but not DAMGO is present (Chakrabarti, Law et al. 1998). Nevertheless, PKA activity showed no effect on opioid receptor desensitization when it was inhibited by its inhibitor (Chakrabarti, Law et al. 1998) or activated by forskolin (Wang and Sadee 2000).
**PKC** Protein kinase C family is a large Ser/Thr kinases family and participates in numerous signaling regulations, such as receptor phosphorylation and desensitization, membrane structure modulation, transcription regulation, and neuron-transmitter release. By these, it affects various cellular events from cell proliferation and synaptic remodeling to learning and memory (Mellor and Parker 1998).

The general structure of the PKCs is composed of a regulatory domain and a highly conserved catalytic domain. Based on their different regulatory domain structure, PKCs are divided into three subfamilies. 1) Conventional PKC (cPKC) group. This group includes PKC\(\alpha\), \(\beta_\text{I}\), \(\beta_\text{II}\), and \(\gamma\). PKCs in this group are sensitive to cellular Ca\(^{2+}\) and defined as classical PKCs. 2) Novel PKC (nPKC). This group includes PKC\(\delta\), \(\varepsilon\), \(\eta\), \(\theta\) and \(\mu\). nPKC activation does not require cellular Ca\(^{2+}\) but binding with diacylglycerol (DAG). 3) Atypical PKC (aPKC). This group of PKCs includes \(\xi\) and \(\lambda/\iota\) (Parker and Murray-Rust 2004). Adjacent to their regulatory domain, PKCs have a pseudosubstrate or autoinhibitory region (Fig. 1.5). The PKCs is allosterically regulated by its pseudosubstrate. Inactive PKCs are kept in a “folded conformation” as interacting pseudosubstrate part to its catalytic domain, and the binding of PKCs activators releases the pseudosubstrate domain from its “kinase core”, thus exposure the catalytic domain of the enzyme (Newton 2001; Amadio, Battaini et al. 2006). PKCs are cytosolic proteins; and after activation, they are translocated to the plasma membrane and phosphorylate the target proteins; this character is used as the indicator of PKC activation (Nishizuka 1995).
The N-terminal moiety contains the regulatory modules: the pseudosubstrate (green); the C1 A and C1B domains which bind phosphatidylinerine and, for all but atypical protein kinase C's, diacylglycerol/phorbol esters (orange); the C2 domain which binds anionic lipids and, for conventional protein kinase C's, Ca$^{2+}$ (yellow); and the PH domain which binds phosphoinositides (purple). The C-terminal moiety contains the kinase domain (cyan). The requirements for the classical cofactors for protein kinase C subclasses are shown on the right: PS, phosphatidylinerine; DG, diacylglycerol; Ca$^{2+}$.

(Newton 2001)
As discussed above, opioid receptors could directly induce or indirectly potentiate intracellular Ca\(^{2+}\) release; therefore, it is reasonable to predict activation of PKC after opioid receptors bind with agonist. Indeed, increasing PKC activity or PKC-dependent opioid receptor signalings has been reported repeatedly (Kramer and Simon 1999; Belcheva, Clark et al. 2005; Zheng, Loh et al. 2008). The involvement of PKC activity in DOR desensitization has been reported by using PKC inhibitors staurosporine and bisindolylmaleimide (GF109203X) (Yoon, Jin et al. 1998; Song and Chueh 1999). This may be because of the directly inducement of [Ca\(^{2+}\)]\(_i\) by DOR itself (Jin, Lee et al. 1992; Smart and Lambert 1996; Yoon, Lo et al. 1999). Ionotropic glutamate receptor NMDA receptor promotes Ca\(^{2+}\) influx, and subsequently activates PKC (Barrios and Liljequist 1996; Zanelli, Numagami et al. 1999; Chiao and Wong 2004). In NG 108-15 hybrid cells and neuroblastoma SK-N-SH cells, activation of NMDA receptor or kainate/AMPA receptors reduces activation of G proteins by DPDPE; whereas PKC inhibitors reverse the effect of glutamate receptor activation (Cai, Ma et al. 1997; Varga, Stropova et al. 1999). However, this decrease in DPDPE-mediated response does not result from DOR phosphorylation, because increase of DOR phosphorylation after NMDA agonist treatment was not observed in this study (Cai, Ma et al. 1997).

MOR was predicted to be putative PKC phosphorylation substrate (Zhang, Yu et al. 1996; El Kouhen, Kouhen et al. 1999; Guang, Wang et al. 2004; Johnson, Oldfield et al. 2006). Increasing PKC activity by phorbol esters enhanced MOR phosphorylation
level (Zhang, Yu et al. 1996; El Kouhen, Kouhen et al. 1999; Guang, Wang et al. 2004). However, whether PMA activation-induced MOR phosphorylation desensitize receptors, and whether agonist-induced MOR phosphorylation is mediated by PKC need to be further investigated (Wang, Arden et al. 1996; Zhang, Yu et al. 1996; Bailey, Kelly et al. 2004; Gabra, Bailey et al. 2007).

Recently, by monitoring K+ channel (GIRK), it has been shown that PMA-activated PKC facilitates morphine-induced MOR desensitization (Bailey, Kelly et al. 2004). Moreover, in brain slices, morphine-induced desensitization is potentiated by stimulation of M3 muscarinic receptors, which is able to directly induce [Ca2+]i release (Hirst and Lambert 1995). Interestingly, this potentiation was only observed when morphine but not DAMGO was used, indicating the agonist-dependent receptor conformation change as the same as is observed in PKA-induced receptor phosphorylation. Moreover, the catalytic domains of PKA and PKC are highly homologous (Xu, Chaudhary et al. 2004) (Fig. 1.6).

However, whether PKC directly phosphorylates opioid receptors is still unclear. Several other protein kinases have been suggested to phosphorylate opioid receptors, including GRKs; and PKC-mediated phosphorylation of GRKs positively regulates its activity (Moyers, Bouton et al. 1993; Kohout, Lin et al. 2001; Krasel, Dammeier et al. 2001). In the human neuroblastoma BE (2)-a C cell, PKC-induced MOR desensitization results from increased GRK2 activity mediated by PKC phosphorylation (Mandyam, Thakker et al. 2002). At the same time, other effectors within opioid receptor signal
Fig. 1.6 Overall structure of the PKCθ-staurosporine complex and comparison with the structure of the PKA-staurosporine-PKI complex.

A, ribbon representation of the PKCθ kinase domain structure. The N-lobe is cyan and the C-lobe is blue. The glycine-rich loop, activation loop, and HM segment are highlighted in red. Staurosporine and phosphorylated residues are shown in stick representation. B, superposition of PKCθ-staurosporine (color coding is the same as described for A) and PKA-staurosporine-PKI structures (yellow ribbons and sticks). Phosphorylated sites in PKA are also shown. Structures were aligned using the central helices from the C-lobe. Both staurosporine-bound kinases display intermediate lobe structures with conformational differences in the glycine-rich loop. (Xu, Chaudhary et al. 2004)
pathways can be phosphorylated by PKC, thus blocking their activity (Murthy, Grider et al. 2000). Nevertheless, particular attention is needed when chemical kinase inhibitors are being used in studies: the lack of effect of one compound does not systematically exclude all the kinase family. For example, Ca\(^{2+}\)-dependent classic PKC inhibitor Go6976 cannot inhibit the heterologous desensitization of chemokine receptor by opioid receptor since only Ca\(^{2+}\)-independent PKC subtypes are involved in this process (Zhang, Hodge et al. 2003).

**ERK1/2, Src** ERK1/2 (extracellular-receptor kinase) belongs to mitogen-activated protein kinase family (MAPK). As discussed before, ERK1/2 is stimulated after MOR activation. By using ERK1/2 inhibitors PD98059, MOR phosphorylation level was demonstrated to be affected by ERK1/2 activity (Schmidt, Schulz et al. 2000). Moreover, DAMGO-induced MOR desensitization was attenuated by ERK1/2 inhibitor-reduced MOR phosphorylation (Polakiewicz, Schieferl et al. 1998; Schmidt, Schulz et al. 2000). However, when purified ERK1/2 was added together with MOR, MOR failed to incorporate \(^{32}\)P, indicating that MOR is not ERK1/2 direct substrate (Schmidt, Schulz et al. 2000). Instead, ERK1/2 may affect MOR phosphorylation through regulating the other kinases like GRKs (Elorza, Sarnago et al. 2000; Thakker and Standifer 2002).

Src kinase family is a non-receptor tyrosine kinase family, which includes seven subtypes (Parsons and Parsons 2004). Among these seven subtypes, c-Src was reported to be activated by all types of opioid receptor (Kramer, Andria et al. 2000; Kam, Chan
et al. 2004; Zhang, Zhao et al. 2009). At the same time, Tyr\textsuperscript{336} within MOR C-terminal was demonstrated to be the c-Src phosphorylation site; and mutating this site together with Tyr\textsuperscript{166} to Ala block prolonged morphine treatment-induced adenylyl cyclase super-activation (Zhang, Zhao et al. 2009). However, whether MOR desensitization can be affect by c-Src-affected MOR phosphorylation need to be further demonstrated.

2.3.2 Regulator of G protein signaling (RGS) proteins

Regulator of G protein signaling (RGS) proteins have been recently discovered to regulate the activity of GPCRs. RGS family is composed of 20 different proteins which all contain a conserved RGS domain that is responsible for its enzyme activity; based on their structures, 20 RGS proteins are subjected into four subfamilies (R4/B, RZ/A, R7/C, and R12/D) (Garzon, Rodriguez-Munoz et al. 2005; Xie and Palmer 2005). By interacting with G\textalpha subunits of Gi Go Gz or Gq, RGS proteins promote the intrinsic GTPase activity of G\textalpha to facilitate the free and activated G\textalpha to hydrolysis bound GTP to GDP. Once GTP-bound G\textalpha switches to GDP-bound G\textalpha, it loses its activity and interacts with G\textbetag\textgamma subunits again (Elenko, Fischer et al. 2003). By this mechanism, RGS proteins can halt the receptor signaling and attenuated drug response \textit{in vivo}.

Several members of RGS-RZ/A, RGS-R4/B and RGS-R7/C have been shown to regulate opioid receptor activities. RGS19 is one of the earliest discovered RGS proteins and belongs to RZ/A subfamily. It enhanced the dissociation of activated G\textalpha\textsubscript{i3} from DOR (Elenko, Fischer et al. 2003) and inhibited MOR response after agonist

So far, regulation of opioid receptor activity by RGS protein is mainly studied in animal models. Therefore, opioid receptor acute tolerance, which is defined as tolerance develops after 24 h one i.c.v. administration of 10 nmol morphine, is proposed as the model of opioid receptor desensitization in vivo (Garzon, Rodriguez-Munoz et al. 2005). Within all RGS proteins studied, RGS-R7 family is responsible for the MOR desensitization (Bahia, Wise et al. 1998; Garzon, Rodriguez-Diaz et al. 2001; Garzon, Lopez-Fando et al. 2003; Sanchez-Blazquez, Rodriguez-Diaz et al. 2003; Garzon, Rodriguez-Munoz et al. 2005; Sanchez-Blazquez, Rodriguez-Munoz et al. 2005). On the other hand, RGS-Rz family differently regulates opioid receptor by protecting the MOR response to desensitize (Garzon, Rodriguez-Munoz et al. 2004; Sanchez-
Blazquez, Rodriguez-Munoz et al. (2005), and the molecular mechanisms involved are still under-investigation.

3 Hypothesis in opioid tolerance development

After long term or repeated usage of opioid analgesic agents, such as morphine, higher doses of opioids were required to achieve the same analgesia effect. This is called tolerance. Among the opioids, morphine produces higher tolerance than the other opioid agents which have equal or higher efficacy (Duttaroy and Yoburn 1995). In addition to tolerance, opioids also produce severe side effects, such as cardio-respiratory depression, seizure, and sedation (Rushton and Sneyd 1997). Increased opioids dose to achieve its analgesic effect after tolerance development eventually augments its lethal side effects, thus seriously limiting its clinical usefulness. Therefore, numerous efforts have been put in elucidating the mechanism of morphine tolerance development both in vivo and in vitro.

Several hypotheses have been proposed to explain the opioid tolerance development in vitro. The first hypothesis suggested the down regulation or alteration of functional MOR density in neuron system resulted in decrease of reaction to opioids (Rothman, Danks et al. 1986; Werling, McMahon et al. 1989; Bhargava and Gulati 1990). However, the discovery of large number of spare receptors (Chavkin and Goldstein 1984; Clark, Nordby et al. 1989; Childers 1991; Connor, Osborne et al. 2004) and no significant change of MOR density after morphine chronic treatment in CNS
(Klee and Streaty 1974; Hollt, Dum et al. 1975; Brady, Herkenham et al. 1989) give an equivocal answer to this hypothesis. Cellular adaptation responses after opioid chronic treatment was considered as another hypothesis in explaining opioid tolerance development (Matsuoka, Maldonado et al. 1994; Lane-Ladd, Pineda et al. 1997; Wolf, Numan et al. 1999; Christie 2008). Such adaption responses may resulted in alterations in neurotransmitter activities and the other receptors signaling (Trujillo and Akil 1991; Trujillo and Akil 1991; Scearce-Levie, Lieberman et al. 2005); thus eventually lead to profound decrease in opioid response after chronic treatment.

Recently, the desensitization and internalization are proposed to be involved in tolerance development (Narita, Mizuo et al. 2002; Johnson, Christie et al. 2005; Martini and Whistler 2007). Morphine induces a higher degree of tolerance than other opioids such as fentanyl or etorphine administered at equieffective analgesic doses (Rezvani, Huidobro-Toro et al. 1983; Duttaroy and Yoburn 1995; Mercadante, Casuccio et al. 1998). Therefore, the morphine tolerance is always the focus in opioids research. Using βArr2 knockout mice, morphine analgesia response was enhanced (Bohn, Lefkowitz et al. 1999) and tolerance development was attenuated in hot-plate tests (Bohn, Gainetdinov et al. 2000). These observations suggest the high involvement of βArrs in morphine tolerance development. Therefore, based on these experiments, the first hypothesis was proposed. In this hypothesis, morphine tolerance development was implicated to directly relate to the MOR desensitization. However, this hypothesis was challenged when tail-immersion tests were used: morphine tolerance development remained in the βArr2 knockout mice, but was attenuated (Bohn, Lefkowitz et al. 2002).
Moreover, as summarized before, compared to the other MOR agonists, morphine inefficiently induces MOR phosphorylation and recruits βArrs. This indicates desensitization in vitro might not be proportionally equal to the tolerance development in vivo.

Since the ability of morphine to induce tolerance appears to conversely correlate to its ability to induce MOR desensitization, Whistler and von Zastrow proposed another hypothesis: the ability of various opioid agonists to produce tolerance is dependent on their “RAVE” values (Whistler, Chuang et al. 1999). “RAVE” stands for relative activity versus endocytosis. Since morphine cannot efficiently promote receptor internalization, it is thought morphine-activated receptors will continue to signal and induce greater adaptations in cells that oppose the effects of MOR activation. This hypothesis is partially supported by using a MOR mutations that facilitate internalization which reduced cellular tolerance (Finn and Whistler 2001). However, with the discovery of morphine-induced MOR rapid desensitization, especially in the neuron system (Borgland, Connor et al. 2003; Schulz, Mayer et al. 2004; Dang and Williams 2005; Walwyn, Wei et al. 2006), this theory needs to be modified. The second hypothesis noticed that after MOR internalization, it could be de-phosphorylized, and recycled back to the cell membrane to function again. This re-sensitization process required MOR internalization; therefore, agonists that induce receptor desensitization but not internalization will eventually leads to tolerance after long term treatment (Koch, Schulz et al. 1998; Koch, Schulz et al. 2001). This theory was supported by co-administration of morphine with a very low dose DAMGO that normally would not
promote receptor internalization but could facilitate morphine-induced MOR internalization (He, Fong et al. 2002). Although the results are still in debate (Bailey, Couch et al. 2003), the close link between desensitization and tolerance is obvious.

In addition to the MOR desensitization, DOR signaling is also suggested to be involved in MOR tolerance development. Using DOR selective antagonist or DOR specific antisense, different groups reported that morphine-induced tolerance and dependence are attenuated (Abdelhamid, Sultana et al. 1991; Fundytus, Schiller et al. 1995; Hepburn, Little et al. 1997). With the help of DOR knockout mice, the participation of DOR in morphine tolerance development is further confirmed. In those mice, morphine tolerance development was completely blocked (Zhu, King et al. 1999). Although the mechanisms of the DOR to modulate morphine chronic effect has not been completely resolved, it is tempting to suggest the modulation effect is due to the heterodimerization of DOR and MOR (Martin and Prather 2001).

4. Aims of this thesis

Since the importance of opioid receptor desensitization in both in vitro and in vivo opioid functions regulation, it has been studied intensively. Current paradigmatic models of GPCR desensitization are based mainly on the involvement of GRKs and βArrs. However, DOR desensitizes immediately after exposure to agonists and is apparently affected by GRKs and βArrs expression levels; meanwhile, the desensitization mechanism of MOR remains equivocal. When Gαi-mediated inhibition
of AC activity is monitored, MOR desensitization is observed only after several hours of DAMGO pretreatment, while receptor phosphorylation and βArrs recruitment occurred within minutes (Barak, Ferguson et al. 1997; El Kouhen, Kouhen et al. 1999). Moreover, contrast to the DOR, the inability of GRKs and βArr2 overexpression to affect MOR desensitization in various cell types further suggests a lack of correlation between MOR desensitization and GRK-mediated receptor phosphorylation and βArrs recruitment (Kovoor, Nappey et al. 1997; Whistler and von Zastrow 1998; Celver, Lowe et al. 2001). These divergent observations of MOR desensitization might be due to the relatively weak interaction between MOR, GRKs and βArr when compared to DOR (Lowe, Celver et al. 2002). In addition, previous studies indicated that Gαi is more efficient in transducing opioid receptor signals than Gβγ-subunits (Prather, Tsai et al. 1994). This is probably the reason why MOR desensitizes in hours when Gαi-mediated inhibition of adenylyl cyclase activity is measured, but desensitizes in minutes when Gβγ-mediated activation of inward rectifying K⁺ channel (GIRK) was monitored (Dang and Williams 2005). Therefore, it is necessary to develop a sensitive and timely method to systematically study MOR desensitization. After establishing the methods, several questions in MOR desensitization need to be addressed: 1. Are receptor phosphorylation and βArrs recruitment also important in regulating MOR desensitization? 2. Since morphine is unable to efficiently induce receptor phosphorylation and βArrs recruitment, does morphine pretreatment still result in MOR desensitization? 3. Regarding the mechanism by which morphine regulates MOR activity — Is this mechanism of morphine-induced MOR desensitization different from the other MOR agonists those can robustly induced receptor phosphorylation and βArrs
recruitment? 4. How does this difference in agonist-induced receptor desensitization mechanisms further imply agonist functions? These results will provide new insights into the MOR and GPCRs desensitization regulation mechanism. In addition, it will provide molecular evidence for studying MOR physical effects in vivo, and may help us to develop new treatments for opioid tolerance and addiction.
Chapter II

Materials and Methods
Materials

Myristoylated PKCα pseudosubstrate peptide (Myr-FARKGALRQ-OH), PKCγ antagonist (Myr-CRLVLASC-OH) and PKCε antagonist (Myr-EAVSLKPT-OH) were obtained from Biomatik Corporation (Cambridge, Canada). PKC inhibitor Ro-31-8425 was purchased from LClab (Woburn, MA). rabbit anti HA was purchased from (Covance, NJ). For the measurement of MOR phosphorylation, the anti-phospho-Ser375-MOR antibody (P375MOR) (Cell Signaling, MA) or the total Phospho-Ser/Thr antibody (Pan) (Zymed laboratories, CA) was used and was normalized to the protein concentration of the samples. For immunoprecipitation assay, mouse anti-HA (1:1,000) (Covance, NJ) or rabbit anti-Gαi2 (1:500) (Zhang, Tetrault et al. 2006) antibodies and rProtein G agarose (Invitrogen, CA) was added to the supernatant and rotated overnight. Anti-phosphor-(Ser) PKC substrate (Cell Signaling, MA), anti-phosphor-Ser (Sigma), PKCε (Cell Signaling, MA), HA and Gαi2 were used in western blotting. Storm 860 and ImageQuant (Amasham, Piscataway, NJ) were used to detect and quantify the fluorescence intensity on western blotting. Ga subunits mutation was made by using site direction myatgenesis kit (Stratagen) on pcDNA3. The mutation was first made at C352 to enable the construct to be resistant to PTX incubation. Then single or combined PKC putative phosphorylation sites were generated following the similar procedures.

Methods

Cell culture
Hemagglutinin (HA) tagged μ-opioid receptor (HA-MOR), HA-MOR in which Ser\textsuperscript{363}, Thr\textsuperscript{370} or Ser\textsuperscript{375} residues was individually mutated to Ala (HA-MORS363A, HA-MORT370A, HA-MORS375A) and HA-MOR with Ser\textsuperscript{363}, Thr\textsuperscript{370} and Ser\textsuperscript{375} residues mutated to Ala (HA-MOR363/370/375) were stably expressed in HEK293 cells as described in our lab previous studies (El Kouhen, Burd et al. 2001). Cells were grown in Eagle’s minimal essential medium (MEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 200 ng/ml G418 sulfate in 5% CO\textsubscript{2} 37°C incubator. Receptor expression level was determined by binding assays. Wild type, β-arrestin2 null (βArr2\textsuperscript{-/-}) and β-arrestin1 and 2 null (βArr1/2\textsuperscript{-/-}) Mouse Embryonic Fibroblasts (MEF) cells (generous gifts from Dr. R. Lefkowitz, Duke University, NC) were cultured in Dulbecco’s modified Eagle’s minimal essential medium (DMEM) with Earle’s salt supplemented with 10% fetal bovine serum. Normally, cells were cultured for 24 hr before agonist treatment.

\textbf{Plasmid construction}

The Gai2C352L, Gai3C351L and GoC352L which are PTX-insensitive mutant were constructed as reported (Zhang, Tetrault et al. 2006). Ga subunits cloned in the pcDNA3 vector were mutated with the site direction mutagenesis kit (Stratagene, CA). The Cys\textsuperscript{352} or Cys\textsuperscript{351} was mutated to Leu in three types of Ga respectively to render the Ga resistant to ADP-ribosylation by PTX. Then single or combined PKC putative phosphorylation sites were generated following the similar procedures. Mutations were confirmed by sequencing. The primer sequence design was following the site-directed mutagenesis guide line which was provided by Stratagene (La Jolla, CA).
Site-Direct Mutagenesis The primers used for mutagenesis were designed accordingly. The sense primer was generated by flanking 20 nucleotides on each side of the site for mutation. The site of mutation is six nucleotides maximally, and the originated coding sequences have been replaced by targeting coding sequences. The antisense primer was generated by complementarily reversing the sense primer. The mutation takes advantage of the PCR techniques. 10ng plasmid containing the coding sequence of un-mutated target gene, 5pmol of the sense and antisense primer, 0.1 mM dNTP and 10 unit Pfu was prepared in 50μl 1 X Pfu reaction buffer. Another three 50μl reaction mix was prepared similarly but with 10 pmol, 15 pmol or 20 pmol primer. The PCR reaction started from 5 min 95 degree which was followed with 18 cycles of 90 sec 94°C, 90 sec 60 °C and 10 min 68 °C. The reaction finished with another 10 min 68 °C. After collecting all the reaction mix in the same tube, and 10 unit DpnI was added, it was incubated in 37 °C for 90 min. After digestion, one volume isopropanol was added and the solution was kept on ice for 10 min. After centrifugation at 13,000rpm 4 °C for 10 min, the pellet was washed with 70% ethanol and air dried. 10ul TE was used to re-dissolve the pellet and the resulting solution was used for transformation. The transformed bacteria was cultured in LB medium individually; the plasmids were purified by using Miniprep kit from Qiagen CA. The successful mutation was identified by sequencing or by enzyme digestion if applicable.

Transfection and infection
Effectene (QIAGEN, Valencia, CA) was used to transfect plasmids into HEK293-MOR cells according to the manufacturer’s protocol. After transfection, cells were incubated at 37°C for 48 h before assays were performed.

Transfection with Nucleofector® Because of the possible stronger coupling of endogenous Gαi to [Ca²⁺], signaling pathway (Chieng, Lee et al. 2008), Nucleofector® was used to transfect PTX-resistant Gαi into HEK293-MOR cells to achieve higher protein expression level. HEK-MOR cells were cultured for 2 days to reach their logarithmic growth phase before transfection. Then cells were harvested and centrifuged at 90 x g at room temperature for 10 min. After completely removing the supernatant, 1 x 10⁶ cells were mixed with 100µl supplemented Nucleofector® solution and 5µg plasmid. Plasmid DNA was transfected into cells by Nucleofector® program and the cells were immediately seeded into 96-well-plate. Transfected cells were incubated in a humidified atmosphere of 5% CO₂ at 37°C for 24hrs prior to [Ca²⁺], assays.

Infection with adenovirus Adenovirus containing MOR (Ad-MOR) was used to express MOR protein in MEF (mouse embryonic fibroblast) cells. The titer of the adenovirus was determined to be ~2.5x10⁹ infectious units (IU)/ml. MEF cells were grown in DMEM with 10%FBS at 6-well-plate for 1 days to be about 50% confluent. Then the media was removed; and Ad-MOR virus was diluted in DMEM with 2% FBS and added to the wells. Multiplicity of infection (MOI) was determined by making virus dose and MOR expression level curve. Desired MOI was used to reach the approximated same receptor expression level. After adding adenovirus and DMEM with 2% FBS for an hour and a half, DMEM with 10% FBS was added back to the MEF
cells, and the cells were incubated at 37°C for 48 hours before the assays were carried out.

**Intracellular Ca²⁺ measurement**

One day before assay, CORNING® black with clear flat bottom 96-well-assay plate (Fisher scientific, IL) was coated with poly-L-Lysine (Fisher scientific, IL). The cells were suspended in their grown media at a density of ~3×10⁴ cells/well and seeded into 96-well plate with 150µl media/well. Then the cells were incubated at 37°C in a humidified atmosphere of 5% or 10% CO₂ at 37°C overnight so as to reach an 80~90% confluent cell monolayer before assay. At the day of assay, 100µl medium/well was removed from plate. To each well, 50µl FLIPR® calcium assay reagent (Molecular Devices Corp) dissolved in 1x reagent buffer (1×HANKs buffer with 20mM HEPES), pH 7.4, with 5mM probenecid was added and the plate was incubated at 37°C for 1 hour. Agonists, inhibitors and other reagents were dissolved in the assay buffer (HBSS: KCl 5mM, KH₂PO₄ 0.3mM, NaCl 138mM, NaHCO₃ 4mM, Na₂HPO₄ 0.3mM, D-glucose 5.6mM, with additional 20mM HEPES, 2.5mM probenecid and 13mM CaCl₂). Using a FLEXstation (Molecular Devices Corp.), the [Ca²⁺]ᵢ fluorescence increases after robotic injections of agonists, inhibitors or other reagents were monitored every 1.52 sec intervals with excitation wavelength at 485 nm and with emission wavelength at 525nm. The [Ca²⁺]ᵢ release normally reached its maximum 15 sec after agonist injection and returned to baseline within 30 sec after injection. The [Ca²⁺]ᵢ fluorescence was measured up to 90 sec after agonist injection. The fluorescence intensity from 3 to 4
wells of cells were averaged and the relative amount of $[\text{Ca}^{2+}]_i$, release was determined by integrating the area under the peak of the $[\text{Ca}^{2+}]_i$, fluorescence averages.

**Immunoprecipitation (IP), co-immunoprecipitation (co-IP), SDS-PAGE and western blotting**

*Immunoprecipitation and co-immunoprecipitation* Cells were grown in 100mm dishes two days before assay to achieve the best condition. At the day of assay, cells in dishes were placed on ice after being treated with drugs as desired. The cells were washed with 4ml ice-cold PBS (137M NaCl, 2.7mM KCl, 10mM NaH$_2$PO$_4$, 2mM KH$_2$PO$_4$, pH7.4) twice before being extracted with proper amount of cell lysis buffer (1% Brij-98 for IP or 1% Triton-100 for co-IP, 50 mM Tris-HCl pH 7.5, 150mM NaCl, 0.25% sodium deoxycholate, 0.1%, 50mM NaF, 1mM dithiothreitol, 0.5mM phenylmethylsulfonyl fluoride, 50mM sodium pyrophosphate, 10mM sodium vanadate and 1×protease inhibitor cocktail from Roche, Indianapolis, IN). After centrifugation at 12,000xg for 5min, the supernatant of the extract was added with SDS-PAGE denaturing buffer (50mM Tris, pH 6.8, 2%SDS, 0.1% bromophenol blue, 10% glycerol), and heated at 65°C for 5min for further analysis with SDS-PAGE and western blotting analysis. For IP or co-IP assay, supernatant was immunoprecipitated with proper first antibodies and rProtein G agarose beads (Invitrogen, CA) at 4°C overnight. Then the beads were washed six times with cell lysis buffer and were extracted with SDS-PAGE denaturing buffer. Then approximately equal amounts of protein were resolved by SDS-PAGE for further analysis.
**SDS-PAGE and western blotting** To further characterize and analyze the protein, protein samples with SDS-PAGE denaturing buffer were fractionated by SDS-polyacrylamide gel electrophoresis. Resolved proteins were transferred to immobilon™ PVDF transfer membrane (Millipore, MA) and blocked with 5% nonfat milk in 0.1% Tween-10 in TBS (TTBS: ). Proteins were incubated with corresponded antibodies in TTBS with 5% nonfat milk, and were further detected.

**SiRNA plasmid construction**

siLentGene™-2 U6 hairpin cloning systems (Human) (Promega, WI) were used to introduce small interfering RNA (siRNA) into cells. Three 21bp siRNA hairpin target sequences including one scramble sequence were designed by a Promega SiRNA designer according to the current guidelines for effective knockdown by this method. The sequences of scrambled control and two βArr2-targeting siRNAs were as follows: 5’-GGGTTAGAGATTCCATCGATC-3’; 5’-GACAAAGTGGACCCTGTAGAT -3’; 5’-AAGGACCGCAAAGUGUUUGUG -3’.

Downstream primer sequence contained target sequence reverse complement-loop sequence-target sequence to create a hairpin structures. It also contained U6 cassette matching and terminator sequence with EcoR V site. After being amplified by PCR, the DNA productions were ligated into psiLentGene™ vectors, amplifying and screening by *E. coli*. The recombination DNA plasmids were transfected into cells to generate siRNA inside the cells.

**PKC subtypes activity assay**
Cells were grown in 100mm dishes two days before assay; and were incubated in FBS free media 16hrs before assay. At the day of assay, cells were treated with drugs, and then the reaction was ceased by washing with ice-cold PBS twice. Then the cells were lysed with cell lysis buffer (1% TritonX-100, 50 mM Tris-HCl pH 7.5, 150mM NaCl, 0.25% sodium deoxycholate, 0.1%, 50mM NaF, 1mM dithiothreitol, 0.5mM phenylmethylsulfonyl fluoride, 50mM sodium pyrophosphate, 10mM sodium vanadate and 1×protease inhibitor cocktail from Roche, Indianapolis, IN) at room temperature for 30 min. Then the total cell lysis was centrifuged and the supernatant was collected while the pellet was discarded. To determine the PKC subtypes activity individually, PKCe, PKRα or PKCγ specific antibody (Cell Signaling, MA) was added into the supernatant and mixed in 4°C for 3hrs. After that, G-protein agarose beads (Invitrogen, CA) 25µl were added into supernatant and antibody mixture; and the mixture was continued to rotate in 4°C for overnight. The next day, beads were washed with PBS for 3 times, then the reaction buffer (Cell signaling, MA) with biotinylated PKA substrate peptides (contains the residues surrounding serine 133 of CREB, peptide core sequence: RRPS*YRK) (Cell signaling, MA) was added together into beads to react with PKC subtypes, which were trapped with G-protein agarose. The reaction continued for 15min in 37°C with rotation, and was ceased by adding 120µl (50mM) EDTA. After rotating in 37°C for 5 min, the mixture was centrifuged at 13,000rpm for 2min at 4°C. Then the supernatant which contained the reacted substrates was collected and 30µl streptavidin beads were added. After rotating under 4°C for 30min, antibody from rabbit specific recognizing phosphor-PKA substrate was added into streptavidin beads (Cell Signaling, MA). Then the streptavidin beads were washed with TTBS (10 mM Tris-HCl, pH 7.5,
150 mM NaCl, 0.05% Tween-20) for three times after rotating at 4°C overnight. In the next, anti-rabbit-488 in 200µl TTBS was added into streptavidin beads as secondary antibody and the beads continued to be mixed and rotated for 2 hrs at room temperature but without light. In the end, beads which trapped phosphor-substrates and antibodies were washed with TTBS for the last three times and 500µl TTBS was added into the beads at the final steps. The beads were mixed and added into 96-well-plate for 100 µl in each well. The PKC subtypes activity level was determined by measuring the antibody fluorescence intensity by α-Fusion plate reader (PerkinElmer Life and Analytical Sciences, Boston, MA).

**Lipid Raft Extraction and gradient fraction preparation**

*Continuous sucrose gradient assay* Currently, sucrose gradient is commonly used to separate lipid rafts in which MOR signaling complex located from other membrane domains (Zheng, Chu et al. 2008). Cells were grown in 150mm dishes two days before assay. At the day of assay, cells were collected with 500mM sodium carbonate 700µl (pH11) after agonist pretreatment. The cells in Na\textsubscript{2}CO\textsubscript{3} were homogenized by passing through 22 gauge, 3-inch needles for 10 times following by sonication using Sonicator Cell Disruptor model W-220F (Heat Systems-Ultrasonic, Inc., Plainview, New York) at output level 4 for 30 sec. The prepared homogenates were mixed with equal volumes of 80% sucrose [in morpholinoethanesulfonic acid (MES)-buffered saline (MBS), pH 6.8] and placed at the bottom of ultracentrifugation tubes. On top of it, 5% to 30% continous sucrose gradients, which were formed by the Gradient Station (BioComp), were placed. Then the gradients were centrifuged at
32,000 rpm for 16 hrs in a SW41 rotor at 4°C, and total 12 fractions were collected with 1ml volume for each sample.

Neutral pH gradient by Optiprep Although isolating lipid raft in pH11 Na2CO3 yielded reliable results, when receptor binding assays were carried in fractions, high PH value significantly affected ligand receptor binding (Bacopoulos 1982). Therefore, a new fraction method using Optiprep (Sigma-Aldrich, Greiner) under PH 7.5 was utilized in my study (Macdonald and Pike 2005).

Cells were grown in 150mm dishes two days before assay. At the day of assay, cells in dishes were placed on ice after treated with drugs as desired. The cells were washed with 6ml ice-cold raft buffer (20mM Tris, 250 mM sucrose, 1mM CaCl2, 1mM MgCl2, PH 7.5) twice, then followed with scraping cells from the plates with 1ml raft buffer with protease inhibitor (0.25% sodium deoxycholate, 0.1%, 50mM NaF, 1mM dithiothreitol, 0.5mM phenylmethylsulfonyl fluoride, 50mM sodium pyrophosphate, 10mM sodium vanadate and 1×protease inhibitor cocktail from Roche, Indianapolis, IN). The cells were then collected by centrifuging at 250 x g (4°C) for 2min, and the supernatant was removed. Then the cell pellets were resuspended with 800µl raft buffer with protease inhibitor again. The cells were homogenized by passing the 22 gauge, 3-inch needle for 15 times first; then were further homogenized by sonication using Sonicator Cell Disruptor model W-220F (Heat Systems-Ultrasonic, Inc., Plainview, New York) at output level 4 for 30 sec. Then the homogeneities were centrifuged at 1000 x g in 4°C for 10 min, and the supernatant was the crude lipid extract. In the next, the same volume of cold 50% Optiprep in base buffer was added to the crude lipid raft extract to make 1.5ml mixture with optiprep final concentration as 25% and put into
ultracentrifuge tube (Beckman, CA). On the 25% Optiprep and lipid raft extract layer, 1ml 20% Optiprep in base buffer was carefully placed and then 1ml 15%, 1ml 10% and 1ml 5% Optiprep were also placed layer by layer; The 0.5ml base buffer without Optiprep was finally place on top of them. The tubes were balanced and put into SW41 rotor bucket to centrifuge at 21,000rpm at 4°C for 90 min (Beckman Coulter, Optima™ L-80XP ultracentrifuge, CA). After centrifugation, 1ml fractions crossing the density interfaces were collected from the top to the bottom. Fraction 1 and 2 are supposed to be the raft fractions, fraction 3 and 4 are supposed to be non-raft fractions.

**Lowry protein assay**

Lowry protein assay was used to determine the protein amount. The proper amount of cell suspension was centrifuged at 12,000 x g for 5min, and the supernatant was discarded. The pellet was resuspended with 1ml 1:1:1 solution (final concentration, 0.33N NaOH, 0.33%SDS) and then was incubated at 60°C for 30min. 100µl of the protein solution was added into 2ml Lowry C buffer (0.01% cupric sulfate, 0.02% sodium potassium tartarate, 2% sodium carbonate, and 0.1N NaOH) with 100µl H₂O. Meanwhile, standard concentration protein control solution in H₂O was added into 2ml Lowry C buffer with 100µl 1:1:1 solution. They were mixed well and incubated at room temperature for 10 min. Then 200µl 50% Folins phenol reagent (Sigma) was added and mixed. After 30min room temperature incubation, the protein concentration was determined by absorbance value at 540nm wavelength against an H₂O blank, and subsequently comparing this absorbance value to the standard protein samples.
Radioligand receptor binding assay

Expression of receptors in both stable and transient expressed cells was determined by using radio-labeled receptor ligands in binding assay. Cells were grown to confluent in 100mm plates and were harvested by PBS-EDTA (0.1M NaCl, 0.01M NaH₂PO₄, 0.04% EDTA, pH7.4). Then cells were washed with PBS for twice and re-suspended in 25mM HEPES buffer (pH7.6). Total binding was determined by co-incubating intact cells with 50,000cpm/1ml [³H]-labeled agonists and 5mM MgCl₂ at 24°C for 90 min. Non-specific binding was determined by co-incubating cells with 10µM antagonists in addition to [³H]-labeled agonists. Then the cells with radio-ligand binding were collected on Whatman GF/B filters. The protein concentrations were determined by the Lowry protein assay. Then the specific binding which represented receptors was determined by calculating the difference between total and non-specific binding together with protein concentrations.

Statistical analyses

At least three independent experiments were conducted to obtain statistical results, presented as means ± standard deviations. Data were analyzed by unpaired Student’s t-tests to determine significance.
Chapter III

Determination of agonist-induced MOR desensitization by measuring MOR-mediated intracellular Ca$^{2+}$ ([Ca$^{2+}$]$_i$) release

MOR agonist pretreatment rapidly desensitizes MOR activity on mediating intracellular Ca$^{2+}$ release.
As discussed in the introduction, although the involvement of receptor phosphorylation and βArrs in GPCRs desensitization has been considered as a dogma, the studies of how they affecting MOR desensitization are still obscure. Those controversial data resulted from different methods which were used measuring receptor desensitization (Connor, Osborne et al. 2004) as Gα-subunit is more efficient in transducing opioid receptor signals than Gβγ-subunits (Prather, Tsai et al. 1994). Therefore, Gα-subunit-mediated signalings are less sensitive to MOR activity change although they generally possess higher efficacy compared to Gβγ-subunits-mediated signalings (Dang and Williams 2005). In addition, since receptor phosphorylation and βArrs recruitment occur in minutes, it is necessary to have a timely method. Therefore, MOR Gβγ subunits-mediated signalings such as activation of GIRK channel, or increasing [Ca^{2+}]_i release are the better choice for MOR activity monitoring. In addition, since MOR agonists are not able to induce [Ca^{2+}]_i release without Gq-coupled receptor co-activation as mentioned in the introduction, MOR agonist pretreatment would not affect the downstream signaling components or depletion [Ca^{2+}]_i store. This character makes the method better than simply recording the decreasing of MOR-activated outward potassium current.

**Part I: With presence of P2Y co-activation, MOR agonist is able to induce [Ca^{2+}]_i release**

**Results**
To detect \([\text{Ca}^{2+}]_i\) release, a FLIPR® calcium assay kit was used. Unlike the Fluo-3 and Fluo-4, the FLIPR® calcium assay reagent is a no-wash fluorescent calcium indicator with minimal background fluorescence signal. Thus this assay provides a sensitive method to monitor \([\text{Ca}^{2+}]_i\) release in live cells with limited perturbation and stress. The dye was incubated with cells in 96-well-plate for 1hr before the assay. Then the baseline was determined by recording the \(\text{Ca}^{2+}\) fluorescence for 30 sec at 525nm before adding the agonists. As discussed in the introductory chapter,, challenging HEK293-MOR cells with MOR agonists, such as morphine or DAMGO, did not induce \(\text{Ca}^{2+}\) release (Fig. 3.1 A). In contrast, a Gq-coupled P2Y receptor agonist ADP evoked a robust cytosolic \(\text{Ca}^{2+}\) fluorescence increase (Fig. 3.1 B). HEK293-MOR cells were washed with \(\text{Ca}^{2+}\) free buffer containing 1 mM EGTA to remove extracellular \(\text{Ca}^{2+}\), thus prevented the \(\text{Ca}^{2+}\) influx. When extracellular \(\text{Ca}^{2+}\) was removed, ADP was still able to evoke cytosolic \(\text{Ca}^{2+}\) increase indicating the \(\text{Ca}^{2+}\) was mobilized from the \([\text{Ca}^{2+}]_i\) store (Fig. 3.2 B). However, when morphine or DAMGO was used together with ADP, the ADP-induced \([\text{Ca}^{2+}]_i\) release was greatly enhanced (Fig. 3.1 C). In addition, this potentiation was blocked by MOR antagonist naloxone (Fig. 3.1 D) and was still observed in the absence of extracellular \(\text{Ca}^{2+}\) (Fig. 3.2 C, D), which indicated this MOR agonists-potentiated \([\text{Ca}^{2+}]_i\) release was specifically mediated by agonists-activated MOR.

Thapsigargin is inhibitor of endoplasmic reticulum \(\text{Ca}^{2+}\)-ATPase (Thastrup, Cullen et al. 1990). By blocking ATP-dependent \(\text{Ca}^{2+}\) pump on endoplasmic reticulum, thapsigargin mediates a rapid \(\text{Ca}^{2+}\) leak and depletes its sensitive stores. To further demonstrate the source of MOR-mediated \([\text{Ca}^{2+}]_i\) release, thapsigargin was used to
Fig. 3.1 MOR-mediated transient cytosolic Ca\(^{2+}\) incensement required Gq-coupled P\(_2\)Y receptor co-activation.
Fig. 3.1 MOR-mediated transient cytosolic Ca$^{2+}$ incensement required Gq-coupled $P_2Y$ receptor co-activation.

Real-time changes in intracellular fluorescence expressed in raw fluorescence units (RFU) were used to assess [Ca$^{2+}$]$_i$ release from HA-tagged HEK293-MOR cells. Fluorescence dye to detect free [Ca$^{2+}$]$_i$ was added 1 hour before compound injection. Fluorescence changes were recorded using a 485-nm excitation wavelength and a 525-nm emission wavelength. After a 30-s baseline reading, agonists in HBSS buffer were added. (A) 1 μM morphine (MS); (B) 200 nM ADP; (C) 200 nM ADP + 1 μM morphine; (D) 200 nM ADP + 1 μM morphine + 30 μM naloxone (nalox) ($n = 3$).
Fig. 3.2 With co-activation of Gq-coupled receptor, MOR-mediated transient cytosolic Ca$^{2+}$ incensement was from intracellular Ca$^{2+}$ reservoir.
Fig. 3.2 With co-activation of Gq-coupled receptor, MOR-mediated transient cytosolic Ca\(^{2+}\) incensement was from intracellular Ca\(^{2+}\) reservoir.

Real-time changes in intracellular fluorescence are expressed in RFU. HEK293-MOR cells were washed with Ca\(^{2+}\) free HBSS and then incubated with chelator EGTA 1mM to bind free extracellular Ca\(^{2+}\) for 5 min before agonists adding (A) 1 μM morphine (MS); (B) 200 nM ADP; (C) 200 nM ADP + 1 μM morphine; (D) 200 nM ADP + 1 μM morphine + 30 μM naloxone (nalox) (n = 3).
depletion thapsigargin-sensitive [Ca\textsuperscript{2+}], store before agonists challenging. As shown in Fig. 3.3, pretreatment of 1 µM thapsigargin for five min significantly decreased morphine-mediated [Ca\textsuperscript{2+}], release; and increased pretreated thapsigargin concentration to 10 µM completely blocked it. In addition, thapsigargin pretreatment also eliminated ADP-mediated [Ca\textsuperscript{2+}], release, indicating it was released from the same [Ca\textsuperscript{2+}], store (Fig. 3.3).

The level of the MOR-mediated potentiation was related to the initial ADP response. When high concentration of ADP was used in the assay, it maximally induced the [Ca\textsuperscript{2+}], release from its intracellular store, and the level of MOR-mediated potentiation was limited. This further confirmed that MOR and ADP induced the release of Ca\textsuperscript{2+} from the same intracellular Ca\textsuperscript{2+} pools. On the other hand, MOR did not potentiate the [Ca\textsuperscript{2+}], release when ADP concentration was too low to efficiently activate P2Y receptors. Thus, in order to optimize the MOR agonist-mediated [Ca\textsuperscript{2+}], release, 0.2 µM ADP was used in present studies to initiate [Ca\textsuperscript{2+}], release. With fixed concentration of ADP, DAMGO- or morphine-mediated [Ca\textsuperscript{2+}], released in a concentration-dependent manner. After subtracting ADP response from total response, the concentration-response curves of DAMGO and morphine on mediating [Ca\textsuperscript{2+}], release were generated (Fig. 3.4). The EC\textsubscript{50} value of DAMGO to potentiate the 200nM ADP response was 19 nM ± 6.9 nM, which was significantly different from that of the morphine, 54 nM ± 9.8 nM (p<0.01, n=4). Furthermore, maximal potentiation induced by morphine was only 64%± 14% of that induced by DAMGO (Fig. 3.4), suggesting that morphine is a partial agonist in invoking [Ca\textsuperscript{2+}], to release.
Real-time changes in intracellular fluorescence are expressed in RFU. HEK293-MOR cells were washed with Ca^{2+} free HBSS and then incubated with chelator EGTA 1mM to bind free extracellular Ca^{2+} for 5 min before agonists added as indicating in the figures. (A) 1 μM thapsigargin followed by 1 μM ADP; (B) 10 μM thapsigargin followed by 1 μM ADP; (C) 1 μM thapsigargin followed by 1 μM ADP with additional 1 μM morphine (MS); (D) 10 μM thapsigargin followed by 1 μM ADP with additional 1 μM morphine (n = 3).
Fig. 3.4 The concentration-response curve of DAMGO and morphine by measuring both agonists potentiated \([Ca^{2+}]_i\) release with co-activation of Gq receptor.

![Graph showing concentration-response curves of DAMGO and morphine](image)

The concentration-response curves of DAMGO (■) and morphine (○) were determined in the presence of 200nM ADP. Total fluorescence response change induced by ADP or by ADP with MOR ligands was quantified by analyzing the areas under the curves with Prism program. ADP response was then subtracted from the response in the presence of ADP and MOR agonists to obtain the DAMGO and morphine responses.
Part II: MOR agonists pretreatment rapidly desensitize MOR without affecting [Ca$^{2+}$]$_i$ store or P2Y receptor activity.

Results

To determine whether pretreatment of MOR agonists induced MOR rapid desensitization, the ability of MOR-mediated [Ca$^{2+}$]$_i$ release after agonists pretreatment was compared to control groups in which only HBSS buffer was pretreated. In control groups, pretreatment of HEK293-MOR with HBSS buffer before addition of 200 nM ADP and 1 µM morphine had no effect on morphine-potentiated [Ca$^{2+}$]$_i$ release (Fig. 3.1 C and Fig. 3.5 B). In desensitization groups, HEK293-MOR cells were pretreated with 100 nM morphine, followed by the addition of ADP and morphine together in order to achieve the same final concentration of ADP and morphine as in the control groups (Fig. 3.5 C, D). In contrast to the control groups, pretreatment with 100 nM morphine for 5 min significantly decreased the release of [Ca$^{2+}$]$_i$ by morphine and ADP to only 35% ± 14% of the control groups (Fig. 3.5 A, B and C, Fig. 3.6 B). Extending the morphine pretreatment time to 30 min further decreased morphine-mediated response to 2% ± 13% that of HBSS-treated groups (Fig. 3.5 A, B and D, Fig. 3.6 B). Furthermore, the rate of MOR desensitization is agonist concentration-dependent. Increasing the concentration of the pretreated morphine accelerated the rate of decrease in the agonist response. Loss of MOR response was observed as quickly as 1 min after 1µM morphine pretreatment; and MOR response was decreased to 0.8% ± 12% that of control groups after only 5 min pretreatment of 1µM morphine (Fig. 3.5 E and Fig. 3.6 A). In contrast to some other reports which suggested that morphine was a poor desensitization inducer
Fig. 3.5 Determining MOR desensitization by monitoring MOR-induced [Ca^{2+}]_i release.
**Fig. 3.5 Determining MOR desensitization by monitoring MOR-induced \([\text{Ca}^{2+}]_i\) release.**

(A–D) Real-time changes in intracellular fluorescence are expressed in RFU. HEK293-MOR cells were pretreated with HBSS or 100 nM morphine for (C) 5 min or (D) 30 min, followed by ADP and additional morphine to achieve final agonists’ concentrations as indicated in the figure. (E) Dose response and time course of morphine-induced receptor desensitization. HEK293-MOR cells were treated as described in (A–D). Total \([\text{Ca}^{2+}]_i\) release after pretreatment was quantitated by calculating the area under curves with a Prism program. Response to ADP was subtracted from total response to obtain the response to MOR. MOR response after morphine pretreatment was compared to MOR response after HBSS pretreatment to obtain the desensitization ratio.
Fig. 3.6. Morphine- and DAMGO-pretreatment induced MOR desensitization.

The ability of 1 μM or 100nM DAMGO (○) and morphine (■) to induced desensitization of wild type MOR in HEK293 cells was examined as described in Fig. 3.5. Total [Ca^{2+}], response of the second injection was quantitatively analyzed by the areas under the curves with Prism program. The total response in control groups of 200nM ADP and 1μM agonist-induced in second injection was referred as 100%; and data were expressed as the percentage of the response in the pretreated group as compare to the control group.
(Kovoor, Celver et al. 1998), by monitoring MOR-mediated [Ca^{2+}]_i release, morphine was shown to induce rapid MOR desensitization.

Agonist-induced MOR desensitization was also rapid; the t_{1/2} of 100nM DAMGO- and morphine-induced MOR desensitization was determined to be 2.2 ± 0.6 min and 3.8±1.1min respectively (Fig. 3.6 B). However, the 100 nM morphine-induced maximum desensitization level was 0.8% ± 12% of control whereas the 100 nM DAMGO-induced maximum desensitization level was 35% ± 9% of control (n = 4; p < 0.01). In addition, when HEK293 cells were pretreated with 1µM morphine, which was an equivalent to 100nM DAMGO at their EC_{90} concentration to induced intracellular calcium release (Fig. 3.4), the t_{1/2} of morphine-induced MOR desensitization was 0.8 ± 0.4 min, which was significantly faster than that of the DAMGO (n = 4; p < 0.01) (Fig. 3.6 A, B).

To examine whether the attenuation in the [Ca^{2+}]_i release could be due to the reduction of ADP activation of the P2Y receptor, ADP response was determined after MOR agonists treatments. HEK293-MOR cells were pretreated with 1 µM morphine or DAMGO for 5 min, and then ADP concentration-response curves were generated with 30 µM MOR antagonist naloxone to block further MOR activation (Fig. 3.7). As shown before, at this MOR agonist concentration, 5 min pretreatment efficiently eliminated MOR activity. In contrast, pretreatment of morphine or DAMGO did not alter the ADP response (EC_{50} of ADP = 8.9 ± 2.7µM with morphine pretreatment and EC_{50} = 8.5 ±
Fig. 3.7 ADP-induced $[Ca^{2+}]_i$ release was not affected by Morphine or DAMGO pretreatment.

HEK293 cells expressing wild type MOR were pretreated with 1µM morphine(■), DAMGO(○) or HBSS(▲) respectively. In second injection, various concentration of ADP together with 30µM naloxone was injected. Then the concentration-response curves of ADP were determined.
3.8µM with DAMGO or EC\textsubscript{50} = 9.4 ± 4.0µM without agonist pretreatment). Moreover, depletion of [Ca\textsuperscript{2+}]\textsubscript{i}, store could also be a possible cause for reduction of MOR-mediated [Ca\textsuperscript{2+}]\textsubscript{i} release. To examine whether the MOR desensitization could be due to reduction of [Ca\textsuperscript{2+}]\textsubscript{i}, stores availability, HEK293-MOR cells were washed with Ca\textsuperscript{2+} free buffer containing 1 mM EGTA to prevented the Ca\textsuperscript{2+} influx, which recharges [Ca\textsuperscript{2+}]\textsubscript{i} stores. Although 10 µM ADP pretreatment for 90 sec significantly decreased the 1 µM thapsigargin-induced [Ca\textsuperscript{2+}]\textsubscript{i} release, 90 sec pretreatments of 10 µM morphine or DAMGO did not alter it (Fig. 3.8). Because pretreatment with MOR agonists neither desensitized the P2Y receptor nor influenced [Ca\textsuperscript{2+}]\textsubscript{i} stores (i.e., morphine did not evoke [Ca\textsuperscript{2+}]\textsubscript{i}, release by itself), the decreased ability of MOR to potentiate ADP-induced [Ca\textsuperscript{2+}]\textsubscript{i}, release reflects desensitization of the MOR.

**Discussion**

Previous reports have considered GRKs and βArrs as critical factors in receptor homologous desensitization (Luttrell and Lefkowitz 2002; Connor, Osborne et al. 2004). However the existence of discrepancy between the kinetics of MOR desensitization and GRK-mediated receptor phosphorylation leading to βArrs recruitment has led to uncertainties in the MOR desensitization mechanism. In the past, such discrepancy was attributed to relatively high levels of MOR expressed in the heterologous expression system. Our lab’s previous reported studies demonstrated that the rate of receptor desensitization could be regulated by reducing the receptor level with β-fumaltrexamine
Fig. 3.8. Thapsigargin-sensitive \([\text{Ca}^{2+}]_i\) reservoir was not altered by morphine or DAMGO pretreatment.
Fig. 3.8. Thapsigargin-sensitive \([\text{Ca}^{2+}]_i\) reservoir was not alter by morphine or DAMGO pretreatment.

(A) Real-time changes in intracellular fluorescence are expressed in RFU. HEK293-MOR cells were washed with Ca\(^{2+}\) free HBSS and then incubated with chelator EGTA 1mM to bind free extracellular Ca\(^{2+}\) for 5 min before agonists added. After cells were pretreated with 1µM morphine (●), DAMGO (○), 10µM ADP (▲) or HBSS (■) respectively, 1µM thapsigargin was added. (B) Thapsigargin-induced total fluorescence change in second injection was calculated as described before; the data were expressed as the raw fluorescence units in bar graph. Student t-test was used to compare the data in treated groups and control group. **: \(p<0.01\); n=3.
(βFNA) treatment or by controlling the level of receptor expressed using an ecdysone-inducible mammalian expression system (Law, Erickson et al. 2000). Since these receptor desensitization studies were carried out by measuring adenylyl cyclase activity which MOR inhibits with a relatively high efficiency, the slow desensitization rate could reflect the presence of large number of “spare receptors” (response is not directly correlated to receptor occupancy) (Pak, Kouvelas et al. 1996). Obviously, the presence of “spare receptors” did not exist when the Gβγ-subunits-mediated [Ca^{2+}]_i response was measured. In current studies, we demonstrate a rapid loss of MOR responsiveness which happens in minutes in regardless of whether morphine or DAMGO is used to pretreat the HEK293 cells. Furthermore, this time course correlates well to the rate of receptor phosphorylation and βArGs recruitment. Therefore, we could address the issue of whether morphine-induced MOR desensitization follows the classical GRK-mediated receptor phosphorylation and βArGs recruitment.
Chapter IV

Determination of the role of receptor phosphorylation and βArrs in agonist-induced MOR desensitization

DAMGO- but not morphine-induced rapid MOR desensitization is receptor phosphorylation- and βArr2-dependent
The classic dogma which uses the β-adrenergic receptor as a model proposed GRKs-mediated receptor phosphorylation and subsequent βArrs recruitment are the critical steps in GPCR regulation. However, due to the different methods used, controversial data were obtained when MOR desensitization was being studied. Besides direct evidence of involvement of receptor phosphorylation and βArrs in MOR desensitization, whether GRKs and βArrs are involved in MOR tolerance development is equivocal. On one hand, the involvement of βArrs in morphine-induced in vivo tolerance development was demonstrated with βArr2 null mice in hot plate assay; however, it was not demonstrated in the tail-flick antinociceptive assays (Bohn, Gainetdinov et al. 2000). Also, the ability of fentanyl but not morphine to induce in vitro tolerance development was not impeded in the GRK3 knockout mice (Terman, Jin et al. 2004). With our current protocol, it is possible for us to measuring MOR activity change in a timely and sensitive way. Therefore, whether GRKs and βArrs are involved in MOR desensitization needs to be studied.

**Part I: DAMGO-, but not morphine-, induced MOR desensitization is MOR phosphorylation dependent.**

Three Ser and Thr sites in the C-terminal of MOR are suggested to be phosphorylated after DAMGO prolong exposure (El Kouhen, Burd et al. 2001). Among these, Ser363 could be phosphorylated without presence of agonist. DAMGO induced MOR phosphorylation is mediated by GRKs (Law, Erickson et al. 2000) and affected the rate and extent of DAMGO-induced MOR internalization (El Kouhen, Burd et al. 2001).
To determine the effect of MOR phosphorylation in receptor desensitization, MOR with individual Ser\textsuperscript{363} (MORS363A) Thr\textsuperscript{370} (MORT370A) and Ser\textsuperscript{375} (MORS375A) mutations, or phosphorylation deficient MOR (MOR363/370/375) in which all three phosphorylation residues were mutated to Ala were stably expressed in HEK293 cells and used in current studies.

As shown in Fig. 4.1, in the absence of agonist, antibody which recognizes Ser/Thr sites phosphorylated protein indicated there was a basal phosphorylation level on wild type MOR. After agonist pretreatment for five min, wild type MOR phosphorylation level was increased 1.5-folds and 2-folds in the presence of 1 µM morphine and DAMGO respectively. Mutating Ser\textsuperscript{363}, Thr\textsuperscript{370} or Ser\textsuperscript{375} individually significantly attenuated DAMGO-induced MOR phosphorylation. However, morphine-induced MOR phosphorylation level was mainly affected by Ser\textsuperscript{375} mutation. In phosphorylation deficient MOR (MOR363/370/375), both basal and agonist-induced phosphorylation was completely blocked. This observation was consistent with the previous report using \textsuperscript{32}P-labeling of the receptor. In contrast to DAMGO, Ser\textsuperscript{375} appears to be the major phosphorylation residues induced by morphine, for mutating this single site completely blocked morphine-induced phosphorylation. However, DAMGO still increased receptor phosphorylation in S375A (Fig. 4.1 A, B). In addition, mutating single Thr\textsuperscript{370} residue attenuated DAMGO-induced MOR phosphorylation without affecting morphine-induced phosphorylation (Fig. 4.1 C).
Fig. 4.1 DAMGO- and morphine-induced MOR phosphorylation in HEK293 cells.
Fig. 4.1 DAMGO- and morphine-induced MOR phosphorylation in HEK293 cells.

HEK293 cells stably expressing HA-tagged mutant or wild type MOR were pretreated with 1µM morphine or DAMGO for 5min. Receptors were immunoprecipitated and receptor phosphorylation was quantitatively analyzed as described in Material and Methods. Student t-test was used to compare the data in morphine- or DAMGO-pretreated group to control group. * : $p<0.05$; ** : $p<0.01$; n=3.
To determine agonist-induced desensitization in phosphorylation site mutant MOR, the assay described in chapter III was used. Among these MOR mutants, S375A but not S363A or T370A mutation decreased 100nM DAMGO-induced MOR desensitization significantly (Table 4.1). The wild type, S363A or the T370A mutants which reduced the agonist activity by 60-65% after 30 min of DAMGO pretreatment; in contrast, the MOR activity with S375A mutant was only reduced by 28% after 100nM 30 min DAMGO pretreatment (Table 4.1). In addition, DAMGO-induced desensitization was observed after as little as 5 min pretreatment, and prolonged pretreatment did not further desensitize MOR (363/370/375) activity; after 30 min 100nM DAMGO pretreatment, 85 ± 14% of control activity remained (Fig. 4.2 A).

Although morphine induced MOR phosphorylation to a lesser extent than DAMGO induced, 100nM morphine still induced rapid MOR desensitization. Moreover, both the rate and extent of morphine-induced MOR desensitization were not affected in the phosphorylation deficient MOR (Fig. 4.2 B). Apparently, morphine-induced MOR desensitization did not require the initial phosphorylation of MOR.

Part II: DAMGO-, but not morphine-, induced MOR desensitization is βArr2 dependent.

Although DAMGO-induced MOR desensitization was attenuated in phosphorylation deficient mutant MOR, a significant desensitization was still observed (Fig. 4.2 A). In addition, morphine-induced MOR desensitization was completely
Table 4.1 Desensitization of wild and mutant types MOR by 100nM DAMGO pretreatment in HEK293 cells.

<table>
<thead>
<tr>
<th>HEK293-MOR</th>
<th>Response</th>
<th>Desensitization</th>
<th>Receptor desensivity,</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC_{50}DAMGO (nM)</td>
<td>K (min^{-1})</td>
<td>Maximum des level, (%)</td>
</tr>
<tr>
<td>WT</td>
<td>19 ± 6.9</td>
<td>0.31 ± 0.12</td>
<td>65 ± 4</td>
</tr>
<tr>
<td>S363A</td>
<td>60 ± 19</td>
<td>0.46 ± 0.32</td>
<td>59 ± 5</td>
</tr>
<tr>
<td>T370A</td>
<td>65 ± 31</td>
<td>0.40 ± 0.11</td>
<td>60 ± 9</td>
</tr>
<tr>
<td>S375A</td>
<td>58 ± 53</td>
<td>0.37 ± 0.19</td>
<td>28 ± 11*</td>
</tr>
<tr>
<td>370/375</td>
<td>11 ± 9.1</td>
<td>0.41 ± 0.10</td>
<td>21 ± 10*</td>
</tr>
<tr>
<td>363/370/375</td>
<td>17 ± 3.6</td>
<td>0.35 ± 0.18</td>
<td>15 ± 14**</td>
</tr>
</tbody>
</table>

Means ± SEM (n ≥ 3); *, p < 0.05; **, p < 0.01; n ≥ 3.

DAMGO-induced [Ca^{2+}]_i release concentration-response curves were determined as described in Fig. 3.4, and EC_{50} values of DAMGO were calculated by log(agonist) vs. response formula. 100nM DAMGO-induced desensitization in wild type or mutant types MOR was examined and data were analyzed as described in figure legend of Fig. 3.5 and Fig. 3.6. K values of DAMGO-induced MOR desensitization were calculated by one phase exponential decay using the data analysis program GraphPad Prism. Maximum desensitization (des) level data were obtained after 30 min agonist pretreatment. Receptor expression level was determined by radioligand binding experiments as described in Material and Methods. Data were showed as the averages of n ≥ 3 experiments.
Fig. 4.2 MOR phosphorylation sites mutant affected DAMGO- but not morphine-induced MOR desensitization.
Fig. 4.2 MOR phosphorylation sites mutant affected DAMGO- but not morphine-induced MOR desensitization.

The ability of 100nM DAMGO and morphine to induce desensitization of wild type MOR (■) and phosphorylation deficient MOR (363/370/375) (○) was examined in HEK293 cells. Total [Ca^{2+}]_i response of the second injection was quantitatively analyzed. The agonist-induced desensitization rate was calculated as described in the legend of Fig. 3.5 and Fig. 3.6. Data were showed as the averages of n≥3 experiments in which HEK293 cells pretreated with 100nM DAMGO (panel A) and 100nM morphine (panel B) respectively to induce MOR desensitization. * denotes p<0.05; ** denotes p<0.01.
receptor phosphorylation independent (Fig. 4.2 B). Besides receptor phosphorylation, βArrs binding is another critical factor in GPCR desensitization. Therefore, it is essential to determine the role of βArrs in DAMGO-or morphine-induced MOR desensitization by transiently transfecting the HEK293 cells expressing wild type or mutant MOR with βArr2-FLAG.

Over-expression of βArr2-FLAG slightly, although not statistically significantly accelerated the rate of 1µM DAMGO-induced wild type MOR desensitization (Fig. 4.3 A). However, in cell expressing the phosphorylation deficient MOR mutant, over-expression of βArr2-FLAG increased 1µM DAMGO-induced desensitization rate significantly, t_{1/2} equal to 0.8±0.5min (p<0.01, n≥ 3) (Fig. 4.3 B). Noticeably, after βArr2-FLAG over-expression, the rates of 1µM DAMGO-induced MOR desensitization was similar in cells expressing wild type or phosphorylation deficient mutant MOR, with t_{1/2} values equal to 0.8 ± 0.5min and 0.7 ± 0.5min respectively. Such data indicated that over-expression of βArr2-FLAG could overcome the reduction in DAMGO-induced MOR desensitization with the phosphorylation deficient mutant, in accordance to other reports indicating GPCR phosphorylation increased the receptor affinity for βArr2 (Lefkowitz 1998). In contrast to DAMGO, the rate and the extent of morphine-induced receptor desensitization were identical in cells with endogenous level or over-expressed level of βArr2 (Fig. 4.4 A, B).
Fig. 4.3 βArr2-FLAG overexpression accelerated 1µM DAMGO induced phosphorylation deficient (363/370/375) but not wild type MOR desensitization in HEK293 cells.
Fig. 4.3 βArr2-FLAG overexpression accelerated 1μM DAMGO induced phosphorylation deficient (363/370/375) but not wild type MOR desensitization in HEK293 cells.

The abilities of DAMGO to induce the desensitization of wild type MOR and phosphorylation deficient MOR (363/370/375) were examined in HEK293 cells transfected with βArr2-FLAG (○) or with mock transfection (■). Cells were pretreated with 1μM DAMGO for the indicated time. Data were showed as the averages of n≥3. *: p < 0.05.
Fig. 4.4 βArr2-FLAG overexpression did not affect 1μM morphine induced either wild type or phosphorylation deficient (363/370/375) MOR desensitization in HEK293 cells.
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The abilities of morphine to induce the desensitization of wild type MOR and phosphorylation deficient MOR (363/370/375) were examined in HEK293 cells transfected with βArr2-FLAG (○) or with mock transfection (■). Cells were pretreated with 1µM morphine for the indicated time. Data were showed as the averages of n≥3.
Both 1 µM DAMGO- and morphine- induced MOR desensitization occur rapidly, which could not sensitively reflect the increase of rate and extent by βArr2 over-expression. As demonstrated in chapter III, lower concentration agonists induced MOR desensitization with slower rates; therefore, 100 nM DAMGO or morphine was used. By examining the rate of 100 nM DAMGO-induced receptor desensitization in cells expressing wild type or phosphorylation deficient mutant MOR, the increase of both rate and extent of DAMGO-induced MOR desensitization by βArr2 over-expression was further demonstrated. As shown in Fig. 4.5 A, over-expression of βArr2 increased the rate but not the magnitude of 100nM DAMGO-induced MOR desensitization in HEK293 cells expressing the wild type receptor. However, over-expression of βArr2 in HEK293 cells expressing the phosphorylation deficient mutant MOR resulted in an increase in both the magnitude and the rate of 100nM DAMGO-induced MOR desensitization (Fig. 4.5 B). As a matter of fact, DAMGO pretreatment reduced receptor activity to the same extent in HEK293 cells expressing either wild type or phosphorylation deficient mutant when βArr2 was over-expressed (Fig. 4.5 A, B). In contrast, 100nM morphine induced receptor desensitization was not affected by the over-expression of βArr2 in HEK293 cells expressing with either wild type or phosphorylation deficient MOR. (Fig. 4.6). The rate and the extent of morphine-induced receptor desensitization were similar in cells with endogenous levels or over-expressed levels of βArr2.

Since over-expression of βArr2 may overcome the effects from endogenous regulatory factors, depletion of endogenous βArr2 is necessary in current studies. To
Fig. 4.5 βArr2-FLAG overexpression accelerated 100nM DAMGO induced both wild type and phosphorylation deficient (363/370/375) MOR desensitization in HEK293 cells.
Fig. 4.5 βArr2-FLAG overexpression accelerated 100nM DAMGO induced both wild type and phosphorylation deficient (363/370/375) MOR desensitization in HEK293 cells.

The abilities of DAMGO to induce the desensitization of wild type MOR and phosphorylation deficient MOR (363/370/375) were examined in HEK293 cells transfected with βArrs2-FLAG (○) or with mock transfection (■). Cells were pretreated with 1µM DAMGO for the indicated time. ∗: $p < 0.05$; **: $p < 0.01$; $n \geq 3$. 
Fig. 4.6 \(\beta\text{Arr2-FLAG}\) overexpression did not affect 100nM morphine induced either wild type or phosphorylation deficient (363/370/375) MOR desensitization in HEK293 cells.
Fig. 4.6 βArr2-FLAG overexpression did not affect 100nM morphine induced either wild type or phosphorylation deficient (363/370/375) MOR desensitization in HEK293 cells.

The abilities of morphine to induce the desensitization of wild type MOR and phosphorylation deficient MOR (363/370/375) were examined in HEK293 cells transfected with βArrs2-FLAG (○) or with mock transfection (■). Cells were pretreated with 1μM morphine for the indicated time. Data were showed as the averages of n≥3.
knock-down the endogenous βArr2, siRNA special targeting βArr2 was designed and transiently expressed in HEK293 cells expressing with either wild type or phosphorylation deficient MOR. However, siRNA over-expression did not affect 1 µM DAMGO-induced MOR desensitization on either wild type or phosphorylation deficient mutant MOR. This might because of the abundant endogenous expression of βArr2, or the inefficiency of siRNA knocking-down (Fig. 4.7).

To further demonstrate the role of βArrs on DAMGO- and morphine-induced MOR desensitization, mouse embryonic fibroblasts (MEF) cells isolated from wild type (WT), βArr2 null mice (βArr2−/−) or βArr1 and βArr2 null mice (βArr1/2−/−) were used. These MEF cells were infected with adenovirus containing MOR (Ad-MOR) to produce similar receptor level expressed as determined by receptor binding assay (Table 4.2).

In these MEF cells, ADP did not evoke [Ca²⁺]ᵢ release, but another broad spectrum purinergic receptor agonist, ATP, evoked the [Ca²⁺]ᵢ release response (Fig. 4.8 A, B). Similar to the observation in HEK293 cells, MOR agonists potentiated ATP-induced [Ca²⁺]ᵢ release in MEF cells (Fig. 4.8 C). Consistent with the HEK293 cells observations, morphine exhibited a partial agonist property in regulating the [Ca²⁺]ᵢ release. Similar partial agonist properties of morphine as compared to DAMGO were observed in all three types of MEF cells (Table 4.2).

Surprisingly, MOR agonists could evoke robust [Ca²⁺]ᵢ release even in the absence of ATP (Fig. 4.9 A). This MOR-induced [Ca²⁺]ᵢ release can be completely
Fig. 4.7 βArr2 siRNA did not affect 1µM DAMGO induced either wild type or phosphorylation deficient (363/370/375) MOR desensitization in HEK293 cells.
Fig. 4.7 βArr2 siRNA did not affect 1µM DAMGO induced either wild type or phosphorylation deficient (363/370/375) MOR desensitization in HEK293 cells.

The abilities of 1µM DAMGO to induce the desensitization of wild type MOR and phosphorylation deficient MOR (363/370/375) were examined in HEK293 cells transfected with βArrs2 siRNA (○) or with scramble sequence (■). Cells were pretreated with 1µM DAMGO for the indicated time. Data were showed as the averages of n≥3.
Table 4.2 EC<sub>50</sub> of morphine and DAMGO for MOR expressed in various MEF cells.

<table>
<thead>
<tr>
<th>MEF</th>
<th>Response</th>
<th>Receptor desensity, (pmol/mg)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; Morphine, (nM)</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; DAMGO, (nM)</td>
</tr>
<tr>
<td>WT</td>
<td>138 ± 48</td>
<td>30.5 ± 1.4</td>
</tr>
<tr>
<td>βArr2-//-</td>
<td>194 ± 50</td>
<td>68.3 ± 2.2</td>
</tr>
<tr>
<td>βArr1/2-//-</td>
<td>61 ± 5</td>
<td>25.3 ± 3.2</td>
</tr>
</tbody>
</table>

Means ± SEM (n ≥ 3)

Wild type, βArr2<sup>−/−</sup> and βArr1/2<sup>−/−</sup> MEF cells were infected with adenovirus containing MOR. Morphine- and DAMGO-induced [Ca<sup>2+</sup>]<sub>i</sub> release concentration-response curves were determined as described in figure legend of Fig. 3.5 and Fig. 3.6. EC<sub>50</sub> values were calculated by log (agonist) vs. response formula using the data analysis program GraphPad Prism. Radioligand binding experiments were performed as described in material and methods. Data were showed as the averages of n ≥ 3 experiments.
Fig. 4.8 ATP- but not ADP-induced \([\text{Ca}^{2+}]_i\) response in MEF cells.
Fig. 4.8 ATP- but not ADP-induced [Ca\textsuperscript{2+}]; response in MEF cells.

Real-time changes in intracellular fluorescence expressed in raw fluorescence units (RFU) were used to assess P2Y receptor-mediated [Ca\textsuperscript{2+}], release from MEF cells. Fluorescence dye to detect free [Ca\textsuperscript{2+}], was added 1 hour before compound injection. Fluorescence changes were recorded using a 485-nm excitation wavelength and a 525-nm emission wavelength. After a 30-s baseline reading, 1 μM ADP (A) or 1 μM ATP (B) was added into MEF wild type (WT) (●), βArr2\textsuperscript{-/-} (■), βArr1/2\textsuperscript{-/-} (▲) respectively (n = 3). (C) MEF wild type cells were added with 1 μM ADP (○) or 1 μM ADP together with 1 μM DAMGO (□).
Fig. 4.9 DAMGO-induced \([Ca^{2+}]_i\) release in MEF WT cells.
Fig. 4.9 DAMGO-induced $[\text{Ca}^{2+}]_i$ release in MEF WT cells.

Wild type MEF cells were infected with adenovirus containing MOR and MOR expressing level was monitored by receptor binding assay as described in experiment procedures. After 30 second baseline reading, MEF WT cells were injected with various compounds as showed by the arrows.
blocked by naloxone (Fig. 4.9 B); and cannot be blocked by removing extracellular Ca\textsuperscript{2+} (Fig. 4.9 C). To investigate whether endogenous P2Y activity was involved in the opioid response, the opioid-induced [Ca\textsuperscript{2+}]\textsubscript{i} release was measured in the presence of the P2Y receptor antagonist suramin (50\(\mu\)M). At this concentration, suramin completely blocked the ATP-induced [Ca\textsuperscript{2+}]\textsubscript{i} release (Fig. 4.9 D), however, did not affect DAMGO-induced [Ca\textsuperscript{2+}]\textsubscript{i} release in MEF cells (Fig. 4.9 E, F). To avoid high concentration of opioid ligands-induced [Ca\textsuperscript{2+}]\textsubscript{i} release, thus resulting in an alteration in the [Ca\textsuperscript{2+}]\textsubscript{i} stores, MEF cells were pretreated with MOR agonists at concentration that did not elicit measurable [Ca\textsuperscript{2+}]\textsubscript{i} release. Therefore, 100 nM DAMGO and morphine were used to pretreat MEF cells. After the MEF cells were pretreated with these two MOR agonists, both 100nM DAMGO and morphine reduced MOR activity in wild type MEF cells. As shown in Fig. 4.10, after 30 min of pretreatment, 100nM morphine- and DAMGO-induced MOR activities were 39 ± 18% and 5 ± 11% of control respectively. However, in \(\beta\)Arr1/2\(^{-/-}\) cells, after 30 min of 100nM DAMGO pretreatment, 1\(\mu\)M DAMGO-induced [Ca\textsuperscript{2+}]\textsubscript{i} release was similar to those observed in the MEF cells not treated by the agonist. This observation supports the phosphorylation deficient mutants studies indicating that \(\beta\)Arrs is critical in DAMGO-induced MOR desensitization. Most likely \(\beta\)Arr2 is the \(\beta\)Arr subtype participated in DAMGO-induced MOR desensitization. For in \(\beta\)Arr2\(^{-/-}\) MEF cells, DAMGO-induced receptor desensitization was attenuated to the same extent as that observed in \(\beta\)Arr1/2\(^{-/-}\) MEF cells. In these \(\beta\)Arr null MEF cells, 91 ± 4% of MOR activity remained after 30 min of 100 nM DAMGO pretreatment (Fig. 4.10 A). On the other hand, the presence of \(\beta\)Arr is not a prerequisite for morphine-induced receptor desensitization. In contrast to DAMGO-induced receptor desensitization, 30
Fig. 4.10 Effect of βArr2 depletion on DAMGO- and morphine-induced MOR desensitization in MEF cells.
Fig. 4.10 Effect of βArr2 depletion on DAMGO- and morphine-induced MOR desensitization in MEF cells.

Wild type, βArr2−/− and βArr1/2−/− MEF cells were infected with adenovirus containing MOR and MOR expressing level was monitored by receptor binding assay. The abilities of 100nM DAMGO and morphine to induce the desensitization of MOR in wild type MEF cells (■) βArr1/2−/− MEF cells (○) and βArr2−/− MEF cells (▲) was examined. Cells were pretreated with 100nM DAMGO or morphine, and agonist-induced desensitization was obtained as described in the legend of Fig. 3.5 and Fig. 3.6. Data summarize the average of n≥3 experiments in which the MEF cells were treated with 100nM DAMGO (A) and 100nM morphine (B) for various time to induce MOR desensitization. *denotes $p < 0.05$ and ** denotes $p < 0.01$. 
mins of 100 nM morphine pretreatment significantly reduced MOR activity by 80 ± 11% in the βArr1/2−/− MEF cells. Although the desensitization rate appeared to be slower when compared to that observed in WT MEF cells, the magnitude of morphine-induced MOR desensitization remained similar (Fig. 4.10 B). These data suggest that morphine-induced MOR desensitization is βArr-independent, whereas DAMGO-induced MOR desensitization is absolutely dependent on βArrs.

Discussion

By taking advantage of using [Ca^{2+}], release as a sensitive indicator of MOR activity, we were able to study the MOR activity regulation mechanism in details as shown in these studies. DAMGO-induced MOR desensitization strictly follows the classic GPCR desensitization mechanism; i.e. it is regulated by receptor phosphorylation and is absolutely dependent on βArrs activity. Mutation of the putative GRK-mediated phosphorylation sites significantly reduced the rate of DAMGO-induced MOR desensitization, and the absence of βArrs in the βArr1/2−/− MEF cells completely blocked the DAMGO-induced MOR desensitization. Since similar maximum rates and magnitudes of DAMGO-induced MOR desensitization were observed in both wild type and phosphorylation minus mutant receptor when βArrs was over-expressed, clearly, βArrs has a basal affinity for non-phosphorylated MOR as reported with other GPCRs (El Kouhen, Burd et al. 2001; Qiu, Law et al. 2003).
Further experiments on the phosphorylation deficient MOR and βArr1/2−/− MEF cells suggested the existence of multiple mechanisms on MOR desensitization. Although similar to DAMGO, pretreatment of morphine caused the rapid loss of receptor responsiveness; our current data indicates that the morphine-induced desensitization appears to be regulated by another mechanism. Furthermore, by over-expressing βArr2 or completely depleting βArrs, it is the first time that βArrs-independent MOR rapid desensitization was shown.

As summarized in introduction chapter, in vitro MOR desensitization was used as a molecular mechanism to explain the tolerance development in vivo (Connor, Osborne et al. 2004). The theory suggested that in vitro MOR desensitization is the basis for in vivo tolerance was supported by the observation with the βArr2−/− mice, in which morphine-mediated analgesia tolerance was completely attenuated as monitored by hot-plate tests (Bohn, Gainetdinov et al. 2000). However, the ability of morphine to induce antinociceptive tolerance as measured by tail-flick tests in the βArr2−/− mice (Bohn, Lefkowitz et al. 2002), and the inability of morphine-activated MOR to recruit βArr indicated factors other than βArr2 participate in morphine-induced tolerance. Our current research observed morphine-mediated βArr-independent desensitization suggests alternative pathways are involved in the in vitro morphine-induced desensitization, and may influence morphine-mediated in vivo tolerance development. The other theory proposed βArrs-mediated receptor internalization is the pathway opioid receptor utilized for resensitization during the receptor recycling process, which can prevent the further loss of MOR responsiveness (Whistler, Chuang et al. 1999; He,
Fong et al. 2002). This theory suggested the severe tolerance development to morphine when compared to the other agonists is due to the agonist’s inability to induce receptor internalization and subsequent resensitization. This theory appears to be supported by our current findings. Since DAMGO is able to induce MOR internalization while morphine cannot, the higher extent of desensitization after 30 minutes of morphine treatment compared to that of DAMGO treatment could reflect that receptor resensitization might be involved in the overall desensitization process.

In previous studies, GRKs over-expression was the most commonly used method to study the effect of phosphorylation on receptor desensitization (Connor, Osborne et al. 2004; Johnson, Oldfield et al. 2006). In order to overcome the difficulties in correlating the phosphorylation states of the receptor and desensitization, previous reports have investigated the effect of receptor phosphorylation on desensitization by truncating the MOR C-terminal after the amino acid residue Ser 363, which is the first phosphorylation site on C-terminal (Qiu, Law et al. 2003). However, the involvement of certain phosphorylation sites or the involvement of receptor phosphorylation in rapid desensitization was still unclear, since the relatively long agonist pretreated time and high agonist concentration resulted in MOR desensitization rate reflecting both the uncoupling of MOR from the G-protein and the internalization of MOR. Our current agonist treatment paradigm, <30 minutes, should minimize the roles of receptor internalization and resensitization in the receptor desensitization process. Clearly, phosphorylation of Ser^{375} participated in the DAMGO-induced desensitization, which is
also demonstrated to involvement in MOR interenalization (El Kouhen, Burd et al. 2001).

Although morphine also induced Ser\(^{375}\) phosphorylation (Schulz, Mayer et al. 2004), the βArr-dependent receptor internalization was not induced by morphine unless either GRK2 or βArr was over-expressed in the system (Whistler and von Zastrow 1998; Zhang, Ferguson et al. 1998). Furthermore, our current studies indicated that phosphorylation-dependent desensitization was not observed when morphine was used in the current study. Morphine-induced MOR desensitization was not affected by the over-expression of βArr, was present in the βArr1/2\(^{-/-}\) MEF cells, and was similar in wild type and phosphorylation minus mutants. This is in contrast to the ability of over-expression of βArr to promote morphine-induced receptor internalization [16]. This might be due to morphine-induced MOR desensitization was fast than that βArrs-mediated one.

Our current studies demonstrate that agonist-dependent desensitization exists, which is consistent with the previous studies on DAMGO- and morphine-induced potassium current desensitization (Johnson, Oldfield et al. 2006). Moreover, our studies clearly demonstrated that morphine could induce receptor desensitization in the absence of receptor phosphorylation and βArr. Since published observations have supported the role of βArr2 in morphine-induced tolerance development (Bohn, Gainetdinov et al. 2000), our current observations might simply reflect the situation with the \([Ca^{2+}]_i\) release. However, depletion of GRK3 \textit{in vivo} only attenuate fentanyl- but not morphine-
induced tolerance (Terman, Jin et al. 2004), and the ability of PKC and PKA inhibitors (Bohn, Lefkowitz et al. 2002; Javed, Dewey et al. 2004; Smith, Javed et al. 2006; Smith, Gabra et al. 2007) or the absence of PKCγ or PKCε to blunt morphine-induced tolerance development \textit{in vivo} (Zeitz, Malmberg et al. 2001; Newton, Kim et al. 2007) suggested agonist-dependent mechanism on opioid tolerance development. Such \textit{in vivo} results are consistent with our observation in the current study, thus an apparent difference exists between DAMGO- and morphine-induced MOR desensitization. Such agonist-dependent desensitization mechanism might contribute to the differences in these agonists’ effects \textit{in vivo}.
Chapter V

Determination the mechanism of morphine-induced MOR desensitization

Morphine-induced MOR desensitization is mediated by PKCε-phosphorylated Gai2
The common pathway for GPCR desensitization which is mediated by GRKs and βArrs (Lefkowitz 1998) is challenged by the existence of agonists whose receptor complexes have low affinities for βArrs and still undergo agonist-induced desensitization (Violin and Lefkowitz 2007). Such agonists might desensitize the receptor via pathways other than those involving GRK and βArrs. For instance, although both DAMGO and morphine induce MOR rapid desensitization, DAMGO was shown to induce receptor desensitization via MOR phosphorylation and βArrs translocation, whereas morphine does not. This is consistent with the lower efficacy of morphine to induce receptor phosphorylation and βArr recruitment relative to agonists such as DAMGO and etorphine (Law, Wong et al. 2000).

Several protein kinases such as PKC (Johnson, Oldfield et al. 2006), PKA (Pitcher, Lohse et al. 1992), and ERK (Polakiewicz, Schieferl et al. 1998) have been suggested to be involved in MOR desensitization. After morphine treatment, increases in PKC or ERK activities have been reported (Feng, Narita et al. 1994; Law, Wong et al. 2000). Although only chronic but not acute treatment of morphine could up-regulate PKA activity (Nestler and Tallman 1988), all three protein kinases have been shown to phosphorylate MOR, thus resulting in receptor desensitization (Zhang, Yu et al. 1996; Bernstein and Welch 1998; El Kouhen, Kouhen et al. 1999; Schmidt, Schulz et al. 2000). Recently, Src activation has also been recognized to be involved in MOR signal regulation (Walwyn, Evans et al. 2007). Whether Src-induced MOR phosphorylation is involved in MOR desensitization needs to be further investigated. Besides directly phosphorylating receptors, these protein kinases were shown to phosphorylate G-protein
subunits or MOR signal components, which could eventually lead to the desensitization of MOR signals (Strassheim and Malbon 1994; Strassheim, Law et al. 1998; Yasuda, Lindorfer et al. 1998; Chakrabarti and Gintzler 2007).

The preference of morphine to use PKC-dependent pathways for signal transduction in vitro and tolerance development in vivo suggest the participation of PKC activity in morphine functions (Feng, Narita et al. 1994; Wang, Bilsky et al. 1999; Bohn, Lefkowitz et al. 2002; Smith, Javed et al. 2002; Zheng, Loh et al. 2008). PKC has also been implicated in morphine-induced MOR desensitization (Johnson, Oldfield et al. 2006; Chu, Zheng et al. 2008). However, the cellular mechanism of how PKC involves morphine function is still unclear. As an important kinase family, PKCs participate in numerous cellular signaling pathways, from short-term neurotransmitter release to long-term cellular adaptation responses (Newton 2001). In vivo studies revealed that morphine function is related to several PKC subtypes. PKCα, PKCγ and PKCε appear to contribute to morphine tolerance (Smith, Gabra et al. 2007), and mice lacking PKCε show an increased response to morphine (Newton, Kim et al. 2007). However, the exact PKC subtype(s) involved in MOR signal transduction, and the mechanisms by which they act, e.g., the kinase targets, remain unknown.

PKC translocates to the membrane on activation (13). Subsequent phosphorylation of membrane proteins could lead to PKC-mediated regulation of GPCR signaling. There are many potential PKC targets that might contribute to receptor desensitization; MOR and G-protein are but two of them. MOR is phosphorylated after
agonist treatment, and GRK-mediated receptor phosphorylation highly correlates with desensitization. Although GRK-mediated MOR phosphorylation does not modulate morphine-induced desensitization, PKC-mediated MOR phosphorylation might; and G-protein phosphorylation has also been implicated in GPCR desensitization (Murthy, Grider et al. 2000).

**Part I: Morphine-induced desensitization of MOR is PKCε-dependent.**

Previous reports indicated that, unlike DAMGO, morphine-induced MOR desensitization is GRK- and βArr-independent. To characterize the mechanism by which morphine induces MOR desensitization, HEK293-MOR cells were incubated with a Src kinase inhibitor (PP2), an extracellular signal-regulated kinase inhibitor (PD98059), or a PKC inhibitor (Ro-31-8425) before inducing receptor desensitization by pretreatment with 100 nM morphine. Among these inhibitors, only 5 μM of Ro-31-8425 attenuated morphine-induced, but not DAMGO-induced, MOR desensitization (Fig. 5.1 A, B). In the presence of the PKC inhibitor, the MOR response decreased by 55 ± 6% after a 30 min morphine pretreatment, which was significantly attenuated from control group (p <0.01, n≥3).

Morphine-induced receptor desensitization is mediated by PKC whereas DAMGO-induced desensitization is not. This could be the result of agonist-dependent signaling or the vulnerability of special agonist-receptor complex to certain regulatory signals. Whether PKC is activated by MOR in an agonist-dependent manner was
Fig. 5.1 Morphine-induced, but not DAMGO-induced, MOR desensitization is PKC-dependent.
Fig. 5.1 Morphine-induced, but not DAMGO-induced, MOR desensitization is PKC-dependent.

Morphine-induced desensitization as measured by assessing agonist potentiation of ADP-mediated [Ca\textsuperscript{2+}]\textsubscript{i} release. 100 nM morphine or DAMGO were used to pretreat HEK293-MOR cells for various time. Desensitization ratios were determined as described in legend of Fig. 3.5 and Fig. 3.6. HEK293-MOR cells were incubated for 3 h with DMSO (Control) (■), 5 μM general PKC inhibitor Ro-31-8425 (○) (**: p < 0.01; n≥3).
investigated. An antibody that recognizes phosphorylated PKC substrates was used to determine PKC activity after morphine pretreatment. When the immunoreactivities of phosphorylated PKC substrates were determined in whole cell lysates, a 5 min pretreatment with 1 μM morphine minimally increased PKC activity to 120 ± 10% that of basal levels, whereas the general PKC activator phorbol 12-myristate 13-acetate (PMA) induced an increase to 160 ± 12% of control activity (Fig. 5.2).

The identity of the PKC subtype responsible for morphine-induced desensitization was determined using membrane-permeable, subtype-specific peptide inhibitors of PKC. These peptides inhibit PKC activity by binding to either the subtype’s catalytic sites or their corresponding anchoring protein RACK (House and Kemp 1987; Mochly-Rosen and Gordon 1998). Although all eleven PKC subtypes are present in HEK293 cells (Kanno, Yamamoto et al. 2006), we focused on the PKC subtypes α, γ and ε, which have been implicated in the development of morphine-induced tolerance *in vivo* (Smith, Gabra et al. 2007). MOR desensitization was completely blocked by the PKCε inhibitor, but not by the inhibitors of PKCα or PKCγ (Fig. 5.3 A). In contrast, DAMGO-induced MOR desensitization was unaffected by any of these inhibitors (Fig. 5.3 B).

The antibody used against the phosphorylated PKC substrate provides indirect evidence for PKC activation, and cannot distinguish the activity of PKC subtypes. Therefore, the subtypes were immunoprecipitated with their respective antibodies and the enzymatic activities within the immunoprecipitates were determined. Among the
Fig. 5.2 Activation of PKC by morphine treatment.
Fig. 5.2 Activation of PKC by morphine treatment.

(A) HEK293-MOR cells were pretreated with Buffer (lane 1), 1µM PMA (lane 2), 1µM morphine (lane 3) or 1µM DAMGO (lane 4) for 5 mins. PKC activity was determined by immunoblotting (IB) with an antibody against phosphorylated PKC substrates. MOR was used as loading controls. (B) Quantitative analysis of immunoreactivities of PKC phosphorylated substrates as determined from IBs. (#: no significant difference; *: \( p < 0.05 \); **: \( p < 0.01 \); n=3).
Fig. 5.3. Morphine-induced MOR desensitization is PKCε dependent
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Morphine-induced desensitization as measured by assessing agonist potentiation of ADP-mediated [Ca^{2+}] release. 100 nM morphine or DAMGO were used to pretreat HEK293-MOR cells for various time. Desensitization ratios were determined as described in legend of Fig. 3.5 and Fig. 3.6. HEK293-MOR cells were incubated for 3 h with DMSO (Control) (■), 50 μM subtype-specific inhibitors of PKCα (◊), γ (□), or ε (▲) before assays (**: p < 0.01; n≥3).
three PKC subtypes examined, only the activity of PKC\(\varepsilon\) was increased by morphine (226 ± 31\%, relative to basal levels); DAMGO only increased PKC\(\varepsilon\) activity to 130 ±14\% of basal activity. Neither morphine nor DAMGO increase the activity of PKC\(\alpha\) or PKC\(\gamma\) (Fig. 5.4).

**Part II: MOR is not PKC substrate within the signaling complex.**

Morphine induces receptor phosphorylation more slowly and to a much lesser extent than does DAMGO. Furthermore, previous data suggest morphine can induce MOR desensitization even after all the putative phosphorylation residues (i.e., Ser/ Thr) on the MOR C-terminal are mutated. These data imply that MOR is not the target of PKC\(\varepsilon\) within the signaling complex. Indeed, no PKC-mediated phosphorylation of the receptor was detected in MOR immunoprecipitates probed on immunoblots with antibody against phosphorylated PKC substrates (Fig. 5.5).

Since activated PKC translocated to the plasma membrane (Newton 2001), it may phosphorylate substrates within the signaling complex. Such phosphorylated substrates, including G-protein subunits or MOR signal components, could eventually lead to the MOR desensitization (Strassheim and Malbon 1994; Strassheim, Law et al. 1998; Yasuda, Lindorfer et al. 1998; Chakrabarti and Gintzler 2007).

As shown in Fig. 5.6, although a 5 min pretreatment with 1 \(\mu\)M morphine pretreatment only increased PKC activity to 120 ± 10\% that of basal levels in whole cell
Fig. 5.4 Determination of morphine-induced PKC subtype activity in whole cell lysates.

HEK293-MOR cells were pretreated for 5 min with HBSS buffer, 1 μM morphine or 1 μM DAMGO. The PKC subtypes were immunoprecipitated with their respective antibodies and the enzymatic activities within the immunoprecipitates were determined as described in Methods and Materials. (*: p < 0.05; ***: p < 0.005; n≥3).
HEK293-MOR cells were pretreated with Buffer (control), 1µM PMA, 1µM morphine or 1µM DAMGO for 5 mins. MOR immunoprecipitated (IP) with HA antibody, and PKC activity was determined by immunoblotting (IB) with an antibody against phosphorylated PKC substrates. Total MOR amount was determined by IB with MOR antibody and used as loading control.
Fig. 5.6 Incensement of PKC activity in MOR signaling complex after morphine treatment
Fig. 5.6 Incensement of PKC activity in MOR signaling complex after morphine treatment

HEK293-MOR cells were pretreated with Buffer (lane 1), 1µM PMA (lane 2), 1µM morphine (lane 3) or 1µM DAMGO (lane 4) for 5 mins. MOR signaling complexes were co-immunoprecipitated (co-IP) with HA antibody, and PKC activity was determined by immunoblotting (IB) with an antibody against phosphorylated PKC substrates. Gαi2 was used as loading control (upper panel). Quantitative analysis of immunoreactivities of PKC phosphorylated substrates as determined from IBs (#: no significant difference; *: p < 0.05; **: p < 0.01; ***: p < 0.005; n=3) (lower panel).
lysates (Fig. 5.2), it increased the amount of phosphorylated PKC substrates present in MOR signaling complexes of anti-HA co-immunoprecipitates 2.0 ± 0.18 fold relative to controls, and this morphine-induced increase was greater than the 1.5 ± 0.16 increase induced by treatment with PMA. Thus, PKC substrates phosphorylated in the presence of morphine were enriched in the receptor signaling complex. In contrast, DAMGO pretreatment did not significantly increase PKC activity, indicating that PKC activation was agonist-dependent.

To specifically identify the PKC subtypes translocated to the receptor signaling complex during agonist pretreatment, lipid rafts (in which MOR signaling complexes are located) were separated from other membrane domains using a continuous sucrose gradient (Zheng, Chu et al. 2008). In control and DAMGO-treated cells, PKCε localized in the last two high-density fractions of the gradient; these fractions correspond to the cytosolic fraction of HEK293 cells. In contrast, morphine treatment induced translocation of PKCε from the last two fractions to the lipid raft fractions (i.e., fractions 3, 4), where MOR and Gαi2 are located (Fig. 5.7). In addition, the amount of PKCε associated with the MOR signaling complex as detected by immunoprecipitation of MOR using anti-HA antibodies increased after morphine, but not DAMGO treatment (Fig. 5.8). Again, these results indicate that morphine induces the activation and translocation of PKCε into the MOR signaling complex.

**Part III: Gαi2 is the PKCε substrate mediating morphine-induced desensitization**
Fig. 5.7 Morphine induces translocation of PKCε to the fraction where MOR and Gαi2 localizing.

Cells were pretreated with 1 μM morphine or DAMGO for 5 mins. Homogenates were fractionated on continuous sucrose gradients. Gαi2 and Gq were used as lipid raft markers; transferrin receptor (TR) as a nonraft marker. Lanes. Left to right: sucrose gradient fractions 1–12.
Morphine treatment increases PKCε associating with MOR. Cells were pretreated with 1 μM morphine or DAMGO for 5 min. MOR signaling complexes were immunoprecipitated with HA antibody and immunoblotted with PKCε antibody; MOR was used as loading control.
Goα is a critical signaling molecule within the MOR signaling complex, and has been proposed as the target of PKC activation and GPCR desensitization (Murthy, Grider et al. 2000). To test this hypothesis, the subtype of Goα used by MOR to invoke [Ca^{2+}]; release need to be identified. Pertussis toxin (PTX) catalyzes the ADP-ribosylation of the α subunits of Gi and Go. This prevents the G proteins from interacting the GPCRs, thus interfering GPCRs signaling (Burns 1988). Endogenous Goα subunits in HEK293 cells were inactivated by pretreatment of PTX while PTX-resistant mutants of Goi2 (Goi2C352L), Goi3 (Goi3C351L) or Goα (GoαC352L) (Zhang, Tetrault et al. 2006) were overexpressed, and [Ca^{2+}], release was monitored after PTX pretreatment. Only the Goi2 mutant restored the morphine-mediated [Ca^{2+}], release in the PTX-treated cells (Fig. 5.9). The inability of overexpressed wild type Goi2 to restore morphine-mediated [Ca^{2+}], release after PTX pretreatment (Fig. 5.10 A) excluded the possibility that the restoration of MOR activity was the result of excessive amounts of the Giα2 protein alone. Moreover, decreases in the responsiveness in the morphine-induced [Ca^{2+}], release in the experiments in which the Goi2 level was attenuated with Goi2 antisense treatment further confirmed the involvement of Goi2 in this MOR signaling process (Fig. 5.10B).

Phosphorylation of Goi2 more than doubled after a 5-min pretreatment with 1 μM morphine (Fig. 5.11). Moreover, the increase in Goi2 phosphorylation was eliminated by PKCε inhibitor but not by inhibitors for the other PKC subtypes, indicating that the Goi2 phosphorylation was PKCε-dependent. The Goi2 sequence
Fig. 5.9 Gαi2 is essential for MOR signaling transduction in HEK293-MOR cells.

PTX-resistant Gαi2 mutants Gαi2C352L (A), Gαi3C351L (B), GαoC352L (C) were overexpressed in HEK293-MOR cells for 48 h. Cells were pretreated with HBSS (Con) (■), PTX 20ng/ml (○) or PTX 100ng/ml (▲) for 16 h before morphine-induced [Ca<sup>2+</sup>]<sub>i</sub> release dose-response assays. Different concentrations of morphine were added with 200nM ADP. Total [Ca<sup>2+</sup>]<sub>i</sub> release after pretreatment was quantitated by calculating the area under curves with a Prism program. Response to ADP was subtracted from total response to obtain the response to morphine. (**: p < 0.01; n=3).
Fig. 5.10  $G_{\alpha 2}$ is essential for MOR signaling transduction in HEK293-MOR cells.

(A) Wild type $G_{\alpha 2}$ ($G_{\alpha 2}$WT) were overexpressed in HEK293-MOR cells for 48 h. Cells were pretreated with HBSS (Con) (■), PTX 20ng/ml (○) or PTX 100ng/ml (▲) for 16 h before morphine-induced $[Ca^{2+}]_{i}$ release dose-response assays. (B) Vector (□), $G_{\alpha 2}$ sense (S) (●) or antisense (AS) (▼) constructs were overexpressed for 48 h before morphine-induced $[Ca^{2+}]_{i}$, release dose-response assays. Different concentrations of morphine were added with 200nM ADP. Total $[Ca^{2+}]_{i}$ release after pretreatment was quantitated by calculating the area under curves with a Prism program. Response to ADP was subtracted from total response to obtain the response to morphine (*: $p < 0.05$; **: $p < 0.01$; n=3).
Fig. 5.11. Gαi2 is phosphorylated by PKCε after morphine treatment.

HEK293-MOR cells were preincubated with specific inhibitors of PKC subtypes α, γ or ε for 3 h before assays. Cells were pretreated with 1 μM morphine. Gαi2 was immunoprecipitated, and Gαi2 phosphorylation was determined using p-Ser antibody. Gαi2 in whole cell lysates was used as a loading control. (top): immunoblots; (bottom): quantitative analysis of immunoblots (#: no significant difference; *: p < 0.05; **: p < 0.01; n=3).
contains five Ser residues predicted to be putative PKCɛ phosphorylation sites (Blom, Gammeltoft et al. 1999; Xue, Ren et al. 2008). These sites were then mutated individually and in combination on the Gαi2C352L mutant. HEK293-MOR cells were transfected with these mutants and pretreated with PTX to eliminate the contribution of endogenous Gαi2 on MOR activity. Relative to the parent strain (i.e., Gαi2C352L), decreases in Gαi2 phosphorylation after PTX and morphine pretreatment were seen in the Gαi2S44A, Gαi2S144A and Gαi2S302A mutants, but not in the other two mutants (Fig. 5.12 A). When these three residues were mutated in combination (Gαi2C352LTM), the increase in Gαi2 phosphorylation after PTX and morphine pretreatment was blocked completely (Fig. 5.12 B).

HEK293-MOR cells overexpressing the Gαi2C352LTM were pretreated with PTX to eliminate endogenous Gαi2 activities. The ability of morphine to induce MOR desensitization in these cells was attenuated significantly (Fig. 5.13 A). In contrast, overexpression of the triple mutant did not affect DAMGO-induced MOR desensitization (Fig. 5.13 B). These data indicate that morphine-induced MOR desensitization results from PKCɛ-induced phosphorylation of Gαi2.

Discussion

Morphine induces a higher degree of tolerance than other opioids such as fentanyl or etorphine administered at equivalent doses (Duttaroy and Yoburn 1995).
Fig. 5.12. Identifying morphine-induced Gαi2 phosphorylation sites.

(A) HEK293-MOR cells were transfected for 48 h with wild type Gαi2 plasmid (WT) PTX-resistant (C352L) Gαi2 plasmid or C352L constructs mutated at serine residues 44, 144, 207, 247 or 302 to alanine. Cells were pretreated with 100 ng/ml PTX for 16 h and with 1 μM morphine for 5 min before determination of Gαi2 phosphorylation as described in legend of Fig.5.11 (B) Cells transfected with wild type Gαi2 plasmid (WT) or C352L mutated at serine residues 44, 144, and 302 (triple mutation; C352LTM) Phosphorylation of the Gαi2 was determined as described previously (*: p < 0.05; **: p < 0.01; n=3).
Fig. 5.13. Morphine-induced MOR desensitization is mediated by PKC-induced Gαi2 phosphorylation.

MOR desensitization was determined by pretreating cells with (A) 100 nM morphine or (B) 100 nM DAMGO for various time as indicated in the X-axis. HEK293-MOR cells were transfected with Gαi2C352L (■) or Gαi2C352LTM (○) for 48 h and pretreated with 100 ng/ml PTX for 16 h before the desensitization assays (*: \( p < 0.05 \); **: \( p < 0.01 \); n=3).
Based on the low efficacy with which morphine recruits βArrs, several hypotheses have been suggested as summarized before. However, a number of in vivo reports indicate that the pathways leading to tolerance development are agonist-dependent, most notably the blunting of fentanyl-induced, but not morphine-induced, tolerance development in GRK3−/− mice (Terman, Jin et al. 2004). The present study indicates that MOR desensitization is also agonist-dependent: Morphine-induced receptor desensitization was PKC-dependent, whereas DAMGO-induced desensitization was receptor phosphorylation and βArrs dependent.

The contribution of PKC to morphine tolerance has been reported extensively (Feng, Narita et al. 1994; Olianas and Onali 1999; Wang, Bilsky et al. 1999; Bohn, Lefkowitz et al. 2002; Smith, Javed et al. 2002; Smith, Javed et al. 2003; Gabra, Bailey et al. 2008), and specific PKC subtypes (α, γ, and ε) have been posited to play a role in morphine function in vivo (Zeitz, Malmberg et al. 2001; Hua, Moore et al. 2002; Fanjun, Junfa et al. 2006; Matsushita, Ishikawa et al. 2007; Newton, Kim et al. 2007; Smith, Gabra et al. 2007). Thus, our observations on the role of PKCε on morphine-induced receptor desensitization parallel those reported for PKCε−/− mice (Newton, Kim et al. 2007). Nevertheless, it is still possible that different PKC subtypes might be involved in acute or chronic morphine actions in different neurons other than those from hippocampus. Indeed, in neurons from the rat locus coeruleus, morphine-activated receptor is desensitized by muscarinic-activated PKCα; although whether morphine activates this PKCα in these neurons remains to be demonstrated (Bailey, Llorente et al.
In vivo studies also have indicated the involvement of PKC\(\alpha\) and PKC\(\gamma\) in morphine analgesia and tolerance (Zeitz, Malmberg et al. 2001; Hua, Moore et al. 2002; Fanjun, Junfa et al. 2006; Matsushita, Ishikawa et al. 2007; Newton, Kim et al. 2007; Smith, Gabra et al. 2007). These discrepancies in the role of PKC subtypes in morphine action could be the result of differential expression of PKC subtypes in neurons expressing MOR. In addition, PKC\(\alpha\) and PKC\(\gamma\) both belong to the conventional PKC subfamily and are activated by Ca\(^{2+}\). In contrast, PKC\(\varepsilon\) is a member of a novel PKC subfamily that is not sensitive to Ca\(^{2+}\) (Newton 2001). The differential requirement of Ca\(^{2+}\) for activation might also account for the different PKC subtype activities in different tissues. Mice knocked-out for the \(\delta\)-opioid receptor (DOR) or the endogenous DOR agonist preproenkephalin fail to develop morphine tolerance, suggesting a possible role for DOR in MOR tolerance development (Zhu, King et al. 1999; Nitsche, Schuller et al. 2002). Although morphine activation of MOR cannot induce [Ca\(^{2+}\)], release, DOR induced [Ca\(^{2+}\)], release in various cell types, including neurons (Okajima, Tomura et al. 1993; Jin, Lee et al. 1994; Thorlin, Eriksson et al. 1998). The distribution of MOR and DOR in the central nervous system (Mansour, Fox et al. 1994) could explain the involvement of different PKC subtypes in the development of morphine tolerance in vivo.

Although previously opioid receptors have been suggested as a molecular target in PKC-mediated phosphorylation, the exact PKC targets involved in morphine-induced receptor desensitization and tolerance development have not been identified (Zhang, Yu et al. 1996). Mutation of morphine-induced phosphorylation sites on MOR were unable
to attenuate morphine-induced MOR desensitization. Also, antibodies specific for PKC substrates failed to recognize MOR in the present study. Clearly, these results do not support the hypothesis that MOR is a PKC target during chronic morphine treatment. The observed reduction in morphine-induced, but not DAMGO-induced, receptor phosphorylation by PKC inhibitors (Zhang, Yu et al. 1996) could be attributed to PKC-mediated phosphorylation and activation of other protein kinases such as GRK or Src (Moyers, Bouton et al. 1993; Krasel, Dammeier et al. 2001) that are known to phosphorylate MOR. Whether morphine-induced MOR phosphorylation participates in either acute or chronic receptor signal transduction needs to be investigated further. Other molecules, such as Gβ, are phosphorylated by PKC and might be related to MOR signaling (Chakrabarti, Regec et al. 2005). A role for Gαi in regulating GPCR desensitization has also been suggested previously (Murthy, Grider et al. 2000), and PKC-mediated Gαi phosphorylation inhibits Gαi activity (Katada, Gilman et al. 1985). Furthermore, phosphorylation of the Gα subunit inhibits its ability to reassociate with Gβγ, thereby impeded G-protein signaling (Kozasa and Gilman 1996). The current study demonstrates that Gαi2 is a target for morphine-activated PKCε and mediates MOR desensitization in HEK293 cells. The importance of PKC-induced phosphorylation of Gαi2 is demonstrated further by the attenuated desensitization seen when the putative PKC sites Gαi2 are mutated.

In summary, MOR desensitization is agonist-dependent: Morphine-induced receptor desensitization was PKC-dependent, whereas DAMGO-induced
desensitization was receptor phosphorylation and βArRs dependent. This phenomenon correlates to that observed *in vivo*. Moreover, instead of MOR itself, Gαi2 was identified as PKC substrate in present studies, and such phosphorylation eventually results in morphine-induced MOR desensitization.
Chapter VI

Crosstalk between MOR and cannabinoid CB1 receptors

Morphine pretreatment heterologously desensitizes CB1 receptor through PKCε-phosphorylated Gαi2
Based on the causation, receptor desensitization is subjected into homologous or heterologous desensitization (Pei, Kieffer et al. 1995; Chuang, Iacovelli et al. 1996). Commonly, GRKs-mediated receptor phosphorylation and subsequent βArrs recruitment result in homologous desensitization, and is thought to be the adaptive response at the level of the receptor itself. In contrast to homologous desensitization, heterologous desensitization usually is resulted from second-messenger-regulated kinases, and may produce larger cellular adaptive response after GPCR activation (Gainetdinov, Premont et al. 2004; Gabra, Bailey et al. 2007).

Increased PKC activity can cause heterologous desensitization (Hosey 1999); at the same time, morphine pretreatment heterologously desensitizes other Gi/o-coupled receptors has also be reported (Rogers, Steele et al. 2000). Therefore, it is possible that the crosstalk between MOR and other GPCRs induced by morphine is through PKC activity. MOR and the cannabinoid receptor CB1 colocalize in the central nervous system and these receptors might interact in vivo (Gaveriaux-Ruff and Kieffer 2002; Pickel, Chan et al. 2004). In addition, CB1 shares most of signal transduction pathways with MOR in vitro and produces analgesia in vivo (Kerr, Hill et al. 1991). CB1 therefore provides a good model to study crosstalk between MOR and other signaling pathways during PKC activation. In this study, the mechanism by which PKC participates in morphine-regulated CB1 activity was examined in vitro. The model derived from these in vitro studies for the interaction between these two Gi/o-coupled receptors might also function in vivo.
Result

Previous data indicate after morphine pretreatment, P2Y receptor activity was not altered (Fig. 3.7). However, P2Y receptor mediates its function via Gq, and it is not known if morphine-induced MOR desensitization is homologous when the activities of other Gi/o receptors are measured. Because MOR-activated PKC\(_\varepsilon\) activity partitioned with the MOR signaling complex (Fig. 5. 6), it is reasonable to hypothesize that morphine treatment might alter the activities of other GPCRs with cellular distributions similar to that of MOR. Previous studies reported possible interactions in vivo and in vitro between MOR and the cannabinoid CB1 receptor (Gaveriaux-Ruff and Kieffer 2002; Pickel, Chan et al. 2004), and like MOR, CB1 is also a Gi/Go-coupled receptor. As with morphine, treatment with the CB1 agonist WIN55,212-2 (Win-2) alone did not elicit \([Ca^{2+}]\), release in HEK293-MOR cells transiently expressing the CB1 receptor, but Win-2 did potentiate P2Y receptor-induced \([Ca^{2+}]\), release (Fig. 6.1). Although possible allosteric effects between MOR and CB1 receptor in vitro have been suggested (Rios, Gomes et al. 2006; Canals and Milligan 2008), neither synergistic nor inhibitory effects were observed when morphine and Win-2 were added together (Fig. 6.2). The absence of an additive response suggests that these receptors might share a common signaling pathway.

To determine the effects of morphine pretreatment on CB1 response, HEK293-MOR cells transiently expressing the CB1 receptor were pretreated with 1\(\mu\)M morphine for 5 min. Then Win-2 was added with ADP and CB1 response was monitored; 10 \(\mu\)M of the MOR-selective antagonist CTOP was added at the same time as the Win-2 and
CB1-induces $[\text{Ca}^{2+}]$, release via the same mechanism as MOR. Time-dependent changes in intracellular fluorescence (RFU) were used to assess $[\text{Ca}^{2+}]$, release. HEK293-MOR cells transiently transfected with CB1 receptor were treated with (A) 3 μM Win-2, (B) 200 nM ADP, or (C) 200 nM ADP + 3 μM Win-2. Data represent the means ± SD from three independent experiments.
No acute inhibitory or synergistic effect between MOR and CB1. HEK293-MOR cells transiently transfected with CB1 receptor were treated with agonists individually or in combination, as indicated. Data represent the means ± SD from three independent experiments. ADP: 200 nM ADP; morph: 1 μM morphine; DA: 1 μM DAMGO; Win-2: 3 μM WIN55,212-2; CTOP: 10 μM CTOP.
ADP to block MOR activity (Fig. 6.3 C). Morphine pretreatment significantly reduced Win-2 potentiation of ADP-mediated \([\text{Ca}^{2+}]\) release (Fig. 6.3 B, C). This attenuation of CB1 activity was prevented by inclusion of 10 \(\mu\text{M}\) CTOP during the morphine pretreatment period (Fig. 6.3 D), indicating that CB1 desensitization resulted heterologously from MOR activation. In contrast, DAMGO pretreatment did not alter the CB1 response (Fig. 6.4).

HEK293-MOR cells transiently transfected with CB1 were then preincubated with PKC\(\varepsilon\) inhibitor; the ability of morphine pretreatment to blunt the Win-2 response was blocked by the inhibitor (Fig. 6.5 A). Furthermore, overexpression of the G\(\alpha_i2\) triple mutant G\(\alpha_i2\)C352LTM rescued the CB1 desensitization induced by morphine pretreatment (Fig. 6.5 B). Taken together, these data suggest that phosphorylation of G\(\alpha_i2\) by morphine-activated PKC\(\varepsilon\) heterologously desensitized CB1 receptor in HEK293 cells.

**Discussion**

Present data indicate inactivation of G\(\alpha_i2\) by PKC-mediated phosphorylation could also reduce the activities of other GPCRs if they share a pool of G\(\alpha\)-subunits with MOR. Functional cross-tolerance between MOR and CB1 *in vivo* has been reported (Gaveriaux-Ruff and Kieffer 2002; Vigano, Rubino et al. 2005), and *in vitro* studies indicated that MOR and CB1 directly antagonize each other’s activity (Rios, Gomes et al. 2006; Schoffelmeer, Hogenboom et al. 2006; Canals and Milligan 2008), which was
Fig. 6.3 Chronic pretreatment of morphine heterologously desensitizes CB1 receptor.
Fig. 6.3 Chronic pretreatment of morphine heterologously desensitizes CB1 receptor

Time-dependent changes in intracellular fluorescence (RFU) were used to assess $[\text{Ca}^{2+}]_i$ release. HEK293-MOR cells transiently expressing the CB1 receptor were pretreated with (A–B) HBSS, (C) 1 μM morphine, or (D) 1 μM morphine + 10 μM CTOP for 5 min, followed addition of (A) 200 nM ADP, (B, D) 200 nM ADP + 3 μM Win-2, or (C) 200 nM ADP + 3 μM Win-2 + 10 μM CTOP. (E) CB1 desensitization after pretreatment with 1 μM morphine or DAMGO was measured at the time indicated in the X-axis (***: $p < 0.005$; n=3).
Fig. 6.4 Morphine, but not DAMGO, induces heterologous desensitization of CB1 receptor.

1µM morphine (■) or DAMGO (○) were used to pretreat HEK293-MOR cells. CB1 receptor response was measured as described in Fig. 6.3. Win-2 max response was determined as subtracting the ADP response from total response. Win-2 response after morphine or DAMGO pretreatment was compared to Win-2 response after HBSS pretreatment to obtain the CB1 receptor desensitization ratio. (***: p < 0.005; n=3).
Morphine-induced CB1 heterologous desensitization is mediated by PKCε-induced Goi2 phosphorylation. (A) Morphine-induced heterologous CB1 desensitization is PKCε dependent. Heterologous desensitization assays were performed as described in Fig. 10. After morphine pretreatment, the Win-2 induced maximum response was compared to the control. Cells were pretreated with PKCε inhibitor (PKCεi) (○) or DMSO (Control) (■) for 3 h before assays. (C) Morphine-induced heterologous CB1 desensitization is Goi2-phosphorylation-dependent. HEK293-MOR overexpressing Goi2C352L or Goi2C352LTM was pretreated with 100 ng/ml PTX for 16 h before assays (**: $p < 0.01$; n=3).
thought to result from the heterodimerization of the two receptors (Rios, Gomes et al. 2006). The current study provides an alternative explanation for the crosstalk between MOR and CB1, i.e., PKC-mediated G\(\alpha\)i2 phosphorylation. Our observations are in accord with a recent study in which only activated MOR and CB1 antagonized each other’s activity (Canals and Milligan 2008).

In mice with decreased Gz protein, cross-tolerance between MOR and CB1 receptors was attenuated (Garzon, de la Torre-Madrid et al. 2009). The Gz-associated protein HINT1/RGSZ translocates PKC\(\gamma\) to MOR in mouse neurons (Rodriguez-Munoz, de la Torre-Madrid et al. 2008); it is reasonable to suggest that decreasing Gz levels also reduce the amount of translocated PKC\(\gamma\). These observations support the possible involvement of PKC in cross-desensitization and cross-tolerance between MOR and CB1. That is, sharing the same pool of G-proteins is necessary and sufficient for morphine-activated PKC to cross-desensitize other GPCRs, and might indeed be the cellular mechanism for some of the interactions observed \textit{in vivo} between MOR and other GPCRs. Moreover, the sharing of the same pool of G\(\alpha\)i2 between MOR and other GPCRs is critical for heterologous desensitization via PKC phosphorylation of G\(\alpha\) subunits: Colocalization within the same microdomain is a prerequisite. This is best illustrated by the inability of morphine-activated MOR to cross-desensitize \(\alpha\)2-adrenergic receptor (Prather, Tsai et al. 1994), which unlike MOR is located outside of the lipid rafts (Chini and Parenti 2004; Zheng, Chu et al. 2008). In contrast, CB1 locates within the lipid rafts (Rimmerman, Hughes et al. 2008), and can be heterologously desensitized by morphine-mediated activation of MOR.
In summary, current study indicates morphine-induced phosphorylation of G\(\text{ai}_2\) by PKC, has significant implications for the responses of other GPCRs that share the same pool of G\(\text{ai}_2\). Since there is a clear distinction among MOR agonists as to whether the \(\beta\text{Arrs}/\text{GRK}\) or PKC pathway is used, future studies on the functional interaction between MOR and other GPCRs will need to consider which signaling and regulatory pathways are involved. Such functional interactions between GPCRs will have to be considered in drug design, specifically, whether homologous or heterologous desensitization among receptors occurs based on the GPCRs and subsequent pathways to be activated.
Chapter VII

Summary
Chronic or repetitive usage of opioid analgesics such as morphine results in the development of tolerance, limiting the clinical application of opioids. Thus opioid receptor desensitization was suggested to be closely related to the in vivo tolerance development; therefore, the mechanism of opioid receptor desensitization has been the focus of increasing numbers of laboratories.

Chronic or repetitive usage of opioid analgesics such as morphine results in the development of tolerance, limiting the clinical application of opioids. Thus opioid receptor desensitization was suggested to be closely related to the in vivo tolerance development; therefore, the mechanism of opioid receptor desensitization has been the focus of increasing numbers of laboratories.

The understanding of GPCR desensitization is based on the GRK-βArrs-mediated desensitization. After agonist binding to GPCR, the receptor was phosphorylated by GRK, and the receptor phosphorylation leads to the recruitment of βArrs, which results in the uncoupling of G protein from receptor complex and the cessation of receptor signaling. However, the existence of discrepancy between the kinetics of MOR desensitization and GRK-mediated receptor phosphorylation leading to βArrs recruitment has led to uncertainties in the MOR desensitization mechanism. To elucidate this, we have developed a novel method to monitor MOR activity. MOR activates [Ca^{2+}]_{i} store need the co-activation of Gq-coupled receptor. By taking advantage of this special characteristic, we are able to measure receptor desensitization with the eliminated influence of agonist pretreatment on downstream signaling pathways. Moreover, since MOR activates [Ca^{2+}]_{i} store through Gβγ-subunits which are less efficient in transducing opioid receptor signals than Gα-subunit, it much more
sensitively reflects MOR activity change, and thus provides an opportunity to study MOR acute desensitization mechanism in detail.

In the current thesis studies, the role of MOR phosphorylation and βArrs are investigated. By using individual phosphorylation site mutant MOR, my studies provide direct evidence of the involvement of receptor phosphorylation in MOR activity regulation. In addition, it is demonstrated that agonist-induced MOR phosphorylation on each individual site did not function equally in the MOR desensitization process. Moreover, this is the first time that it has been clearly demonstrated βArrs is absolutely required in DAMGO-induced MOR desensitization: eliminating βArrs expression prevents the development of DAMGO desensitization in the cellular model; this correlates to the observation of βArrs’ function in opioid tolerance in vivo.

Furthermore, during the thesis studies, it was interesting to find out that MOR desensitization mechanism is agonist-dependent. Unlike DAMGO, high potent tolerance inducer morphine-induced MOR desensitization is not affected by receptor phosphorylation and the absence of βArr. PKC, especially PKCε, is highly involved in MOR desensitization in HEK293 cells. Such agonist-dependent desensitization mechanism could be due to different abilities of agonists on activating downstream signals or different affinities of agonist-receptor complexes to certain regulator. Present studies demonstrate PKCε is specially activated by morphine but not DAMGO, thus initiates the agonist-dependent desensitization.
In addition, by using phosphorylation sites triple mutant Gαi2C352LTM, this study provides the first instance that directly establishes the involvement of Gαi proteins’ phosphorylation in MOR desensitization as well as the first time to demonstrate the exact mechanism of morphine-induced MOR desensitization. Previously, it was considered PKC-mediated MOR desensitization was due to the receptor phosphorylation. Present data show that morphine-, but not DAMGO-, induced MOR desensitization is through PKCɛ-phosphorylated Gαi2 subunit. Unlike phosphorylation at the receptor level, which causes homologous desensitization, phosphorylation at Gα subunit produces more profound impacts in other receptor cellular signaling pathways. Such functional interactions between GPCRs will have to be considered in drug design.

Our group are among the first few groups to notice the agonist-dependent phenomena in MOR signaling. This agonist-dependent signaling corresponds to the agonist’s functions in vivo. We hypothesis that agonist-dependent signaling results in distinct cellular adaptation response, such as βArrs recruitment or Gαi phosphorylation; thus eventually leading to the different ability of agonist on inducing tolerance. The mechanism of agonist-induced MOR desensitization could be a good predictor for the agonist properties; and should be considered in the future drug design for a better analgesia with less potential to induce tolerance development.
References


