μ-Opioid Receptor Regulation: Desensitization and Re-sensitization

By

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Abstract:

Tolerance and dependence can result from long-term exposure to opiates. The cause for these negative side effects has been linked to changes at the cellular level in the central nervous system (CNS). While the exact mechanism behind the development of tolerance and dependence is unclear, there is growing evidence linking acute receptor desensitization and internalization to these processes.

In this study, several properties of μ opioid receptor (MOR) regulation were examined using whole cell and intracellular recordings. The results show that the rank order of opiates was different when comparing the magnitude of hyperpolarization and their ability to cause desensitization in locus coeruleous (LC) neurons. The relative ability of opiates to cause endocytosis in HEK293 cells correlated well with the degree of desensitization in LC neurons. This strong correlation suggests that the processes are closely linked. It also suggests that agonist efficacy is not necessarily a predictor of the ability of opiates to cause MOR desensitization or internalization.

When tested in naïve LC neurons, morphine can cause homologous desensitization. After exposure to high concentration of [Met]^{5}enkephalin (ME), morphine induced desensitization was occluded.

Following chronic morphine treatment, MOR regulation was altered as follows. First, the rate of desensitization was increased. Second, recovery from desensitization was always incomplete, even after a brief (1-2 min) exposure to agonist. When brain slices from controls were treated with inhibitors of PKC or monensin, agents known to compromise g-protein coupled receptor (GPCR) re-sensitization, desensitization was

increased and recovery was significantly reduced. These results indicate that after chronic morphine treatment, MOR desensitization is potentiated and receptor re-sensitization is compromised.

Chronic morphine treatment also facilitated the manifestation of heterologous desensitization of the α_2–adrenoceptor induce response. This process occurs through the mitogen activated protein kinase (MAPK) pathway and is dependent of receptor endocytosis.

Together, these results indicate that acute MOR desensitization and the adaptive processes associated with the development of tolerance are linked. They also suggest that acute desensitization involves multiple, perhaps redundant components. This adds to the complexity of the process, making it difficult to characterized the mechanism(s) behind desensitization and cellular tolerance, particularly in neurons.

Abbreviations:

CNS	Central nervous system
DRG	Dorsal root ganglion
LC	Locus Coeruleous
PAG	Periaqueductal gray area
GPCR	G-protein coupled receptor
MOR	μ opioid receptor
GRK2	G-protein coupled receptor kinase 2
Arr-3	ß-arrestin2
AP2	Adaptor protein 2
ERK	Extracellular signal-regulated kinase
PKC	Protein kinase C
PKA	cAMP dependent protein kinase A
CAMK II	Calcium calmodulin dependent kinase II
VDCC	Voltage dependent calcium channel
GIRK	G-protein coupled inward rectifying potassium channel
PP2A/PP2B	Protein phosphatases 2A/2B
PI3K	Phosphoinositide-3 kinase
DERM-BTR	Dermorphine Bodipy Texas Red
DERM-A488	Dermorphine Alexa®Flour 488
DAMGO	[D-Ala^2,MePhe^4,Gly^5-ol]enkephalin
ME	Methionine Enkephalin
M6G	Morphine-6-ß-glucaronide

MP	Morphine
MD	Methadone
NA	Noradrenaline
MTA	Morphine treated animals

Introduction:

Opiates such as morphine are very important analgesics used for pain management. After extensive usage, however, their therapeutic property is negated by their adverse side effects. Although the mechanism is unclear, long-term use of opiates leads to the development of tolerance, where higher doses are required to produce the desired effect.

Chronic exposure to opiates also causes compensatory changes at the cellular level, in response to drug exposure, to reestablish physiological homeostasis (reviewed in [1]). It is not understood how changes at the cellular level directly contribute to its pathology of dependence, but when drug intake is ceased, these compensatory adaptations cause the precipitation of withdrawal. All together, these adverse effects limit the clinical use of opiates.

Studies using animal models suggest that morphine analgesia, tolerance, and dependence are mediated through the Mu-opioid receptor (MOR) [2,3]. When MORs are constantly stimulated, particularly by an efficacious opiate such as Methionine-enkephalin (ME), there is a gradual loss of MOR function, called desensitization. This observation has led investigators to implicate acute desensitization to be the precursor for opioid tolerance. As such, the mechanisms behind the process of acute desensitization may be the same ones that lead to the development of tolerance. This study will examine the process of acute MOR desensitization and re-sensitization in native neurons. By understanding how MORs are regulated in neurons during sustained stimulation by opiates, the mechanism leading to the development of tolerance will be better understood.

This will facilitate the development of drugs that can produce therapeutic effects without causing the negative side effects.

BACKGROUND:

Opioid receptors

Opiates induce their physiological effects by activating their respective GPCRs. Currently, there are three subtypes of opioid receptors: μ, ∂, and k opioid receptors [4]. As members of the GPCR super-family, opioid receptors have seven transmembrane domains with an extracellular amino terminus and intracellular carboxyl terminus. When activated by ligand, opioid receptors, like other GPCRs, function as the GDP-GTP exchanger for the heterotrimeric G-protein. Activation of the heterotrimeric G-protein cause it to dissociate into G_{α} and a $G_{\beta\gamma}$ subunits. Together, they function as the second messengers, interacting with a series of effectors, transforming extracellular stimuli into intracellular signals [5-7]. A simple illustration of MOR-mediated signaling is shown in Figure 1.

Opioid receptors are coupled to $G_{i/o}$, the family of inhibitory G-proteins that act to reduce cellular activity and signaling. Activated G_{α} binds to and inhibits adenylyl-cyclase (AC) function, preventing the synthesis of cAMP. The $G_{\beta\gamma}$ subunit binds to and inhibits the activation of voltage-dependent calcium channels (VDCC), blocking neurotransmitter release [8,9]. The $G_{\beta\gamma}$ subunit also binds to and activates G-protein-coupled inward rectifying potassium (GIRK) channels [10]. The activation of these channels leads to channel opening, inducing an outward potassium conductance and causing the membrane potential to become hyperpolarized.

With persistent stimulation, MOR-mediated signaling is reduced, or desensitized. This is an adaptive process that allows the cell to regain homeostasis during intense and sustained stimulation[11-16].

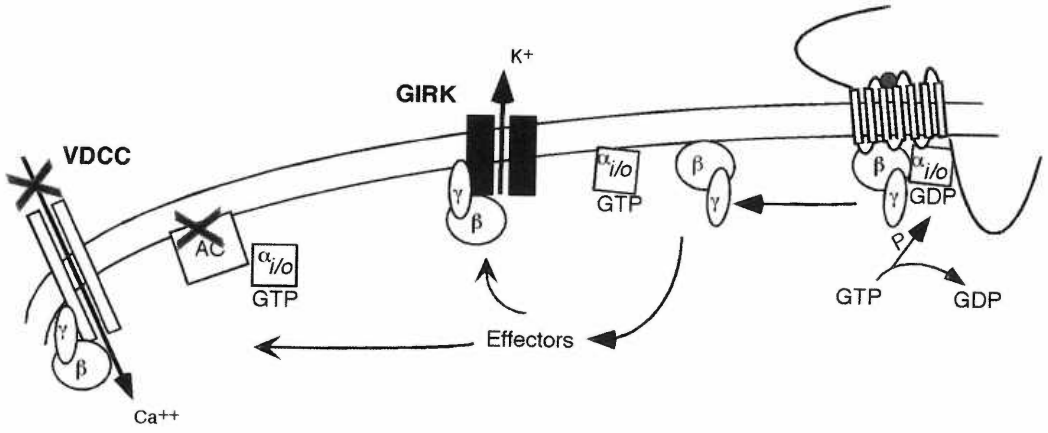


Figure 1. MOR-mediated signaling. MOR activates $G\alpha_{i/o}$ G-proteins. These G-proteins inhibit voltage-dependent calcium channels (VDCC) and adenylyl cyclase (AC). They activate the G-protein-coupled inward rectifying potassium channel (GIRK) and other effectors that act to reduce neurotransmitter release and cellular excitability.

MOR Desensitization

GRK2, ß-arrestin mediated MOR Desensitization

The mechanism behind GPCR regulation has been studied in a variety of systems, using a variety of effectors as the measure for receptor function. One of the widely accepted mechanisms for GPCR regulation involves receptor phosphorylation, sequestration, and internalization [17-19]. For the MOR, the process of receptor regulation is initiated by the phosphorylation of the ligand-bound receptor by G-protein-coupled receptor kinase 2 (GRK2). Under basal conditions, the inactive GRK2 resides mostly in

the cytoplasm. Activation of MOR activates $G_{\beta\gamma}$, which also binds to GRK2. In conjunction with its interaction with charged phospholipids, GRK2 is activated and trafficked to the plasma membrane [20-23] (Figure 2). At the plasma membrane, GRK2 binds to and phosphorylates the ligand-bound receptor [24-27]. The phosphorylation of specific serine and threonine residues by GRK2 on the second intracellular loop and the carboxyl terminus of the ligand bound receptor increases the receptor binding affinity for ß-arrestin2 [24].

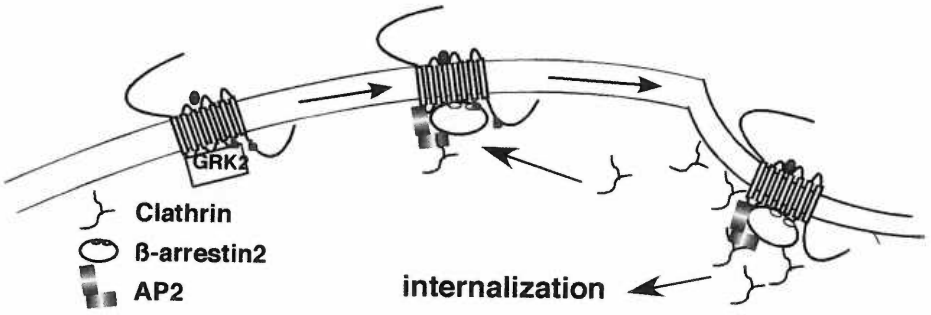


Figure 2. MOR internalization. Receptor phosphorylation by G-protein coupled receptor kinase 2 (GRK2) increased the receptor binding affinity with ß-arrestin2. ß-arrestin2 recruits adaptor protein-2 (AP-2), which leads to receptor sequestration into clathrin-coated pits. The receptors are then internalized.

ß arrestin2 recognizes and binds to the GRK2-phosphorylated MOR on the second and third intracellular loops. These are also the sites for receptor-G-protein interaction; therefore, ß-arrestin2 binding prevents further G-protein activation by physically inhibiting their interaction with the receptors [28-30]. Apart from preventing further receptor mediated signaling, ß-arrestin2 binding to MOR also leads to receptor sequestration and downregulation. This process is initiated by the recruitment of AP2, an adapter protein, to the ligand/receptor/ß-arrestin complex. AP2 binding facilitates the

interaction of the receptor-ß-arrestin complex to the clathrin-coated pits, leading to receptor sequestration and internalization by a dynamin-dependent mechanism [31-33].

Figure 2 shows that the binding of ß-arrestin2 and subsequent receptor internalization mark the end of MOR signaling, but it is still unclear where in the process the loss of receptor function occurs.

Alternative Pathway for MOR Desensitization

Current studies are providing more evidence supporting the notion of alternative mechanisms for MOR desensitization. Biochemical studies indicate that MOR directly interacts with calcium-calmodulin-dependent kinase II (CAM kinase II). Phosphorylation of the receptor by CAM kinase II reduces the coupling of MOR with the G-protein. Cyclic-AMP dependent protein kinase A (PKA), as well as protein kinase C (PKC), has also been demonstrated to be involved in the process of MOR desensitization [34-38]. Even though these studies showed that MOR could be phosphorylated by these kinases, there is no direct evidence showing that MOR phosphorylation by these protein kinases leads to receptor sequestration and internalization.

Time course of MOR desensitization

Studies trying to determine the time course of MOR desensitization have yielded inconsistent results [36,39,40]. Part of the observed differences can be attributed to the different systems used in the studies, as well as differences in assays used to determine receptor functions [41]. It is also possible that the onset of receptor desensitization involves more than one component or mechanism, making it difficult to determine when and how

receptor function is attenuated during sustained stimulation. Acute receptor desensitization can be assayed at different steps along the regulatory process, starting with receptor phosphorylation and ending with receptor internalization.

MOR Phosphorylation and Internalization

During sustained stimulation with a high concentration of agonist, MOR phosphorylation can occur in minutes, reaching the maximum level within 15-20 minutes [41-44]. Studies examining receptor endocytosis indicate that a significant level of receptor internalization can be detected after 2 min [45]. Together, these studies show that the initiation of the desensitization process occurs rapidly. Thus far, there is no direct evidence linking receptor phosphorylation or internalization to the loss of receptor function.

Loss of MOR function

Sustained stimulation of MOR leads to a reduction in the maximal response. The rate at which this occurs varies with the assays used to detect the loss of receptor function. One measure of MOR function is to assay for G_α inhibition of AC. With this assay, it took 30 minutes to several hours before significant receptor desensitization was detected [41,43,46,47]. Because receptor reserve plays an important role in the manifestation of GPCR desensitization, the large discrepancy in the time course of desensitization can be attributed to the differences in the levels of receptor expression among the studies, the expression systems used in them, or both [41].

MOR function can also be assessed through its activation of GIRK channels or the inhibition of VDCCs [11,39,48,49]. When the activation of the GIRK channel was used as a measure for receptor function, significant desensitization of the maximal response was detected after a 5 min exposure to the supra-maximal concentration of agonist [12-16,39]. Similar results were seen with the inhibition of calcium channels as the measure of MOR function [49].

Although receptor internalization in neurons has not been demonstrated to be linked to the observed loss of MOR function, there is a high correlation between MOR desensitization and receptor internalization [11,15]. Apart from removing receptors from the plasma membrane, the process of receptor internalization is also crucial for the recovery of MOR from acute desensitization [36,50-52]. Recent evidence suggests that MOR endocytosis can also promote intracellular signaling via ERK1/2[29].

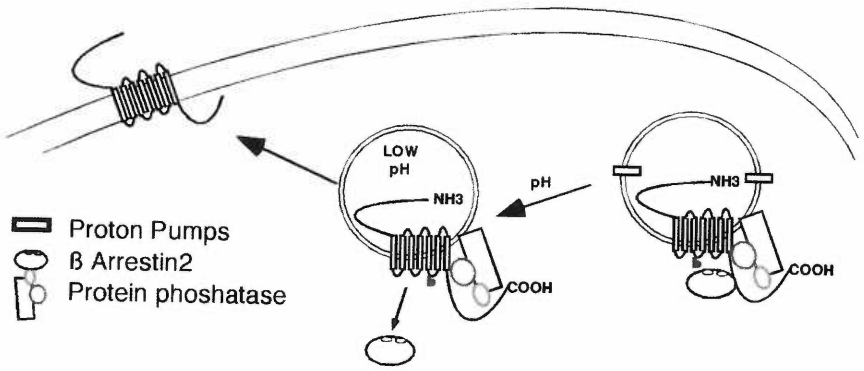
MOR Re-sensitization

Endocytosis of desensitized GPCRs is an important component of the receptor regulation process. It is this stage of receptor regulation that dictates the fate of the receptors. Once internalized, receptors are trafficked to the early endosomes, where they are sorted and transported to the appropriate regulatory pathway [53-55]. There are three possible post endocytic fates for internalized GPCRs as determined by specific amino acid sequences in their carboxyl termini: (1) rapid recycling, (2) delayed recycling, and (3) degradation [50,53,56,57].

Rapidly recycled GPCRs are sorted and transported to endosomal compartments. The low pH in the endosome causes the receptors to change conformation and dissociate

from ß-arrestin. The dissociation of ß-arrestin allows protein phosphatases (PP2A/PP2B) to dephosphorylate the desensitized receptors [58,59]. After the receptors are dephosphorylated, the functional receptors are transported to and reinserted into the plasma membrane [24,41,45]. This process of receptor re-sensitization is illustrated in Figure 3.

Figure 3: MOR re-sensitization. After receptor internalization, MOR is sorted to the endosome. The acidic environment causes the receptor to change its conformation, allowing ß-arrestin2 to



dissociate from the MOR. The receptor is then dephosphorylated by protein phosphatases and becomes re-sensitized. The functional MOR is re-inserted into the plasma membrane.

Delayed-recycling GPCRs are sorted in the early endosomes, then trafficked to intracellular compartments resembling the perinuclear compartments [60]. The mechanism for this process of receptor recycling is not fully understood.

GPCRs that are destined for receptor degradation are trafficked to lysosomal compartments, where they undergo enzymatic degradation [56,61,62]. When the receptor is destined for receptor degradation, it is ubitiquinated during the endocytic process. GPCR ubitiquination is the marker for protein degradation. This process is dependent on ß arrestin as a scaffolding protein [33,63-66].

Regulation of MOR Cycling

The post-endocytic fate for GPCR is determined by the specific sequence of amino acids at their carboxyl terminus. As expected, this is a very precise regulatory process, where variation of one amino acid or phosphorylation state can significantly alters the time course and/or fate of the desensitized receptor [50,54,62,67-69].

For the rat MOR, there are two splice variants, MOR1 and MOR1B, with identical sequence homology except for the last 24 amino acids at the carboxyl terminus [36,70]. Studies of the rat MOR1 indicate that it is a rapidly recycling receptor [36,41,45,71]. Recently, Tanowitz et al. have shown that, MOR1 contains a sequence of amino acids, LENLEAE, that enables the internalized receptors to be sorted and trafficked to the recycling pathway [66]. Although both splice variants of MOR recycle and are re-sensitized after acute desensitization, differences in their carboxyl termini produced very different rate of recycling. The MOR1B splice variant has a shorter carboxyl terminus and thus has a faster recycling kinetic. The short C-terminus enhances MOR1B interaction with clathrin-coated pits, enabling faster rate of receptor internalization and thus faster receptor re-sensitization [36,70]. The MOR1 splice variant of the rat opioid receptor is different from MOR1B in that it has a longer carboxyl terminus with a threonine residue at position 394. Phosphorylation of threonine 394 on MOR-1 slowed internalization and delayed recovery following acute desensitization [40].

MOR activation of ERK1/2

Activation of GPCRs can lead to the activation of ERK1/2. The mechanism by which this occurs is still controversial. One early study suggested that receptor endocytosis was necessary for the activation of ERK1/2 [29]. It was proposed that ß-arrestin2 functioned as the scaffolding protein, recruiting cSRC to the plasma membrane, during the process of receptor endocytosis. There, cSRC phoshorylated Ras, converting it into the active form Ras-GTP. Ras-GTP then recruited and activated Raf-1. Raf1, which is a mitogen-activated protein kinase kinase kinase (MEKK), activates MEK1, which phosphorylates and activates ERK1/2.

For the MOR, it was also demonstrated that ERK1/2 activation can occur in a receptor-endocytosis-independent manner [30,72]. While this process is independent of receptor endocytosis, functional dynamin was necessary for the activation of ERK1/2. Recent work also showed that morphine, a MOR agonist that does not cause receptor endocytosis, is capable of inducing ERK1/2 phosphorylation in vivo [73].

Acute desensitization and tolerance

Acute desensitization of MOR has been implicated to be the precursor of opioid tolerance in a variety of expression systems and animal studies [74-77]. Recent studies using molecular manipulations present conflicting results regarding the mechanisms leading to morphine anti-nociceptive tolerance [75,78,79].

Studies from mice lacking ß arrestin2 demonstrate that these mice have increased morphine sensitivity after acute morphine administration, and morphine anti-nociceptive

tolerance, as determined via a hot plate paw withdrawal assay, was attenuated after chronic treatment [75].

Taking a different approach, Whistler et al. demonstrated that by inducing receptor desensitization i.e. receptor internalization morphine anti-nociceptive tolerance was inhibited [78]. This observation was the foundation for the RAVE hypothesis. RAVE, Relative Activity Versus Endocytosis, suggests that opioid tolerance occurs as a result of overstimulation. When a ligand is capable of eliciting high level of activity but does not cause desensitization, the sustained stimulation will cause compensatory changes in the cell that counteract the receptor-mediated signaling. As such, morphine, which is a potent analgesic that does not cause MOR desensitization, does cause dramatic opioid tolerance.

Whereas the RAVE hypothesis predicts that the inhibition of receptor endocytosis would increase morphine anti-nociceptive tolerance, results from the ß-arrestin2 knockout model suggest that inhibiting MOR desensitization abolishes morphine anti-nociceptive tolerance. One possible explanation for the discrepancies between the two studies is that different assays were used to determine morphine anti-nociceptive tolerance. As demonstrated by Bohn et al., morphine tolerance is differentially expressed in the supra-spinal reflex, which is the pathway for pain recognition in the tail-flick assay used by Whistler et al. [80]. When this technique was used to measure the pain reflex, morphine tolerance was delayed in ß-arrestin2 knockout mice but was apparent after days 4 and 5. When ß arrestin2 knockout mice were treated with chelerythrin, a protein kinase C (PKC) inhibitor, morphine anti-nociceptive tolerance was inhibited [81]. The mechanism by which chelerythrin inhibits the induction of tolerance is unclear.

All together, these results show that MOR regulation is a complex process that may affect several levels of receptor-mediated signaling. The fact that different cells from different areas of the CNS are differentially affected by chronic morphine treatment adds to the complexity of the mechanisms behind opioid tolerance.

Acute MOR Desensitization in Neurons

Much of the current understanding of the mechanisms behind MOR regulation came from studies using molecular and biochemical approaches. These studies have provided many models for the process of MOR regulation. However, limited resolution and assay sensitivity have made it difficult to detect rapid and subtle changes in receptor function; therefore detailed and functional characterization of the mechanisms involved remain unclear.

In native neurons, MOR couples to GIRK, VDCC, or both. MOR coupling to GIRK and VDCC is fast and are sensitive to receptor reserve; therefore, real-time examination of receptor function in native neurons is possible by using electrophysiology as the tool to assay for MOR function [39,82]. Studying MOR regulation in native neurons also provides a more precise understanding of receptor function and regulation in vivo. To date, however, the number of studies looking at MOR regulation in native neurons is limited.

Electrophysiological recordings done from locus coeruleus (LC) neurons showed that exposure to supra-maximal concentration of ME (30μM) caused a maximum response that gradually declined during the sustained application of drug [13]. Depending on the preparation and recording conditions, a 5 min exposure to ME (30μM) caused a decline of 30-50% of the maximal GIRK-mediated response. This component of acute desensitization is primarily homologous and shows significant recovery in 20-30 minutes [12-15]. Many attempts were made to block the induction of desensitization using various protein kinase and phosphatase inhibitors. Treatment of LC neurons with general

phosphatase inhibitors such as okadaic acid or mycrocystin failed to alter the induction of MOR desensitization. Microcystin did, however, attenuate receptor recovery [14].

Recent work by Blanchet et al. indicates that extended desensitization of MOR with DAMGO leads to the manifestation of heterologous desensitization such that the α_2-adrenoceptor-mediated GIRK current was attenuated following MOR desensitization [16]. Although the mechanism was not identified, it was proposed to occur at the effectors rather than the receptors.

Examinations of MOR desensitization in dorsal root ganglion (DRG) neurons indicate that sustained stimulation (4 hrs) with DAMGO reduced DAMGO inhibition of VDCC. The mechanism behind this decline is dependent on phosphoinositide 3-kinase (PI3K) and ERK1/2 and occurred at the channels, not the receptor [83].

A summary of the results indicates that MOR desensitization in native neurons occurs rapidly after exposure to a maximal concentration of efficacious agonist. Desensitization of MOR for 5-10 min is mostly homologous (in current-clamp recordings), and MOR re-sensitization is nearly complete after 20-30 min. Following extended desensitization, heterologous desensitization of the α_2-adrenoceptors can occur. In sensory neurons, acute desensitization of MOR inhibition of VDCCs is mediated via a PI3K- and ERK1/2-dependent mechanism. Under this condition, acute desensitization occurs at the effector rather than the receptor. These results generate as many questions as they answered. For instance, how does acute desensitization occur? What role does receptor reserve play in shaping the rate of desensitization? How would ligands with different efficacy and potency affect this process? How would MOR regulation be altered following chronic morphine treatment? What is the mechanism behind

heterologous desensitization of the α_2-adrenoceptors? The studies presented here will address many of these questions.

Working hypothesis:

MOR desensitization and internalization have been implicated to cause morphine analgesic tolerance [75,79]. Different opiates have different potency and efficacy for MOR. These pharmacological properties have been proposed to be good predictors of the likelihood that a drug will cause tolerance and dependence.

- The efficacy and potency of the agonist represent its propensity for causing receptor desensitization.

Currently, there have been no studies done in native neurons showing loss of MOR function following extended exposure to morphine. Studies using expression systems provided contradictory results. Under basal conditions, most studies showed that morphine does not cause desensitization or MOR endocytosis [15,78,84,85]. Borland et al., however, did show that morphine could cause rapid desensitization in AtT20 cells [11].

- Morphine does cause MOR desensitization.

Chronic morphine treatment increases GRK2 and ß arrestin2 expression in the neurons of the LC [86]. These are important regulatory proteins for MOR regulation [87]. When GRK2 is overexpressed, MOR desensitization is facilitated [84].

- Increased receptor desensitization causes receptor-mediated tolerance following chronic morphine treatment.

Heterologous desensitization of the α_2-adrenoceptor can result from ME- or DAMGO-induced MOR desensitization. These opioid peptides have been shown to be potent inducers of MOR internalization. The process of MOR internalization has been shown to occur through ß-arrestin- and dynamin-dependent mechanisms. These processes also activate ERK1/2, which can modulate cellular signaling.

- ERK1/2 activation following MOR desensitization causes heterologous desensitization of the α_2-adrenoceptor-induced potassium conductance.

METHODS

Electrophysiological recordings

Whole-cell recordings were done in horizontal brainstem slices (225-250 μm) containing the LC prepared from adult male Sprague Dawley rats (140-200g) (Charles River) as previously described [88]. Extracellular solution contained (mM):126 NaCl, 2.5 KCl, 2.4 $CaCl_2$, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 21.4 $NaHCO_3$, 11.1 glucose, equilibrated with 95%O_2-5%CO_2 at 35°C. Whole-cell recordings were made using Nomarski optics and infrared illumination. Recordings were made with an Axoclamp-2A amplifier (Axon Instruments, Foster City, CA) in current-clamp or voltage-clamp mode. Pipettes (2-3 MΩ) were filled with internal solution containing (in mM): 115 MES (2-[morpholino]-ethane-sulfonic acid) Potassium salt, 20 KCl, 1.5 $MgCl_2$, 1 BAPTA, 5 HEPES, 4 Mg-ATP and 0.4 Na-GTP, pH= 7.3.

For intracellular recordings, pipettes (30-50 MΩ) were filled with 2 M KCl and recordings were made with an Axoclamp 2A (Axon Instruments, Foster City, CA). Data was acquired at 100Hz (Chart version 4.0, MacLab System; DInstruments, Castle Hill, Australia) and analyzed in Axograph (version 4.5, Axon Instruments) when necessary.

All the experiments were performed at 35°C, and, unless otherwise stated, pharmacological agents were applied by bath perfusion. All experiments with ME were done in the presence of the peptidase inhibitors bestatin (10 μM) and thiorphan (1 μM). In some cases, fluorescent agonists were applied at high concentrations using pressure ejection from a patch pipette placed in the slice within 50 μm from the recording site.

Desensitization Protocols

Four different protocols were used to measure MOR desensitization of the hyperpolarization response in LC neurons (Figure 4). One method compared the amplitude of the hyperpolarization produced by an EC_{50} concentration of [Met]^5-enkephalin (ME, 300 nM) before and after exposure to a high desensitizing concentration of ME ([13]; figure 4A). The amplitude of the opioid response was calculated as the average value of the membrane potential during the opioid perfusion minus the average resting membrane potential before the application. This protocol was particularly good for studying desensitization induced by ME because it cleared rapidly from the preparation after washout, so that an EC_{50} concentration of ME could be tested within 2-3 min. In order to examine desensitization of other opioid agonists, including DERM-BTR, a new protocol that did not require rapid agonist washout was used (Figure 4B). Previous work has shown that, following MOR desensitization, the maximal amplitude of the opioid response was depressed [14]. This protocol takes advantage of that observation and measures the maximal response to ME (10 μM) at various times during a prolonged application of other agonists applied at submaximal concentrations. The applications of ME (10 μM) were limited to 1.5 min with a 5 min washout, to reduce the induction of desensitization by the ME applications alone. Decreases in the amplitude of the maximal ME response produced by a prolonged application (25min) of the test agonist were taken as a measure of desensitization. At the end of the agonist treatment, naloxone (1 μM) was used to reverse the opioid-induced hyperpolarization. Once the potential returned to baseline, a maximal concentration of the α_2-adrenoreceptor agonist UK14304 (3 μM) was tested to control for both rundown of the signal pathway during the experiment and for

heterologous desensitization. This method was used for measuring desensitization induced by agonists that did not wash from the preparation. A third method measured the decline in the amplitude of the hyperpolarization induced by a high agonist concentration (Figure 4C). This decline was taken as a sign of desensitization. At the end of each experiment, a maximal concentration of the α_2-adrenoreceptor agonist, UK14304 (3 μM) was applied. The hyperpolarization induced by UK14304 was reversed by superfusion with the α_2–adenoceptor antagonist yohimbine (1-10μM).

Examination of heterologous desensitization was done in coronal sections (260-290μM) under voltage clamp. Membrane potential was held at -50)--60mV.

MOR endocytosis

MOR endocytosis was determined by feeding experiments as described elsewhere [89]. Human Embryonic Kidney (HEK) 293 cells stably expressing flag-tagged MOR were grown in DMEM supplemented with 10% Fetal Bovine Serum and propagated in the presence of 700 μg/ml G418 (all tissue culture reagents were purchased from Life Technologies, MD). For the experiments, cells were grown on poly-D-lysine coated coverslips (Fisher Scientific, PA) to about 60% confluence. They were incubated with 1:500 dilution of M1 mouse anti-FLAG antibody (Sigma, MO) for 5 min at 37°C, then with agonists. At the end of incubation period, the antibody was stripped off by a brief 1 min rinse at 37°C with a hypertonic acid solution (DMEM adjusted to pH 4 with acetic acid + 0.5 M NaCl + 5 mM EDTA). This treatment disrupted the extracellular calcium-sensitive M1-epitope interaction and the residual membrane-bound agonst. Cells were fixed in 4% paraformaldehyde in PBS, permeabilized in 0.1%Triton X-100, and stained

with Alexa®Fluor 488 goat anti-mouse antibody (Molecular Probes, Inc., Eugene, OR) or Cy5 goat anti-mouse antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Cover slips were mounted in Mowiol®(Sigma, St Louis, MO).

Image acquisition and analyses: Images were acquired under a confocal microscope equipped with a krypton-argon laser coupled with a Bio-Rad MRC-1000 and an Optiphot II Nikon microscope. Cells were visualized under a Plan Apo 60X objective lens (1.4 NA, oli). The filters used for scanning Alexa488 were 488 nm for excitation and 522 nm for emission, and for Cy5 were 647 nm for excitation and 680 nm for emission. Pictures were taken from 4-5 fields from each coverslip and analyzed by Scion image for Windows software (version Beta 4.0.2, Scion Corp, Frederick, MD). The fluorescence intensity of about 50 individual cells (10 cells/field) was determined for each coverslip and one mean fluorescence intensity value was obtained. For each condition, a duplicate in one experiment and 3-5 separate experiments were performed.

Statistics and curve fitting

Data analysis was done with PRISM Analysis software. Values are given as mean±SEM. For all experiments, $p < 0.05$ was considered as a significant difference. Multiple group comparisons were made with one-way ANOVA analysis or repeated measure ANOVA analysis followed by Bonferroni's or Dunnett's multiple comparison tests. Two-group comparisons were made using paired or unpaired t tests. Statistical analysis, curve fits,

and correlations were performed with GraphPad Prism (GraphPad Software, Inc, San Diego, CA).

MATERIALS: [Met]^5-enkephalin, dermorphin, bestatin and yohimbine were obtained from Sigma (St. Louis, MO). Naloxone and UK14304 were obtained from RBI (Natick, MA). Thiorphan was obtained from Bachem (Torrance, CA). Dermorphin-BTR and dermorphin-A488 were prepared as described in Arttamangkul et al.., 2000). Morphine, methadone, normorphine, and etorphine were obtained from NIDA.

Chapter 1

μ-Opioid Receptor: Ligand-dependent Activation of Potassium Conductance, Desnsitization, and Internalization

Veronica A. Alvarez, Seksiri Arttamangkul, Vu Dang, Abdallah Salem, Jennifer L. Whistler, Mark von Zastrow, David K. Grandy, John T. Williams.
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Mu-opioid receptor (MOR) desensitization and endocytosis have been implicated in tolerance and dependence of opioids. The efficiency of each process is known to be agonist-dependent; however it is not known what determines the relative efficiency of various agonists at either process. In this chapter, homologous MOR desensitization in locus coeruleus (LC) neurons and MOR internalization in HEK293 cells were examined using a series of agonists. The results show that the rank order of this series of agonists was different when comparing the magnitude of hyperpolarization and the ability to cause desensitization in LC neurons. Endocytosis of MOR was also examined in HEK293 cells, using the same agonists. The relative ability to cause endocytosis in HEK293 cells correlated with the degree of desensitization in LC cells. This strong correlation suggests that the two processes are closely linked. The results also suggest that agonist efficacy is not necessarily a predictor of the ability to cause MOR desensitization or endocytosis. Identification and characterization of the biophysical properties of agonists that favor desensitization and internalization of receptors will lead to a better understanding of opioid signaling.

MOR desensitization

The response to an EC_{50} concentration of ME (300 nM) was significantly reduced after treatment with a high concentration of ME (30 μM, protocol figure 4A, figure 5A, upper trace). The amplitude of the EC_{50} response measured ten minutes after washout of the high concentration of ME (MEpost) was about half of the initial EC_{50} response value (MEpost/MEpre= 0.46±0.05, n= 4, figure 5). The amplitude of the MEpost response

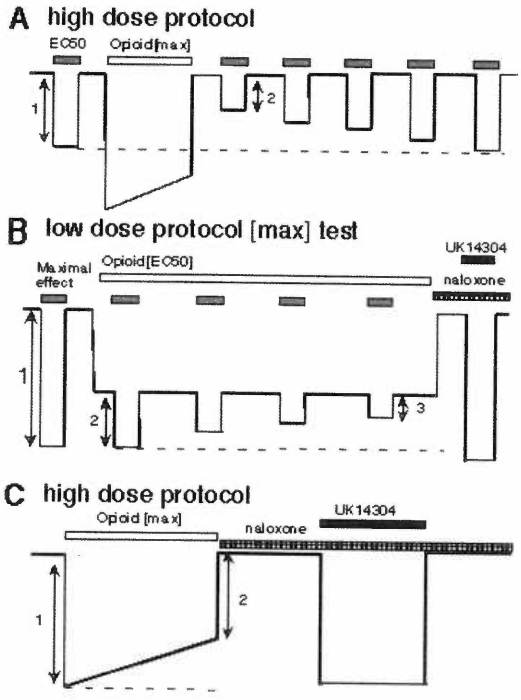


Figure 4. Protocols used in recordings from LC neurons to measure desensitization. A, an EC_{50} concentration of ME (300 nM) was used as a test before (1) and after (2) treating the tissue with a maximal concentration of agonist. The decrease in amplitude of the hyperpolarization immediately after washout was taken as a measure of desensitization. Over time, the amplitude of the hyperpolarization in response to the EC_{50} test returned toward control values. B, the hyperpolarization induced by a maximal concentration of ME (10 μM) was tested before (1) and during (2) the continuos superfusion with an EC_{50} concentration of opioid agonists. The decline in the peak hyperpolarization (3) was taken as a sign of desensitization. The hyperpolarization induced by the EC_{50} concentration of agonist was reversed with naloxone (1 μM). The hyperpolarization induced by a maximal concentration of UK14304 (3 μM) was determined for each experiment. C, a maximal concentration of opioid agonist was applied and the difference between the peak hyperpolarization (1) and the amplitude of the hyperpolarization after 15 min (2) was taken as a sign of desensitization. In each experiment, naloxone was used to reverse the opioid induced hyperpolarization and the hyperpolarization induced by UK14304 was determined.

recovered over a period of 40 min in accordance with previous reports [13]. The same amount of desensitization and similar kinetics of recovery were obtained with both intracellular and whole cell recording configurations. Thus, the recording conditions did not apparently interfere with the rapid desensitization process.

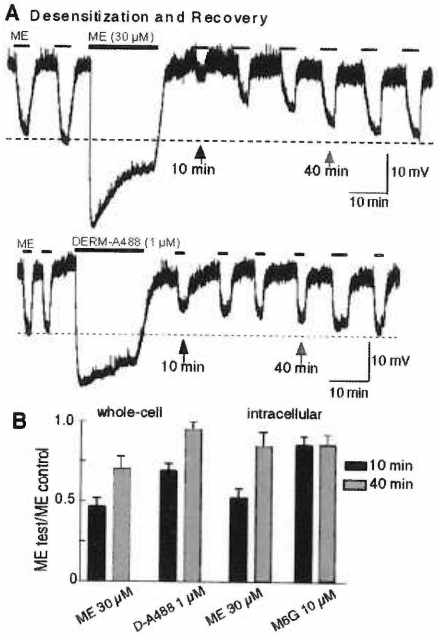


Figure 5. Induction of and recovery from desensitization. In this and other figures the recordings are of membrane potential. In some recordings, the presence of spontaneous oscillations in membrane potential resulted in 'noise' in the trace. The oscillations and thus the noise was abolished in the presence of opioid agonists. A, an EC_{50} concentration of [Met]^{5}enkephalin (ME), 300 nM, was tested every 5 min before and after the application of a maximal concentration of ME (30 µM, upper trace) and DERM-A488 (1µM, lower trace, applied by pressure ejection). The amplitude of the EC_{50} MOR response was significantly decreased after the desensitizing treatment with ME (upper trace) but only slightly reduced after DERM-A488 (lower trace). Desensitization recovered over 30-40 min. B, summarized data from desensitization experiments done with whole cell (left) and intracellular (right side) recordings. The decrease in amplitude of the hyperpolarization following desensitization is presented as a fraction of the amplitude of the initial response to ME (300 nM). Black bars are the ratio obtained after 10 minutes application and gray bars are the ratio after 40 minutes.

This protocol was also used to examine the desensitization of two other agonists that washed quickly from the tissue, an active metabolite of morphine, morphine 6-glucuronide (M6G, 10 μM), and a fluorescent analog of dermorphin (DERM-A488, 1 μM, applied by pressure ejection). The effect of ME (300 nM) was determined 10 and 40 min after washout of the high concentration of these compounds. The amount of desensitization induced by DERM-A488 (1 µM, n=6) was present but significantly smaller than that induced by ME and there was little or no desensitization caused by M6G (10 µM, n=6, figure 5B).

Desensitization by DERM-A488 and DERM-BTR

When the same experiment was used to examine the desensitization induced by another dermorphin analog, DERM-BTR, the washout was too slow to permit measurements of

desensitization. In fact, this protocol was not suitable for testing many agonists. Compounds that washed slowly from the tissue were tested with a protocol that used submaximal concentrations and desensitization was measured using maximal ME concentration applied at various intervals during the prolonged treatment (Figure 4B).

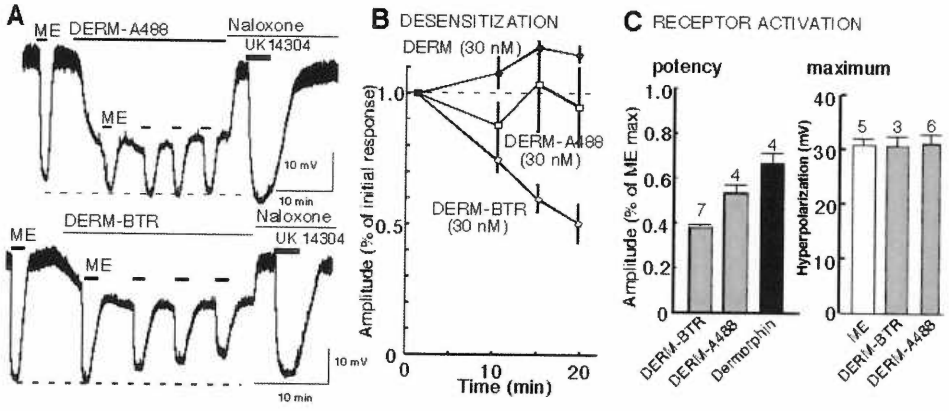


Figure 6. DERM-BTR induced desensitization in LC. A, examples of experiments done with the low-dose protocol using DERM-A488 (30 nM, top) and DERM-BTR (30 nM, bottom). The maximum hyperpolarization induced by ME (10 μM) was not changed during treatment with DERM-A488, whereas the maximum hyperpolarization was reduced in the presence of DERM-BTR. B, summarized data showing the amplitude of the ME induced hyperpolarization during the treatment of slices with dermorphin, DERM-A488 and DERM-BTR (n=5 for each experiment). The amplitude is plotted as a fraction of that observed during the first application of ME in the presence of the low concentration of each dermorphin analog. C, a summary of the acute effects of dermorphin, DERM-A488 and DERM-BTR all applied at 30 nM (left). Right side indicates the maximal hyperpolarization induced by DERM-A488 (1 μM, applied by pressure ejection) and DERM-BTR (1 μM, applied by pressure ejection) is the same as that induced by ME (10 μM).

Using this protocol, DERM-BTR but not DERM-A488 caused significant desensitization (Figure 6). There was a significant decline in the maximal hyperpolarization caused by ME that had been induced by DERM-BTR (30 nM, 50±7% after 20 min). Neither DERM-A488 (30 nM, 20 min) nor dermorphin (30 nM, 20 min) changed the amplitude of maximal ME hyperpolarization (P>0.05, one-way ANOVA), indicating that there was no desensitization when applied at this low concentration. Although the EC_{50} concentration of DERM-A488 did not cause desensitization, a maximal concentration did

(Figure 5). This indicates that the desensitization was dependent on the concentration applied. It appears that concentration was not the only factor in causing desensitization, however, because DERM-BTR (30 nM) induced desensitization even though it caused a hyperpolarization that was smaller than that caused by both DERM-A488 (30 nM) and dermorphin (30 nM, Figure 6C). Thus, DERM-BTR was more potent at causing desensitization.

MOR endocytosis

Previous work with DERM-A488 and DERM-BTR showed that these agonists differ substantially in their internalization properties even though these agonists had similar binding affinity ($K_i \sim 2.5$ nM) and biological activity ($EC_{50} \sim 30$ nM, [90]). Although the only distinction between these analogs is the fluorescent dye attached to the C-terminus, only DERM-BTR was internalized by CHO cells expressing MOR. Given the current observation that these agonists also desensitized MOR responses differently, one prediction was that the ability of the two fluorescent agonists to cause MOR internalization would be different. The ability of the dermorphin analogs to stimulate receptor internalization was examined directly by measuring the endocytosis of flag-tagged μ-opioid receptors (flag-MOR) stably expressed in HEK cells (Figure 7). These cells express functional receptors, and receptor endocytosis was a reliable indicator of arrestin-dependent regulation of downstream signaling [24,84].

In the absence of opiates, these cells exhibited constitutive endocytosis of approximately 34±3% of the total membrane receptors, over a 30 min incubation period,

at 37°C (figure 7B,D). Both dermorphin analogs caused receptor internalization, but the concentrations of agonist required for internalization were very different. While DERM-A488 at an EC_{50} concentration (30 nM) did not cause endocytosis above constitutive

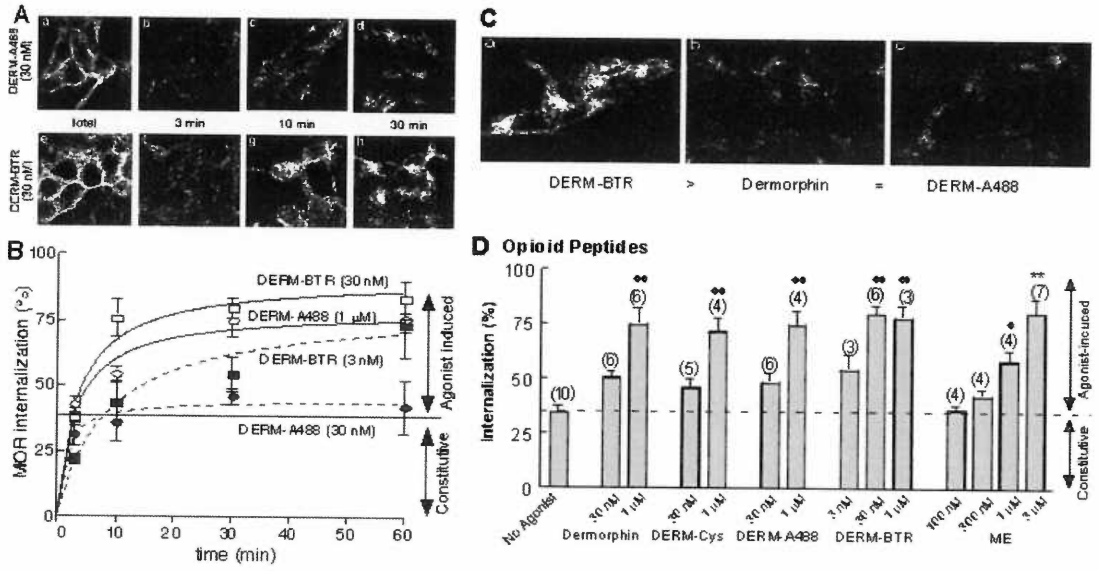


Figure 7. Endocytosis of flag-tagged MOR in HEK293 cells by opioid peptide agonists. A, examples of experiments examining the endocytosis induced by DERM-A488 (top) and DERM-BTR (bottom). The far left images (a and e) show total receptor binding using anti-flag antibodies. In b-d and f-h, the cells were incubated with DERM-A488 and DERM-BTR for the period indicated. At each time point, the anti-flag antibody remaining on the plasma membrane was stripped off so that only internalized label remained. The results show more internalization during treatment with DERM-BTR. B, summarized data showing the time course of internalization induced by different concentrations of DERM-A488 and DERM-BTR. C, examples of the maximal internalization caused by DERM-BTR, dermorphin and DERM-A488 all applied at 30 nM for 30 min. D, summary of many experiments with the dermorphin analogs and ME after a 30 min incubation period at 37°C. Each of the opioid peptides caused internalization that was dependent on the concentration. Also shown is constitutive internalization, that is the amount of receptor that was internalized in 30 min in the absence of any opioid agonist (no agonist).

levels (45±3%), a very low concentration of DERM-BTR (3 nM) caused a constant increase of receptor internalization that reached maximal levels over a 60 min incubation (80±8%, Figure 7B). Higher concentrations of both fluorescent agonists produced rapid and maximal receptor internalization with a similar time constant (DERM-A488, 1 μM, τ

= 3.1±1 min, max= 78±6 % and DERM-BTR 30 nM, τ= 3.5±1 min, max= 91±6 %). Maximal agonist-induced endocytosis reached about 80% of the total membrane receptors, and it was not further increased with longer incubation times, presumably due to receptor recycling.

These results indicate that DERM-BTR itself was not only internalized, as it has been previously shown [90], but it also caused efficient receptor endocytosis. In fact, DERM-BTR was the most potent agonist studied for promoting receptor endocytosis,

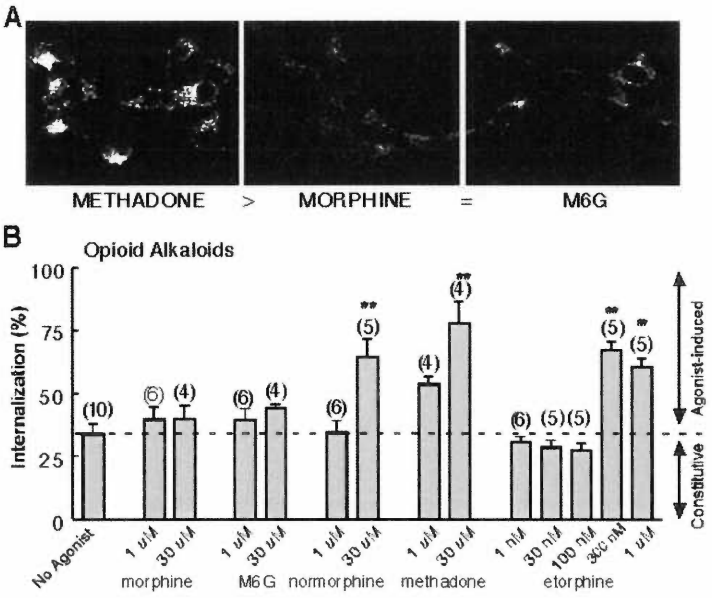


Figure 8. Methadone but not morphine caused endocytosis of flag-tagged MOR in HEK293 cells. A, representative images from endocytosis experiments. Fluorescence stains internalized receptor after 30 min exposure to 30 μM methadone (left), morphine (middle) and M6G (right). B, summarized data from several endocytosis experiments testing exposure to alkaloid opioids for 30 min at 37°C. Normorphine, methadone, and etorphine caused internalization that was dependent on the concentration. Morphine and M6G did not cause MOR endocytosis above the constitutive level (dotted lines), even at high concentrations.

more potent than dermorphin itself or dermorphin plus the cysteine linker (DERM-Cys, Figure 7D). The endocytosis induced by DERM-A488 was similar to dermorphin and DERM-Cys in that it caused receptor endocytosis only when applied at maximal concentrations (Figure 7). ME-induced internalization was also apparent only when incubated at a high concentration (3 μM). The results of the two experiments indicate

that DERM-A488 at high concentration can both desensitize and internalize MORs, whereas DERM-BTR does each at low concentration.

The internalization of a number of well characterized alkaloid agonists were examined under the same experimental conditions, in order to put the results obtained with the two fluorescent dermorphin analogs into perspective (Figure 8). In agreement with previous reports, morphine and M6G failed to promote MOR internalization even at very high concentrations. Normorphine at 1 μM did not induce receptor internalization but when the concentration was increased to 30 μM, 65 ± 6% of the total membrane receptors were internalized. Methadone and etorphine both caused internalization that was dependent on the concentration of agonist used. No etorphine-induced endocytosis was observed at concentrations up to 100 nM, whereas at 300 nM a maximum effect was observed (figure 8B). Methadone at 1 μM caused some endocytosis and a maximal response was observed at 30 μM. Thus the results are completely consistent with the work of others and suggest that the difference in endocytosis induced by DERM-A488 and DERM-BTR results from an agonist specific property conferred by chemical modification of this opioid peptide.

Desensitization by alkaloid opioid agonists

In order to correlate the degree of desensitization with the ability of agonists to induced endocytosis, the desensitization measured in LC neurons was determined using the same opioid agonists. Given that the washout of most agonists was slow, a combination of the low-dose (Figure 4B) and high-dose (Figure 4C) protocols were used.

With the low-dose protocol, morphine (1 μM) and M6G (1 μM) failed to induce changes in the maximal ME response (Figure 6). The results with M6G are consistent with those obtained with the high dose protocol (Figure 5B). These results confirmed that neither morphine nor M6G was capable of inducing desensitization.

Methadone caused both receptor internalization and desensitization. Perfusion with methadone (1 μM) reduced the amplitude of maximal ME response by 50±5% (n=5) after 20 min (Figure 9). There was no difference between the mean amplitude of the first ME response (before exposure to methadone) (30±1.5 mV) and the amplitude of first pulse of ME response (32±2 mV) immediately (3 min) after the onset of superfusion with methadone. However, in the continued presence of methadone, a reduction was detected as early as 10 min and declined continuously over 20 min. (Figure 9).

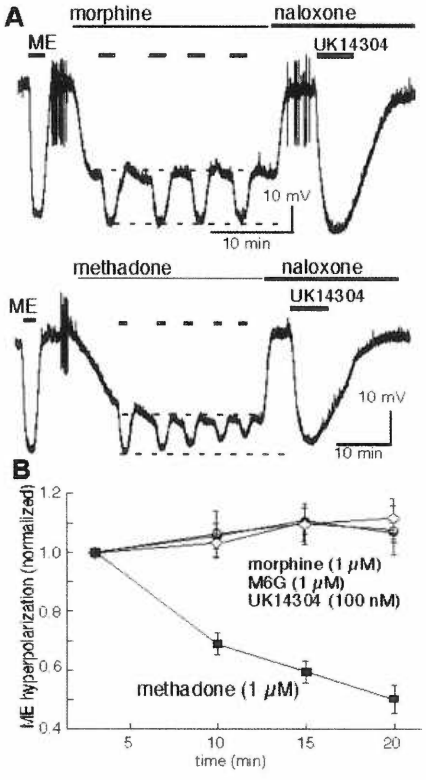


Figure 9. Methadone induced desensitization using the low-dose protocol. A, examples of experiments using the low dose protocol with morphine and methadone. The amplitude of the ME-induced hyperpolarization was not changed during treatment with morphine (3 μM, top trace) but decreased progressively during superfusion with methadone (3 μM, bottom trace). B, summarized data showing the decline in the ME-induced hyperpolarization in the presence of methadone and the lack of any change in the presence of morphine (3 μM), M6G (3 μM) and UK14304 (100 nM).

Desensitization was also measured using the decline in the hyperpolarization during a prolonged exposure to a high agonist concentration (Figure 4C). This protocol can be used for any agonist, regardless of the washout

kinetics. The decline in the hyperpolarization was measured at the end of a 15 min application of morphine (30 μM, n=4), methadone (10 μM, n=4), etorphine (1 μM, n=4) and ME (10 μM, n=5, Figure 10). Morphine was the only agonist that did not cause desensitization. The decline in response during the 15min test was the same for each of the other agoinsts (about 10 mV). Naloxone (1 μM) was used to reverse the effect and a maximal concentration of the α_2-adenoceptor agonist, UK14304 (3 μM), was applied to control for rundown or heterologous desensitization (Figure 10). In one set of experiments, UK14304 (3 μM) was tested prior to application of any opioid agonist. The amplitude of the UK14304-induced hyperpolarization in these experiments was the same as that observed after desensitization to opioids (Figure 10C, n=4 for each experiment).

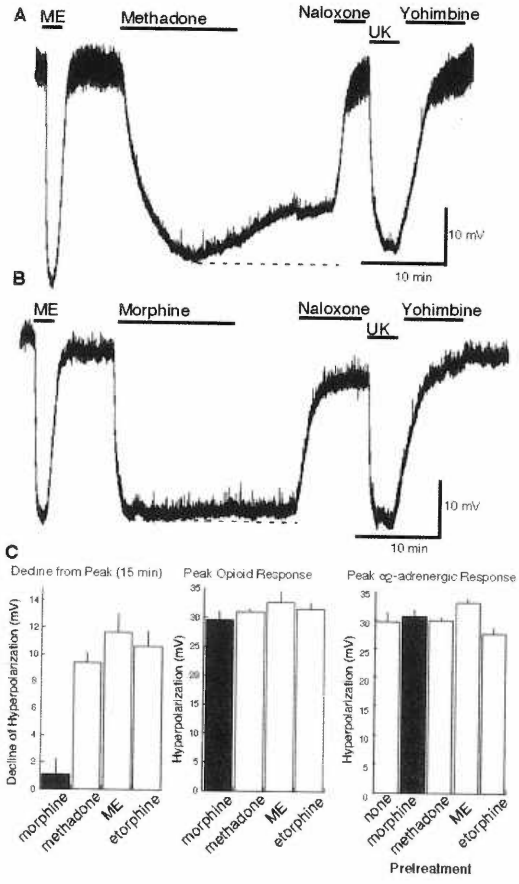
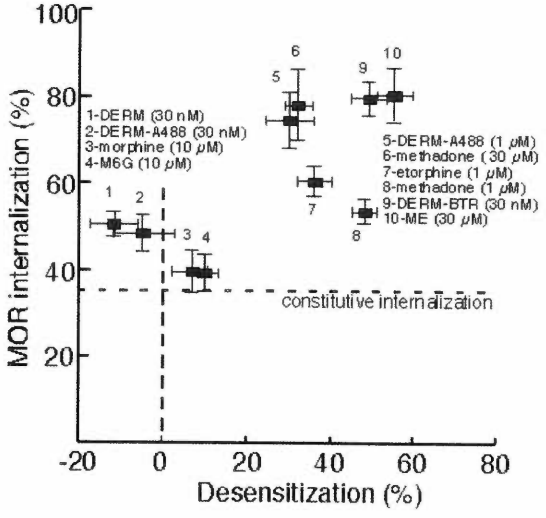


Figure 10. Methadone-induced desensitization using the high dose protocol. A, an example of the desensitization caused by a high concentration of methadone (30 μM). The peak hyperpolarization caused by methadone was about the same as the hyperpolarization induced by ME (10 μM) and UK14304 (3 μM). The decline in the hyperpolarization during the 15 min application was taken as a sign of desensitization. B, An example of the same experiment using a high dose protocol desensitization induced by morphine. C, left plot, summarized data showing the amplitude of the decline in hyperpolarization during a 15 min application of several agonists. This decline was about 10 mV for methadone, ME and etorphine but less than 2 mV for morphine. Middle plot shows the peak amplitude of the hyperpolarization caused by each agonist. Right plot shows the amplitude of the hyperpolarization induced by UK14304 (3 μM), before (none) and after treatment with each of the indicated opioid agonists. There is no sign of heterologous desensitization induced by any of the opioid agonists.

Correlation between endocytosis and desensitization

A summary of the results obtained with LC neurons in brain slices and HEK cells is illustrated in Figure 11. Desensitization measured in locus coeruleus neurons showed a positive correlation with agonist-induced receptor endocytosis in HEK293 cells. Independent of the assay used to measure desensitization, whether it was a change in EC_{50} or a change in maximal hyperpolarization, agonists that failed to induce desensitization also failed to produce endocytosis. Furthermore, desensitization was only observed at agonist concentrations that also caused internalization of at least 70% of total membrane receptors.

Figure 11. A comparison of MOR endocytosis and desensitization. Summarized data from all endocytosis and desensitization experiments. The percentage of the total MOR internalized by a given concentration of agonist (taken from Figures 7&8) was plotted as a function of amount of desensitization achieved when tested in LC neurons (expressed as percentage of the maximal opioid hyperpolarization). A significant correlation was found (non-paramentric Spearman r = 0.77, two tailed *P* = 0.01269). The lowest possible value on the y-axis was (35%) is the level of constitutive endocytosis.



Chapter 2:

Morphine Does Cause MOR Desensitization

Vu C. Dang and John T. Williams

Morphine has been widely accepted as the opioid receptor ligand that does not cause receptor desensitization or internalization. The sustained signaling by morphine has been postulated to cause cellular adaptations that mediate tolerance and in the absence of morphine result in drug withdrawal. This study reports that morphine can cause acute desensitization in locus coeruleus (LC) neurons when tested before prior application of any other opioid agonist. This finding challenges the notion that the inability of morphine to cause MOR desensitization is the cause of morphine analgesic tolerance. It also suggests that the development of tolerance to morphine may also be dependent on the regulatory processes at the level of the opioid receptor.

Morphine-induced desensitization of MOR

Whole-cell recordings from LC neurons were used to measure the outward potassium conductance caused by morphine. Morphine (10-30 μM) caused an outward potassium current of 110±10 pA (n=18). Sustained application of morphine (5, 10 and 15 min) resulted in a peak current that declined over time (Figure 12A). This indicates that there is a loss of MOR function. The rate and magnitude of desensitization were slower and smaller than that induced by [Met]^{5}enkephalin (ME) (Figure 12B). Application of morphine for 5min did not cause a significant reduction of the maximal current. After10 min in morphine, the peak current decreased to 82±5% (paired t test; P<0.016; n=7) and after 15min, the current was reduced to 65±4% (paired t test; P<0.0001; n=12).

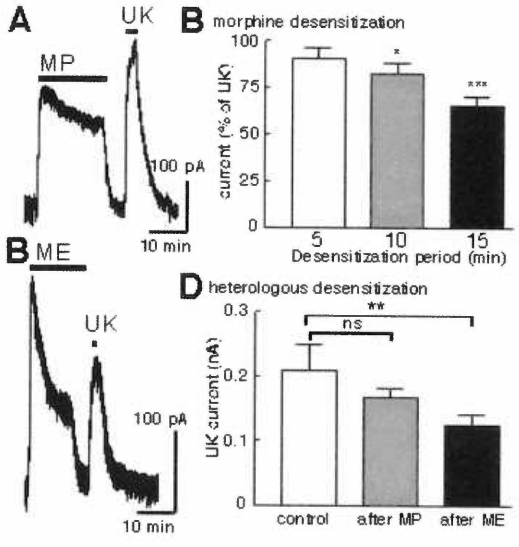


Figure 12. Morphine (MP) caused acute homologous desensitization of MOR. A, The morphine-(15 μM) induced current declined over time and did not affect the peak current induced by UK14304 (3 μM). B, Summary of the decline in current induced by MP applied for 5, 10, and 15 min. C, ME (30 μM) induced heterologous desensitization. D, Summary showing the current induced by UK14304 (3 μM) in control, after a 10 min treatment with MP (15 μM) and ME (30 μM).

Morphine-induced desensitization is homologous. As shown in Figure 1B, following 10 min desensitization by ME (30μM), the α_2-adrenoceptors response was significantly reduced. Before any desensitization treatment, UK14304 (3μM), a potent α_2-adrenoceptor agonist, induces an outward current of 227±35pA (n=10). Following ME induced desensitization, the maximal α_2-adrenoceptor-induced response was reduced

to 124±16pA (n=17). After 15 min desensitization with morphine, the α_2-adrenoceptor-mediated response was 167±14pA (n=12). This is not significantly different from the control response (P>0.106). This is consistent with previous reports showing that ME can cause heterologous desensitization but exposure to morphine does not affect α_2-adrenoceptor-mediated signaling [16].

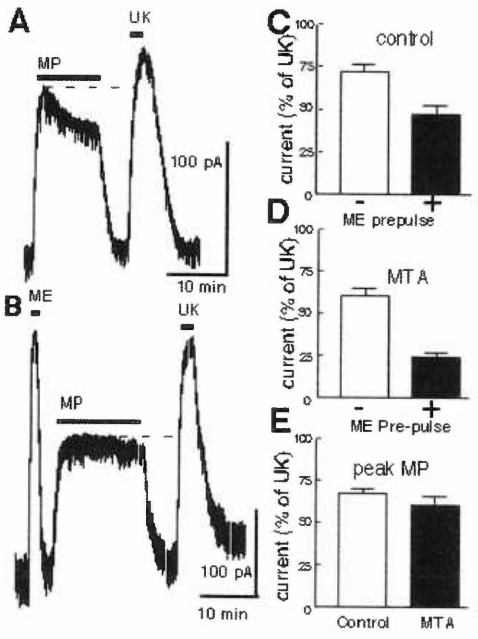


Figure 13. Brief application of ME reduced the current activated by morphine and blocked morphine-induced desensitization. A, In control, morphine activated current was 81±10% of the maximum UK14304 current. B, Following application of ME (10 μM, 1 min), the current induced by morphine was reduced to 52±7% of the UK14304 current. D,E, Summary showing the reduction of MP response following brief exposure to ME in slices from control D and morphine treated animals (MTA, E). F, Summary of peak MP current for in control and MTA without prior exposure to ME.

Morphine treatment does not reduce morphine efficacy

Chronic morphine treatment has been reported to reduce the maximal morphine-activated potassium conductance [82]. In the present study, the outward current induced by morphine in slices from control and morphine-treated animals were not significantly different (Figure 13). The maximal morphine-activated potassium current was 90±14 pA (P>0.174, n=9).

Chapter 3:

Chronic Morphine Treatment Reduces Recovery from Opioid Desensensitization

Vu C. Dang and John T Williams. *Journal of Neuroscience 24, 7699-706 (2004).*

Tolerance and dependence result from long-term exposure to opioids, and there is growing evidence linking acute receptor desensitization to these more long-term processes. Receptor desensitization encompasses a series of events leading to the loss of receptor function and internalization. This study examines the onset of receptor desensitization and recovery from desensitization in locus coeruleus neurons, recorded in brain slices taken from animals that have been chronically treated with morphine. After chronic morphine treatment, MOR regulation was altered as follows. First, the rate of desensitization was increased. Second, recovery from desensitization was always incomplete, even after a brief (1-2 min) exposure to agonist. This contrasts with experiments in controls where recovery from desensitization following a brief exposure to agonist was complete within 20 min. Finally, morphine N-6-glucaronide, a metabolite of morphine that was ineffective at causing desensitization in control, induced significant desensitization in slices from morphine-treated animals. When brain slices from controls were treated with inhibitors of PKC or monensin, agents known to compromise G-protein coupled receptor re-sensitization, desensitization was increased and recovery was significantly reduced. The results indicate that receptor re-sensitization helps to maintain signaling during periods of intense and sustained stimulation. Following chronic morphine treatment, MOR desensitization is potentiated and receptor re-sensitization is compromised.

Onset of Desensitization

Studies examining the desensitization and recovery of MOR have relied on the decline of the maximal response as the hallmark of desensitization. When LC neurons were exposed to a saturating concentration of ME (30 μM for 5 min), the maximal hyperpolarization gradually declined over time [12-16,91]. To determine if desensitization occurred prior to the initial decline of the maximum response, slices were given a brief pulse (1-2min) of ME (10μM). An EC_{50} concentration of ME (300 nM) was tested before and immediately after the desensitizing pulse (figure 14). After the desensitizing pulse, the hyperpolarization caused by the test concentration of ME was reduced to 50±11% of control (Figure 1). When additional desensitizing pulses were given, the test response was further desensitized (Figure 14B). The shift in the concentration response curve is plotted and shows a decrease in the sensitivity to ME, without a significant decline in the maximum hyperpolarization (Figure 14D). This experiment shows that the onset of desensitization was rapid and occurred well before the initial decrease of the maximal response.

Given the rapid onset of desensitization, the next set of experiments assessed the extent of opioid receptor desensitization, using different periods of drug application. For these experiments, LC neurons were treated with ME (30 μM) for 5, 10, and 20 min. The decline in the maximal hyperpolarization was not significantly changed between the desensitizing treatments. The responses were 80±5 (n=9), 71±5 (n=9), 74±3 (n=7) % of the maximum hyperpolarization after desensitization for 5, 10 and 20 min respectively. The concentration response curve following a 10min desensitization period illustrates the decrease in maximal hyperpolarization and an increase in the EC_{50} to 1.6±2.2 μM. These

values are similar to that reported in a previous study that used a 5 min desensitization period (EC_{50} control 283 nM, desensitized 1.1 μM) [14]. Thus, desensitization can be detected after a very short exposure to a saturating concentration of agonist, and the extent of desensitization, measured as the decline in maximal hyperpolarization, reached a limit after a 5-min treatment.

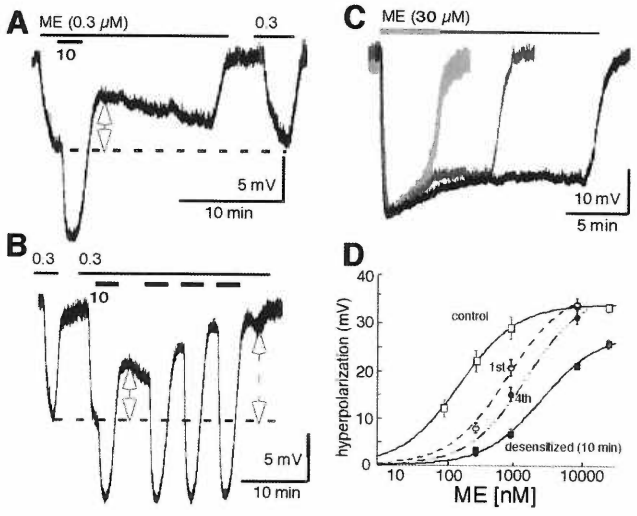


Figure 14. The onset of MOR desensitization. Loss of receptor function is a rapid process. A, 1-2 min pulse of 10μM ME given on top of the EC50 concentration (300nM) reduced the amplitude of hyperpolarization to 50±11%. B, Addition of desensitizing pulses of 10μM ME further reduced the amplitude of hyperpolarization induced by 300nM ME. C, Extended perfusion with 30μM ME for 5,10, or 20min, the maximal hyperpolarizations were reduced to 80±5 (n=9), 71±5 (n=9), and 74±3% (n=7) respectively. D, Estimated concentration response curve showing the loss of sensitivity of MOR to ME. 1st represents the shift in sensitivity to ME after a single pulse of 10μM ME. 4th represents the shift in sensitivity after 4 pulses of 10μM ME. Desensitized represent changes in sensitivity after a 10 min desensitization treatment.

Chronic Morphine Treatment Facilitates Desensitization and Reduces Recovery

The extent and rate of recovery from desensitization, induced by ME, were examined in LC neurons from animals that have been treated with morphine for 6-7 days. With short applications of ME (10 μM for 90-120 s), the hyperpolarization induced by 300nM ME was reduced to 29±2% of the pre-pulse (n=3) (Figure 15A). This decrease is significantly larger than that found in controls. Chronic morphine treatment did not however alter the magnitude of desensitization measured by the decrease in the maximal

hyperpolarization. The maximal hyperpolarization to ME (30 μM) declined to 74±10% of the peak after a period of 10 min, not significantly different from control animals (Figure 15B).

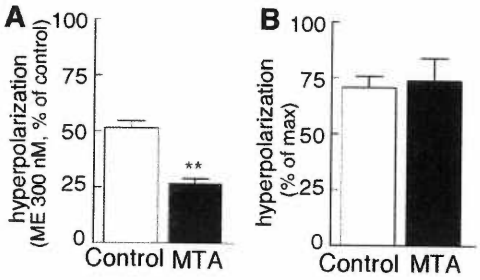


Figure 15. Morphine Treatment Potentiate MOR Desensitization. When the same experiment, as shown in Fig. 14A, was repeated in slices from morphine treated animals, a 10μM ME desensitization pulse caused a greater reduction of the 300nM ME response. A, Data summary. While the desensitization after a single pulse of 10μM ME was greater after morphine treatment, 10 min desensitization with 30μM ME reduced the maximal hyperpolarization to 74±10% (B).

Chronic morphine treatment reduced the recovery from acute desensitization. Recovery following a 2 min desensitization treatment was complete within 20 min when recordings were made from cells in slices from control animals. In these experiments, after 5 min, the hyperpolarization induced by ME (300 nM) was 71±7% of the pre-pulse and was 94±3% of the pre-pulse after 25 min (n=10).

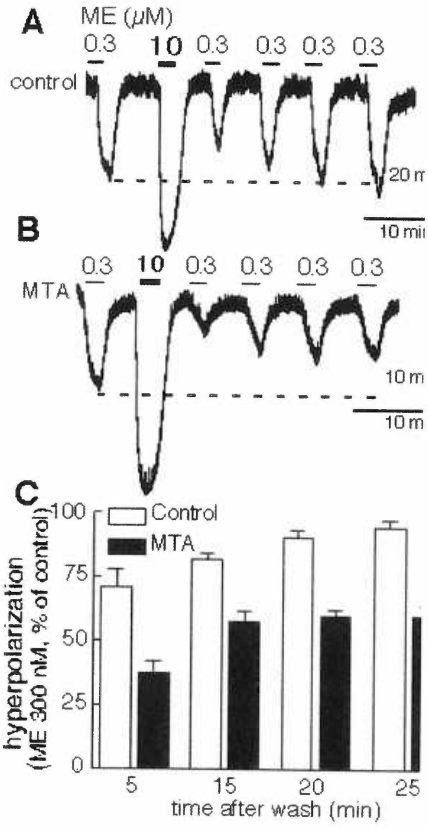


Figure 16. Recovery from Rapid Desensitization. After 1-2min desensitization pulse, the loss of receptor function recovers rapidly and recovery is complete in control animals (94±3% after 25min) (A). B, Following chronic morphine treatment, receptor recovery is incomplete. After 25 min, the 300nM-ME response is only 60±7% of the pre-pulse. C, Data summary.

Following chronic morphine treatment, recovery of the hyperpolarization induced by ME (300 nM) following a 2 min desensitization period was 44±6% after 5 min (n=4) and only 60±7% after 25 min (n=5) (Figure 16).

Recovery following a 10 min desensitization treatment was similarly altered by chronic morphine treatment. After washing for 30 min, the hyperpolarization induced by ME (300 nM) was 59±5% in slices from morphine-treated animals (n=9), compared to 82±4% in slices from untreated animals (n=5-10) (figure 17). These results indicate that chronic morphine treatment facilitates acute desensitization, decreases receptor re-sensitization, or both, such that receptor recovery was attenuated or incomplete.

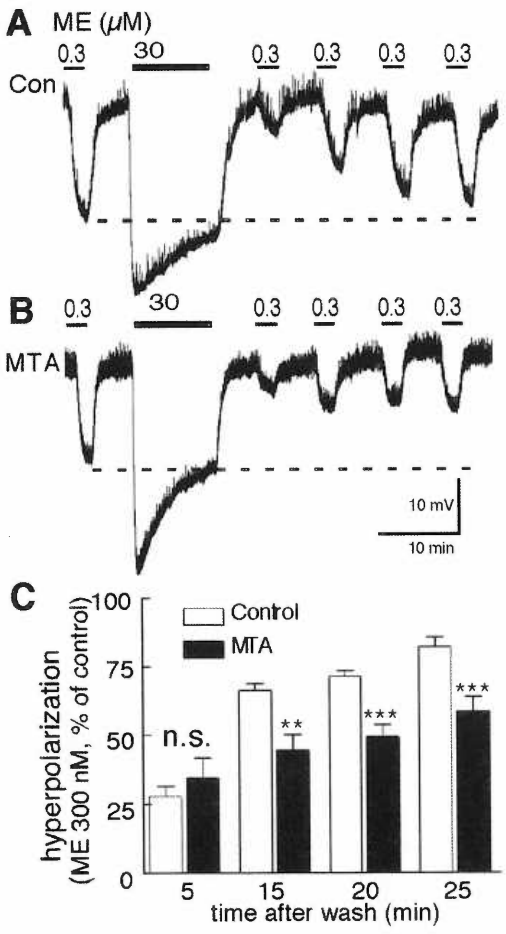


Figure 17. Recovery from 10min desensitization. Although 10min desensitization with 30μM ME caused the same magnitude of desensitization in control and morphine-treated animals, the recovery from desensitization is different. A, From control animals, receptor recovery is faster and more complete (82±4% after 30min). B, After morphine treatment, 59±5% recovery is seen after 30min. C, Data summary.

Morphine-6-ß-D-glucuronide-induced desensitization

Morphine-6-ß-D-glucuronide (M6G), a metabolite of morphine, does not cause desensitization in LC neurons or receptor internalization in HEK293 cells expressing MOR [15]. This observation was confirmed by comparing the hyperpolarization caused by ME (300 nM) before and after treatment with

a saturating concentration of M6G (10 μM) for 5 (n=5-8), 10 (n=5-6) and 20 min (n=4-5). After washing out the M6G, no significant desensitization was seen in slices from control or placebo treated animals (Figure 18). In slices from morphine-treated animals, M6G (10 μM, 5 min, n=6) reduced the hyperpolarization induced by ME (300 nM) to 66±5% of control. When the M6G treatment period was increased to 10 min (n=7-9), the test response was reduced to 55±4%. Recovery from desensitization was not observed even after 45 min (n=4, Figure 18). These experiments further indicate that acute MOR desensitization is facilitated or receptor recovery is attenuated after chronic morphine treatment, or both.

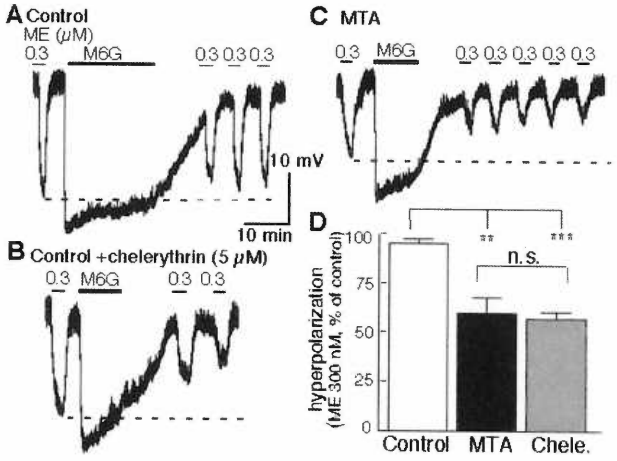


Figure 18. Morphine N-6-Glucaronide induced desensitization. LC slices were perfused with 10μM M6G to induce desensitization. After washout of M6G, 300nM ME was used to probe for loss of MOR function. To ensure complete washout of M6G, only data points taken 30min after the washout were used for data analysis. 1μM naloxone was given whenever the reestablishment of baseline was unclear. A, In control animals, after 20min M6G induced desensitization, the 300nM ME response was 94±3% of the pre-pulse. B,Treatment of LC slices with chelerythrin, to inhibit GPCR re-sensitization, unmasks M6G induced desensitization. After the 10min desensitization, with 10μM M6G, the 300nM ME response only 57±3% of the pre-pulse. C, Morphine treatment, similarly, reveals M6G induced MOR desensitization. 10min desensitization with M6G reduced the 300nM ME response to 60±7% of the pre-pulse. D, Data summary of the 300nM-ME-induced hyperpolarization recorded 30min after the washout of M6G.

Disrupting Receptor Recycling Increases Desensitization and Attenuates Recovery

Monensin and PKC inhibitors have been shown to disrupt GPCR re-sensitization [36,41,58,59,92]. To determine the role of receptor re-sensitization in the process of MOR

regulation, experiments were done in the presence of monensin or PKC inhibitors. The PKC inhibitor chelerythrin (5-10 μM) significantly increased MOR receptor desensitization (Figure 19). Following a 2 min desensitization period, the hyperpolarization induced by ME (300 nM) was reduced to 31±6% of the pre-pulse (n=6-8). After 25 min, recovery was only 68±8, compared to 94±3% in untreated slices. Similar results were seen when staurosporin (50-100 nM) was used to inhibit PKC. Following the 10 min

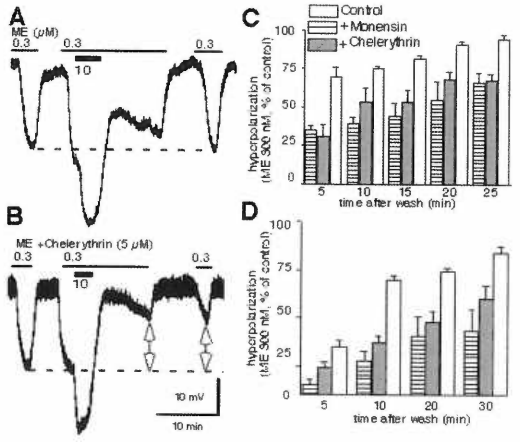


Figure 19. Inhibiting receptor re-sensitization reduced receptor recovery. A, Same experiment as shown in Fig.14A, 1-2 desensitization has fast and complete recovery. B, Rapid desensitization in the presence of chelerythrin was greater and recovery was reduced. C, Summary of receptor recovery data from the rapid desensitization. D, Summary of receptor recovery after 10 min desensitization with 30μM ME).

desensitization treatment, 22±6% (n=5)

recovery was seen after 5min of washing. After 30 min of washout, the ME (300nM)-induced hyperpolarization was 59±5% of the pre-pulse (n=6). When monensin was used to disrupt receptor recycling, similar results were obtained. The hyperpolarization caused by ME (300 nM) was reduced to 35±3% immediately after a 2 min desensitization period, and the recovery was only 66±6% after 25 min (n=4).

Given that disrupting receptor resensitization potentiates desensitization and reduces recovery, the magnitude of desensitization would be predicted to increase. Treatment with either chelerythrin or monensin increased the magnitude of desensitization. In chelerythrin, desensitization with ME (30 μM, 10 min) reduced the

maximal hyperpolarization to 58±4% of maximum, compared to 71±5% (n=6) in controls. In the presence of monensin, the hyperpolarization induced by ME (30 μM) declined to 54±12% of the maximum (n=5). In LC slices from morphine-treated animals, this effect of monensin was even greater, decreasing the hyperpolarization to 39±5% of the maximum after 10 min (n=6, Figure 20).

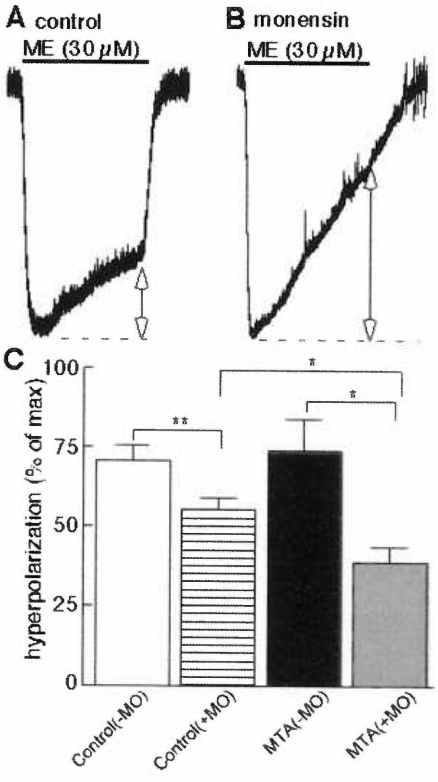


Figure 20. Inhibition of receptor re-sensitization increases the magnitude of desensitization from control and morphine treated animals. A and B are representative traces from two experiments done from morphine-treated animals. A, 10 min desensitization with 30μM ME, after morphine treatment, reduced the maximal hyperpolarization to the same level as control animals (see Fig.2). B. 10 min desensitization with 30μM ME in the presence of monensin (1-5μM) reduced the maximal hyperpolarization to 54±12 (n=4) and 39±5% (n=6) in control and morphine treated animals, respectively. C, Data summary.

The recovery, following a 10 min desensitization period in the presence of monensin or chelerythrin was also significantly reduced (Figure 21D). In monensin-treated slices, the hyperpolarization induced by ME (300 nM) was only 37±13% of the pre-pulse 30 min after washout (n=5). Recovery in chelerythrin-treated slices was only 56±7% after 30 min (n=8).

PKC Inhibitors Unmask M6G-induced MOR desensitization in Control Animals

It was difficult to determine if M6G can cause desensitization in control animals because of the slow washout of M6G from the brain slice. It took between 10-20 min for the membrane potential to return to baseline after washing M6G (10μM). Although there was no significant decline in the response induced by ME (300nM) 30 min after washing M6G, it was possible that it did cause desensitization that recovered during the slow wash out. To determine if M6G can cause desensitization, experiments were conducted in the presence of chelerythrin. By disrupting receptor resensitization, any desensitization that did occur would be detectable. After treatment of the slice with chelerythrin (5μM), M6G (10μM, 10 min) caused a significant decrease of the hyperpolarization caused by ME (300nM, Figure 19B). The hyperpolarization induced by ME (300nM) was only 57±3% of the pre-pulse, 30 min after the washout of M6G. Thus, as was observed in slices from morphine-treated animals, treatment with chelerythrin reveals M6G-induced desensitization.

Homologous Desensitization

Recent work by Blanchet and Luscher found heterologous desensitization of α_2-adrenoceptors following MOR desensitization [16]. In the present study, heterologous desensitization of α_2-adrenoceptors was tested by measuring the hyperpolarization caused by a sub-maximal concentration of NA (30 μM) before and after MOR desensitization. NA (30 μM) induced 24±2mV hyperpolarization before and 20±3 mV (n=5) after a desensitization treatment with ME (30 μM, 10 min, in the presence of monensin). In

addition, the hyperpolarization induced by a maximal concentration of UK14304 (3 μM), a full agonist at α_2-adrenoceptors, did not change after desensitization with ME (30μM for 20min). The hyperpolarization induced by UK14304 was 26±1mV following desensitization, which is 83±5% of the maximal opioid response. This observation is consistent with previous reports indicating that heterologous desensitization makes up only a small component of the total amount of desensitization using the same recording condition [82]

Opioid Tolerance is Homologous Following Chronic Morphine Treatment

To determine whether the cellular adaptations responsible for the altered MOR responsiveness would extend to other G-protein-coupled receptors, the hyperpolarization induced by α_2-adrenoceptors was examined. There was no significant difference in the EC_{50} or the maximal hyperpolarization induced by noradrenlaline (NA, Figure 21). In naïve and placebo-treated animals, NA (10 μM) caused a 12±3 mV hyperpolarization (n=5). The maximal hyperpolarization was 19±3 mV (NA, 50 μM). After chronic morphine treatment, NA (10 μM) caused a 15±2 mV hyperpolarization, and the maximal response was 25±2 mV (n=4).

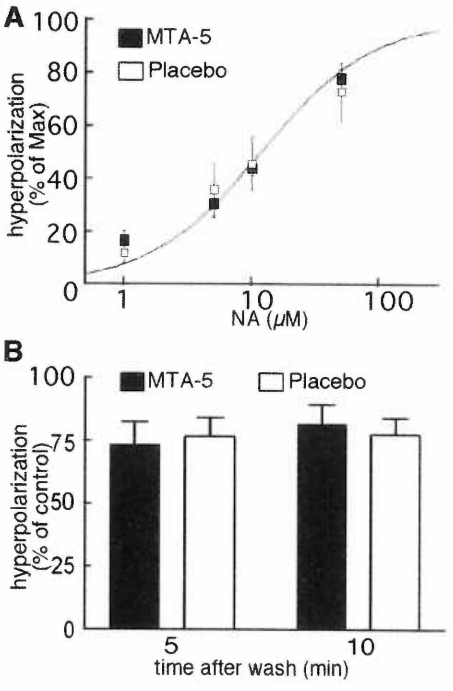


Figure 21. Morphine treatment did not alter α_2-adrenoceptor function. A, Concentration response curve for NA slices from animals treated with placebo and morphine pellets. The amplitude of the hyperpolarization is

plotted as the percentage of hyperpolarization induced by a maximal concentration of UK14034 (3 μM). B, Morphine treatment did not change the desensitization caused by NA. After a desensitizing pulse of NA (50 μM, 5 min) NA, the hyperpolarization induced by NA (10 μM) was the same in the morphine treated and the placebo-treated animals.

Given that desensitization of MORs was increased following chronic morphine treatment, it was possible that desensitization of other similarly coupled receptors may also be affected. This possibility was tested with the use of a saturating concentration of NA to desensitize the α_2-adrenoceptors. The hyperpolarization induced by NA (10 μM) was tested before and after treatment with a saturating concentration of NA (50 μM, 5 min). The hyperpolarization induced by the test pulse, after the 5 min desensitizing treatment, was 77±7% and 74±9% of the control, in slices from placebo- (n=4) and morphine-treated animals (n=5), respectively. Thus, chronic morphine treatment did not result in a generalized increase in the desensitization of the α_2-adrenoceptors.

Chapter 4:

ERK1/2-mediated Heterologous Desensitization of the α_2-adrenoceptors Following MOR Desensitization

Vu C. Dang and John T. Williams

Under voltage-clamp whole-cell recording conditions, MOR desensitization by DAMGO or $[Met]^5$-enkephalin leads to the development of heterologous desensitization of α_2-adrenoceptor-mediated potassium conductance. In this study, experiments were conducted to determine mechanism behind the development of heterologous desensitization. The results indicate that activation of ERK1/2 mediated heterologous desensitization following MOR receptor desensitization. Inhibition of cSrc, the upstream activator of ERK1/2, prevented the development of heterologous desensitization. This process is dependent on receptor internalization because only agonists that can cause MOR desensitization and internalization can cause heterologous desensitization. This observation indicates that the induction of acute MOR desensitization can cause changes in LC neurons that alter cellular signaling.

MOR and α_2-adrenoceptors Receptor Activated GIRK Currents

Maximal concentration of ME (10μM) activates MOR and opens the GIRK channels, causing an outward potassium current of 231±38pA. This is a maximal current for MOR activation GIRK because higher concentrations of ME (30μM) caused the same magnitude of outward current. When multiple pulses of ME (10μM) were applied to the LC neurons, the maximal currents were not significantly different. This result indicates that brief exposure to high concentrations of agonist does not alter GIRK conductance. Stimulation of the α_2-adrenoceptor activates the same GIRK channels as MOR. Stimulation with UK14034 (3μM), a full and selective α_2-adrenoceptor agonist, caused an outward current of 208±40pA (88±3% of the ME response) (Figure 22A). This observation is consistent with previous studies measuring GIRK-mediated currents by MORs and the α_2-adrenoceptors [15,82]

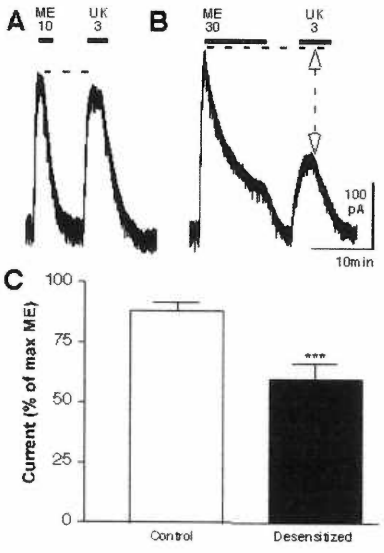


Figure 22. MOR-induced heterologous desensitization. A, Prior to MOR-induced desensitization, UK14304 (3μM) caused an outward potassium conductance that is 88±3% of the maximal ME (10μM) response (n=7). B, After 10 min desensitization of MOR with ME (30μM), the UK14304 (3μM) response was 55±3% (n=17) of the max ME response. C, Data summary (***p<0.0001).

ME-induced desensitization reduced the α_2-adrenoceptor-mediated GIRK current

Stimulation of the MOR with ME (30μM) induced an outward potassium current that desensitized over time [12,14,16,91]. Previous studies have shown that when desensitized for 5 min, the ME (30μM)-induced desensitization is primarily homologous. Recent work by Blanchet et al. indicates that when LC neurons were stimulated for 15 min with ME (30μM), the α_2-adrenoceptor-mediated GIRK current was significantly reduced (heterologous desensitization). This result indicates that extended desensitization by ME can alter intracellular signaling. These experiments were done in horizontal brain slices, where the dendritic arbor of the LC neurons is more intact and the receptor reserve is more preserved [16].

In order to remove the electrotonic-coupling property of LC neurons and to assure reliable voltage-clamp recordings, experiments were conducted from LC neurons in coronal brain slices. Under this condition, ME (30μM) elicited an outward current of 220±20pA which desensitized to 98±10pA (46±4%) after 10 min. Following the MOR desensitization, the current induced by UK14034 (3μM) was 124±16pA. This is significantly smaller than the control response (60±7%) (heterologous desensitization) (Figure 22).

Inhibition of ERK1/2 Prevents Heterologous Desensitization

Activation of GPCRs has been shown to activate ERK1/2 signaling cascade. To determine if this signaling cascade could contribute to the manifestation of heterologous

desensitization, experiments were done in the presence of ERK1/2 inhibitor PD98059. PD98059 (10μM) inhibited the induction of heterologous desensitization without affecting the homologous desensitization. As shown in Figure 23, following ME-

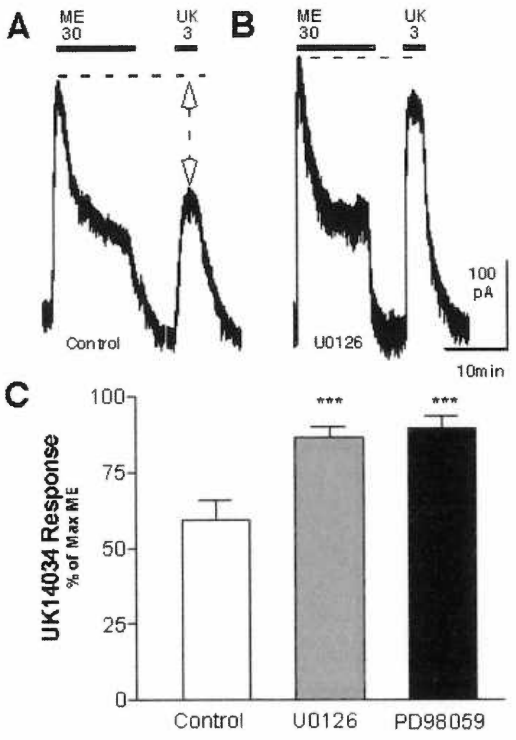


Figure 23. ERK1/2 inhibitors rescued the α2-adrenoceptor response. A, A representative experiment showing heterologous desensitization after ME (30μM)-induced MOR desensitization. B, When the same experiment is conducted in the presence of U0126 (10μM), a ERK1/2 inhibitor, the UK14304 (3μM) response was 87±4% of the max ME (30μM) response (n=11). C, Data Summary with PD98059 a chemically different ERK1/2 inhibitor (n=5) (***P<0.0001).

induced MOR desensitization, the α_2-adrenoceptor current was 167±30pA (n=5) (89±4% of maximum ME response). To confirm this effect, a chemically different ERK1/2 inhibitor, U0126, was used to block ERK1/2-mediated signaling. Similarly, U0126 (10μM) prevents the induction of heterologous desensitization. UK14034 (3μM)-stimulated current was 167±15pA (n=11) (87±4% of maximal ME response) (Figure 23B).

ERK1/2 activation is via a cSrc-dependent mechanism

Previous studies have shown that activation of GPCRs can activate ERK1/2 through two mechanisms. One is through Gi protein activation of ERK1/2 via PKC activation [93]. The second is through cSRC activation following receptor endocytosis. To determine which of these signaling pathways is responsible for causing heterologous desensitization, experiments were done with a PKC peptide inhibitor or PP2, a specific cSRC inhibitor.

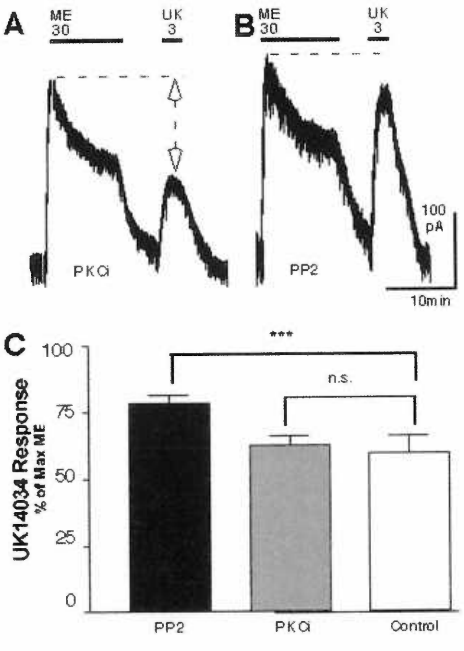


Figure 24. cSrc inhibitor prevents heterologous desensitization. Both PKC and cSrc can activate ERK1/2 following GPCR activation. To determine the pathway, experiments were done in the presence of PKCi (100μM), a peptide PKC inhibitor of PP2 (10μM), a cSrc inhibitor. A, PKCi fail to block heterologous desensitization (n=9). B, PP2 significantly reduces heterologous desensitization (n=11). C, Data summary (n.s. P>0.164, ***P<0.0001).

To block PKC activation, LC neurons were loaded with PKC peptide fragments (100μM), which bind to the catalytic site of PKC, inhibiting its auto-phosphorylation as well as protein substrates activation. As shown in Figure 24, PKC inhibition did not prevent the induction of heterologous desensitization. After ME-induced MOR desensitization, the α_2-adrenoceptor-induced current was 131±18pA (n=10) (68±10% of ME response).

When LC neurons were treated with PP2, a cSRC inhibitor, prior to ME-induced desensitization treatment, heterologous desensitization was inhibited (Figure 24B).

Following MOR desensitization, UK13404 (3μM)-induced current was 180±22pA (n=11) (79±10% of the ME response). These results indicate that the induction of heterologous desensitization is initiated by the activation of cSRC, which activates ERK1/2 via a Ras pathway.

Methadone-induced heterologous desensitization

The activation of cSRC, through GPCRs activation, has been shown to be dependent on receptor internalization. To determine whether receptor-internalization-dependent activation of ERK1/2 was responsible for the heterologous desensitzation of the α_2-adrenoceptor current, experiments were done using morphine or methadone as desensitizing drugs.

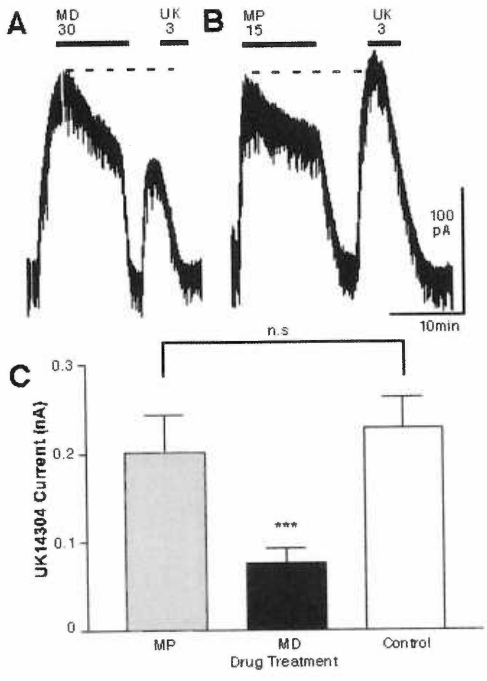


Figure 25. UK14304 (3μM) activation of the α2-adrenoceptor causes a potassium conductance of 230±35pA. A, After Methadone (MD. 30μM)-induced MOR desensitization (15min) and UK14304 (3μM) response was reduced to 77±16pA (n=11). B, Morphine-(MP) induced desensitization (15min) did not cause heterologous desensitization. The UK14304 current following MP-induced desensitization was 201±42pA (n=5). Data summary (n.s. P>0.663, ***P<0.0007).

With acute treatment, morphine has been shown to activate ERK1/2 via a receptor-endocytosis-independent pathway.[94]. Acute as well as chronic morphine treatment increased ERK1/2 phosphorylation in the LC [73]. Morphine, however, is ineffective at causing

MOR endocytosis [11,15,84]. To determine if morphine-induced ERK1/2 activation would lead to heterologous desensitization, LC neurons were perfused with morphine (15min, 15μM) and then reversed with naloxone (1μM). As shown in Figure 25B, morphine-induced desensitization did not cause a significant reduction of the UK14034 (3μM)-activated current. After morphine (10-15μM) desensitization, the α_2-adrenoceptor-mediated potassium current was 201±40pA.

Methadone is also a partial agonist, but unlike morphine, methadone is a potent inducer of MOR endocytosis. When methadone (15-30μM) was used as the desensitizing drug, the maximal UK143034 (3μM) current was only 77±15pA. This is 48±8% of the control response. Together, these results indicate that receptor-endocytosis-dependent activation of ERK1/2 initiates the signaling cascade that leads to the development of heterologous desensitization.

DISCUSSION:

GIRK Activation and Desensitization

Striking differences were found among the opioid agonists with respect to the relative ability to activate GIRK-mediated hyperpolarization and to trigger desensitization. The rank of order for desensitization of MOR-activation of potassium current in LC neurons was DERM-BTR > etorphine > methadone ≈ ME > dermorphin ≈ DERM-A488 > morphine ≈ M6G. The desensitization was dependent on the concentration of agonist applied. At roughly EC_{50} concentrations, DERM-A488 (30 nM) and etorphine (1 nM, not shown) did not result in detectable desensitization, whereas at maximal concentrations both induced significant desensitization. DERM-BTR, however, caused both desensitization and internalization at a low concentration (30 nM).

Based both on our experiments and previous literature, the rank of order for receptor activation is etorphine ≈ dermorphin > DERM-A488 ≈ DERM-BTR > ME ≈ methadone > morphine. These differences in the rank of order are particularly relevant because it has been suggested by studies in heterologous expression systems that the rate at which μ-opioid agonists induce rapid desensitization is dependent on agonist efficacy [95]. Other studies, however, found that differences in the ability of certain agonists to induce MOR internalization in transfected fibroblasts were not positively correlated with either potency [96] or apparent efficacy [30]. Here we examined the relationship between efficacy and desensitization in neurons expressing endogenous μ-opioid receptors. Hence, the relationship between efficacy and desensitization was studied under

conditions where expression levels of receptors and signaling molecules were not manipulated.

The fluorescent opioid peptides were powerful tools for addressing the question of the relationship between agonist efficacy and desensitization and/or internalization of receptors. Created from the same opioid agonist, dermophin, DERM-BTR and DERM-A488 share several characteristics, such as EC_{50} and binding affinity (Kd= 2.3 nM and 2.5 nM for DERM-A488 and DERM-BTR, respectively); [90] but were very different in their ability to desensitize and internalize the receptor. DERM-BTR had a much greater ability to induce MOR desensitization and internalization. This observation questions the idea that receptor activation alone is required for rapid receptor desensitization.

Conjugation of Bodipy Texas Red (BTR) to dermorphin resulted in a very hydrophobic peptide. Given that this is the primary difference between DERM-BTR and DERM-A488, it is reasonable to propose that the unique desensitization properties of DERM-BTR could be related to its hydrophobicity. At least three possible explanations could account for the results. First, DERM-BTR could accumulate in the proximity of the plasma membrane and create very high local increases in agonist concentration that could saturate receptors causing desensitization and endocytosis. This explanation, however, should result in the maximal activation of a physiological response. Second, the hydrophobic properties of DERM-BTR could enhance clustering, dimerization of the receptor, or both, which could facilitate desensitization and endocytosis. Third, DERM-

BTR could slow recycling of internalized receptors such that an apparent increase in endocytosis and desensitization could result from failure to recycle.

In both the brain slice and HEK293 cell experiments, the onset and recovery from application of DERM-BTR was slower than DERM-A488. Although this observation may be related simply to differences in hydrophobicity, it may also suggest that the receptor binding kinetics is different for the two ligands. A slower dissociation rate might imply that DERM-BTR remains bound to receptors, even after endocytosis, thereby impairing receptor recycling. This notion is supported by the observation that a low concentration of DERM-BTR induced a progressive accumulation of MOR in the intracellular compartment (Figure 7B). The slow but constant rate of receptor accumulation might suggest a reduced rate of receptor recycling.

Desensitization and internalization

Desensitization was tested using different protocols that included exposure to maximal or submaximal agonist concentrations all yielding consistent results. A strong correlation was found between desensitization observed in LC neurons and internalization observed in HEK293 cells. Ideally, both internalization and desensitization should be studied in the same neurons; however, to date imaging experiments in brain slices have been limited technically. Although the correlation between cell types does not definitively demonstrate a link between these processes, it does not disprove a connection.

The opioid agonists, normorphine, methadone, etorphine, dermorphin, DERM-Cys, and DERM-A488 all induced MOR desensitization and internalization in a concentration-dependent manner. Interestingly, supramaximal concentrations of these agonists were often required to cause significant desensitization and endocytosis. An important example is etorphine, which is both potent and efficacious at activating a number of different effectors, but required relatively high concentrations to induce both desensitization and endocytosis. This observation suggests that higher receptor occupancy may be required for internalization and desensitization than for activation of other effectors. The exception to this statement is DERM-BTR.

Morphine-induced MOR Desensitization

Studies in brain slices often use ME primarily because of the rapid wash into and out of the preparation. The maximal current induced by ME was often tested before commencing desensitization experiments [15]. Recent work has shown that even brief exposure to ME (10 μM) caused a rightward shift of the concentration curve, which was taken as a sign of significant desensitization [97]. In the present study, when the application of morphine was preceded by a brief treatment with ME, the current induced by morphine was reduced and desensitization was not observed (Figure 13A,B). The desensitization induced by morphine therefore is very sensitive to receptor activation, especially by an efficacious opioid like ME. For this reason, morphine induced-desensitization has eluded detection.

Although the onset of desensitization is slow compared to that caused by ME, 15 min exposure to morphine (10-30μM) does lead MOR desensitization. It is possible that morphine, being a partial agonist for the activation of GIRKs, is also a partial activator of important protein kinases that cause MOR desensitization. Therefore sustained stimulation is required to produce a cumulative effect that results in MOR desensitization. It is also possible that the interaction of morphine with MOR forced the receptor to be in the conformation that is a poor substrate for protein kinases, ß arrestin or both. This idea is supported by the observation that increased expression of GRK2 potentiates MOR desensitization and internalization.

Despite its ability to cause acute desensitization, chronic morphine treatment did not reduce morphine efficacy. Together, these results challenge the current understanding of the mechanism behind morphine anti-nociceptive tolerance. They suggest that the mechanism of tolerance may involve changes at the level of MOR-mediated signaling, as well as receptor regulation.

Receptor Internalization and Receptor Recycling

The most widely studied mechanism underlying the regulation of G-protein coupled receptors, more specifically the MOR, comprise a stepwise process that begins with phosphorylation of the receptor by G-protein receptor kinase [24,28,53]. The phosphorylated receptor has an increased affinity for ß-arrestin, resulting in the displacement of G-proteins [24]. The ligand/ß-arrestin/receptor complex is then thought to enter the endocytic pathway. Like the $ß_2$-adrenoceptor, the MOR has been shown to

recycle rapidly, from the endosomal compartment back to the plasma membrane. Although these studies showed that this process is important for receptor recycling, there was no evidence directly linking this process to the observed loss of receptor function [41,43].

Complete recovery from desensitization found after a short desensitizing treatment indicates that receptor re-sensitization can be a fast process. The observation that recovery was compromised by monensin, an agent shown to interfere with receptor recycling, suggests that the rapid turnover includes receptor sequestration and reinsertion of functional receptor back to the plasma membrane. Therefore, the initial steps of acute desensitization rapidly decrease receptor coupling or number, resulting in a shift of the concentration response curve to the right. With continued depletion of receptors, the maximum response declines and reaches a steady state after about 5 min. At this point, the rate of receptor re-sensitization and recyclingand the rate of receptor desensitization are at equilibrium, such that there was no further decline in the hyperpolarization when the desensitization period was increased to 10 or 20 min.

Another possibility is that different populations of the receptors are responsible for each of the components of desensitization and recovery. The two splice variants of rat MOR (MOR-1 and MOR-1B) have different carboxyl termini and have different rates for receptor desensitization and recovery [36,70]. Phosphorylation of threonine 394 on MOR-1 slowed internalization and delayed recovery following desensitization [40]. The short C-terminus found on MOR 1B enhances its interaction with clathrin-coated pits,

enabling faster rate of receptor internalization and recovery [36,70]. Thus the rapid recovery from desensitization may result from one splice variant and the slow or incomplete recovery from a second form of the receptor. Currently, it is not known which of the splice variants is expressed in LC neuron.

It is also possible that a completely different mechanism is responsible for the slower rate of recovery found after a more prolonged period of drug exposure. A recent study in LC neurons suggested that acute desensitization induced by the stable opioid peptide ligand DAMGO resulted in desensitization that was manifested at the G-protein-gated potassium channel [16]. Previous work and the present results indicate that neither acute desensitization nor chronic morphine treatment caused a dramatic decrease in the potassium conductance activated by the alpha-2-adrenoceptor.

Experiments done in vivo also indicate that there are alternative pathways for the regulation of MORs. For instance, in ß-arrestin-2 knockout animals, the development of morphine tolerance was initially delayed, but became apparent later in treatment. When these mice were treated with chelerythrin, a PKC inhibitor, morphine tolerance was attenuated [81]. These results suggest that there are two pathways mediating the effect of morphine tolerance, one that is ß-arrestin-2 dependent and one that is PKC-dependent. Although it is not clear how the results obtained in vivo, in the knockout animals, relate to the present results, it is clear that tolerance to morphine is regulated at multiple levels.

Desensitization and Receptor Reserve

Chronic morphine treatment results in a rightward shift of the concentration response curve to DAMGO [82]. A similar rightward shift is seen in LC neurons from control animals, following a brief application of ME (10 μM). With repeated short applications, the rightward shift is increased without a change in the maximal response. Sustained applications of ME (30 μM) produced a gradual decline of the maximum hyperpolarization. This gradual decline in responsiveness, as well as the reduction of the maximal response, is similar to that seen from experiments where the receptor reserves have been depleted by treatment with ß-chlornaltrexamine or ß-funaltrexamine [11,41,82]. These results suggest that the process of receptor desensitization is rapid and occurs as a continuum. The loss of receptor function closely correlates with the loss of receptor reserves. As such, the rightward shift in the concentration response curve to ME is a sensitive assay for the initial decline in receptor reserves. After chronic morphine treatment, the rightward shift of the concentration response curve persists. This observation indicates that the receptor reserve is compromised and receptor re-sensitization was impaired such that a subset of internalized receptors were either not recycled or recycled at a considerably slower rate. The reduced receptor reserve, in addition to the lack of recovery from acute desensitization, would increase the degree of receptor-dependent tolerance.

The regulation and trafficking of μ-opioid receptors has been shown to be agonist-dependent [11,15,84,85,98]. Most agonists, when applied at saturating concentrations, cause receptor desensitization and internalization. Morphine and its metabolite, M6G,

are the exceptions in that they are ineffective at inducing receptor desensitization and internalization under basal conditions [15,95] (but also see [11]). When GRK2 and/or ß-arrestin are overexpressed, on the other hand, internalization of μ-opioid receptors can be induced by morphine [24,84]. Chronic morphine treatment has been shown to elevate the expression level of GRK2 and ß-arrestin in the locus coeruleus [86]. These molecular adaptations can contribute to the increased in desensitization caused by M6G in slices from morphine-treated animals.

When brain slices from control animals were treated with chelerythrin, M6G-induced desensitization was detected. This observation suggests that M6G can induce desensitization of MORs under basal condition. There are two possible explanations for the inability to detect the loss of receptor function under control conditions. One is that M6G-induced MOR desensitization recovers rapidly; therefore the loss of receptor function was not seen due to the slow washout. It is also possible that M6G is not as efficient as M at inducing MOR desensitization. As a result, the rapid rate at which receptor re-sensitization occurs was sufficient to maintain the receptor reserve. When receptor re-sensitization was disrupted by treatment with chelerythrin, the loss of receptor function was apparent.

Heterologous Desensitization

Desensitization of MOR-mediated signaling has been shown to be mostly homologous [12-15,91]. In a recent study, it was demonstrated that extended stimulation of

MOR with DAMGO could lead to heterologous desensitization of the α_2-adrenoceptor-mediated activation of GIRK channels [16].

Early studies of MOR have used current-clamp recordings to measure MOR function following receptor activation by ligand. From these studies, it was clear that MOR desensitization is mostly homologous. Even after extended desensitization (20min) by ME (30μM), the α_2-adrenoceptor response evoked by UK13404 (3μM) was not significantly affected (Dang and Williams, unpublished data).

The first reports of MOR-induced heterologous desensitization indicate that 15 min exposure to ME or DAMGO can reduce the α_2-adrenoceptor-mediated-response in LC neurons[16]. Other works examining MOR regulation, using voltage clamp recordings from LC neurons, reveal variable results. Reports from these studies indicate that 5 min desensitization of MOR receptor with ME (30μM) is primarily homologous [12,14]. There are several possible explanations for the discrepancies between these studies. A study done by Fiorello et al. was done with intracellular recording, and the desensitization was stimulated for only 5 min compared to 15 min done by Blanchet et al.. Another possible explanation for the differences may be the age of the animals used in the studies. Blanchet et al. used very young animals. Although, it has not been determined whetherGPCR or MOR regulation and signaling is different in developing animals, it remains a plausible possibility.

For this study, MOR-evoked heterologous desensitization was assessed in coronal brain slices prepared from adult rats. Under these experimental conditions (see Methods), desensitization of MOR with ME (10min, 30μM) caused heterologous desensitization of the α_2-adrenoceptor response through ERK1/2 activation. Following

chronic morphine treatment, where ERK1/2 phosphorylation is elevated, heterologous desensitization was facilitated. While the exact mechanism has yet to be determined, the results suggest that this process is dependent on receptor endocytosis. This process has been proposed to activate ERK1/2 via a cSRC-dependent pathway [29].

A recent report has demonstrated that desensitization of MOR inhibition of VDCC was mediated by phosphoinositide 3-kinase cascade and ERK1/2 [83]. This desensitization occurs at the channels. Although the exact mechanism is still unclear, evidence suggests that activation of ERK1/2 reduced the $G_{\beta\gamma}$, interaction of VDCC, negating the inhibition of channels. As such, the effect of MOR desensitization is heterologous to GPCRs that are coupled to $G_{i/o}$. There is no direct evidence demonstrating alteration in GIRK-channel conductance as a result of acute MOR desensitization. The fact that heterologous desensitization can be inhibited by kinase inhibitors while the homologous desensitization was not significantly altered indicates that the effector in this case was not altered.

Conclusion:

MOR function, desensitization, and internalization were examined in this study. Receptor function and desensitization were measured from LC neurons, and receptor internalization was assessed in Hek293 cells. The results indicate that there is no correlation between the efficacy or potency of a drug with its propensity to cause receptor desensitization. There is, however, a high correlation between the ability of a drug to cause receptor internalization and receptor desensitization.

Receptor desensitization can be measured as declined in maximal response. The results of this study show that the process of MOR desensitization is complex and involves multiple levels of regulation. Desensitization can occur after a very brief exposure to high concentrations of drugs. Under control conditions, this loss of receptor function recovers completely in 25 min. After chronic morphine treatment, desensitization was facilitated and re-sensitization was impaired, such that complete recovery was not observed. Disruption of receptor re-sensitization in LC neurons from control animals showed increased receptor desensitization and incomplete receptor recovery. This indicates that one mechanism by which chronic morphine treatment alters receptor function is by reducing receptor reserve, disrupting receptor re-sensitization, or both.

Sustained stimulation of MOR with ligand that can cause robust receptor internalization leads to the manifestation of heterologous desensitization of α_2-adrenoceptor. Following chronic morphine treatment, this process is facilitated. Blocking ERK1/2 activation prevents MOR reduction of α_2-adrenoceptor response.

Although the kinetic is slow, morphine can cause desensitization of MOR. Sustained exposure to morphine causes a significant reduction of the maximal response. Even though morphine can cause acute MOR desensitization, chronic morphine treatment did not reduce morphine efficacy.

These results indicate that the process of MOR regulation is a complex process and involves multiple levels and mechanisms.

Future Directions:

In order to advance our understanding of the mechanisms behind tolerance and dependence, the process of MOR desensitization needs to be better characterized. The first step forward would be to determine the cause for the loss of receptor function during the process of desensitization. The next step would be to determine if MOR is differentially regulated in different areas of the central nervous system and how this may contribute to the development of tolerance and dependence. To pursue these questions, I propose the following aims:

- To determine if arr-3 is the cause for the loss of receptor function (desensitization).

The initial set of experiments will to determine if MOR desensitization is lost in neurons from the arr-3-knockout mice. MOR activation in neurons from the CNS inhibits VDCC activation and activates potassium conductance through the GIRK channels. Whole-cell voltage clamp recordings will be made from brain slices containing LC neurons and periaqueductal gray area (PAG), where the process of MOR has been previously characterized. Reduction of the maximum GIRK current during sustained MOR stimulation will be used as the measure of receptor desensitization. This preparation will allow us to study the regulation of the receptors under conditions that are close to their endogenous state. Using electrophysiological techniques will also provide a more precise and real-time measure of receptor function.

Result 1: If MOR desensitization is lost in neurons from these animals, it would suggest that arr-3 is responsible for the loss of receptor function during sustained stimulation. This can be confirmed by re-introducing arr-3 into the neurons. This can be done by viral transfection or electroporation in a culture preparation. Arr-3 can also be re-introduced into neurons through the whole-cell recording pipette. Desensitization experiments will be repeated to determine if MOR desensitization is rescued. This will show whether arr-3 is directly responsible for the loss of MOR function in the receptor desensitization process. To ensure that arr-3 is re-introduced into the neurons being recorded, arr-3 molecules will be attached with a HA-tag. After each recording, tissues or cells will be fixed and immunohistochemistry will be conducted for confirmation.

Result 2: If MOR desensitization is not lost or altered in these animals, it would suggest that arr-3 is not directly responsible for the loss of MOR function during receptor desensitization. If the kinetics or magnitude of desensitization and recovery were altered (reduced), it would indicate that there is more than one component to the process of MOR desensitization. This possibility will be explored first by pharmacologically disrupting the signaling of potential protein kinase candidates. These include PKC, PKA, CaMKII, ERK1/2, and GRKs.

Suppressing arr-3 expression reduced morphine analgesic tolerance but did not alter opioid dependence in mice [75]. There are several possible explanations for this observation. The simplest would be that the mechanisms leading to opioid tolerance and

opioid dependence are two distinct mechanisms, and that the mechanism behind morphine analgesic tolerance is dependent on arr-3. It is also possible that morphine differentially alters neuronal signaling or that MOR is regulated through distinctly different mechanisms in neurons from the CNS and in sensory neurons. Another possible explanation is that the mechanism leading to the desensitization of MOR coupling to VDCCs is different from that of GIRK channels. MOR is coupled to VDCCs in sensory neurons, whereas in the CNS, they are coupled to both VDCCs and GIRK channels.

To determine how MORs are regulated in sensory neurons (in the dorsal root ganglion and the trigeminal ganglion), neurons from the periaqueductal gray area, and the locus coeruleus nucleus will be used. Neurons from the LC [49,99-103] and the PAG [104-106] are chosen because they have been shown to participate in the expression of opioid dependence. The following aims will be pursued:

- To assess the time course and mechanism of MOR desensitization of opioid inhibition of VDCCs in sensory , LC, and PAG neurons.

Cell cultures from the DRG, LC, and PAG will be prepared from arr-3 knock out animals and their wild-type littermates. Using voltage-clamp recording, the time course for MOR desensitization and receptor function recovery will be determined for each cell group. The results from these experiments will indicate whether there is a difference in the

mechanism of MOR desensitization among sensory neurons and the neurons from the LC and PAG, as well as the involvement of arr-3 in this process.

- To determine if the mechanism of MOR desensitization is different for GIRK channels activation and for inhibition of VDCC.

The same methodology as above will be used. MOR desensitization and recovery will be assessed in PAG and LC neurons. Receptor function will be measured through MOR activation of GIRK currents and the inhibition of VDCC. Regardless of the results, we will be able to determine how intra-cellular signaling is mediated as well as the way in which MOR is regulated by neurons from different areas of the CNS.

- To determine the effects of chronic morphine treatment on sensory, LC, and PAG neurons.

Wild-type littermates and arr-3 knock-out mice will be treated with morphine for five days. After morphine treatment, acutely dissociated neurons will be prepared from the DRG, LC, and PAG. Whole-cell recording will be made to measure GIRK activation and VDCC inhibition. Result from each groups will be compared to determine differences in MOR regulation between wild-type and arr-3 knock-out animals.

Conclusion:

The major aims for this proposal are to determine the role of arr-3 in the process of MOR desensitization and to determine if the mechanism behind opioid tolerance is distinct from that of opioid dependence. To completely accomplish these aims will require the use molecular and biochemical techniques along with electrophysiology to identify the mechanisms and components involved in these processes. Regardless of the results, the experiments proposed will greatly add to the current understanding of MOR regulation and the extent to which it is involved in the long-term pathology of tolerance and dependence. Being able to isolate the mechanisms behind opioid tolerance and compare them with those behind dependence will facilitate the treatment for addiction.

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