Desensitization of functional μ-opioid receptors increases agonist off-rate

John T. Williams

Vollum Institute, Oregon Health & Science University, Portland, Oregon 97239, USA
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Corresponding author:

John T. Williams, Vollum Institute, Oregon Health & Science University, Portland, Oregon 97239, USA.

Phone: +1 503-494-5465, Fax: +1 503-494-4723, Email: williamj@ohsu.edu

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Abbreviations: CNV-NLX, carboxynitroveratryl-naloxone; CYLE, carboxynitrobenzyl-tyrosine-[Leu^5]-enkephalin; DAMGO, [D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin; DERM, dermorphin; GIRK channel, G protein-coupled K+ channel; GPCR, G protein-coupled receptor; LC, locus coeruleus; LE, [Leu^5]-enkephalin; ME, [Met^5]-enkephalin; MOR, mu opioid receptor; NLX, naloxone...
Desensitization of µ-opioid receptors (MORs) develops over 5-15 min following application of some but not all opioid agonists and lasts for 10s of min following agonist removal. The decrease in function is receptor selective (homologous) and could result from (1) a reduction in receptor number or (2) a decrease in receptor coupling. The present investigation used photolysis of two caged opioid ligands in order to examine the kinetics of MOR-induced potassium conductance before and following MOR desensitization. Photolysis of a caged antagonist, caged-naloxone (CNV-NLX), blocked the current induced by a series of agonists and the time constant of decline was significantly decreased following desensitization. The increase in the rate of current decay was not observed following partial blockade of receptors with the irreversible antagonist, β-CNA. The time constant of current decay following desensitization was never more rapid than 1s, suggesting an increased agonist off rate rather than an increase in the rate of channel closure downstream of the receptor. The rate of GIRK current activation was examined using photolysis of a caged agonist, [Leu]-enkephalin (CYLE). Following acute desensitization or partial irreversible block of MORs with β-CNA there was an increase in the time it took to reach a peak current. The decrease in the rate of agonist-induced GIRK conductance was receptor selective and dependent on receptor number. The results indicate that opioid receptor desensitization reduced the number of functional receptors and the remaining active receptors have a reduced agonist affinity.
INTRODUCTION

Numerous studies have examined the reduction in μ-opioid receptor (MOR) signaling (desensitization) following the application of a saturating concentration of opioid agonists (Williams et al., 2013). Desensitization is homologous and recovers over a period of 30-60 min. One consistent observation is that the extent of desensitization is never complete. There are two potential reasons for the inability to observe a complete block of signaling. One is that some receptors may escape or recover very quickly from desensitization, and the second is that desensitized receptors remain functional but with reduced efficiency. Given the complexity of the downstream signaling events mediated by G-protein coupled receptors, multiple mechanisms could underlie the decline in functional measures (Williams et al., 2013). It is therefore necessary to examine the agonist/receptor interaction more directly before and following desensitization.

A previous study reported that agonist/receptor affinity was increased in living HEK293 cells expressing Flag-MORs following treatment (2 min to 2 hours) with saturating concentrations of some, but not all, opioids (Birdsong et al., 2013). The increase in agonist affinity was measured as a decrease in agonist off rate (K-off) by directly observing the dissociation of a fluorescently labeled peptide agonist. The decrease in K-off was resistant to pertussis toxin treatment and persisted in MEF cells from arrestin knockout animals. Thus persistent agonist occupation induced a long lasting state of the receptor having high affinity for agonist. This state was not dependent on downstream signaling, partially recovered over a period of 45 min following the removal of agonist and was interpreted to be the desensitized receptor. However, these experiments did not measure receptor activity. If the desensitized high affinity receptors were functional, although less efficient, it should be possible to measure a change in ligand-receptor interaction kinetics using a functional assay.

To study the agonist/receptor interaction following desensitization directly in neurons, the present study examined the kinetics of MOR-induced activation and inactivation of potassium conductance in locus coeruleus neurons in brain slices.
The experiments used two caged opioids, caged nalo xone (CNV-NLX) and caged [Leu]-enkephalin (CYLE). Both caged compounds have little or no affinity for the opioid receptor and can be applied at a high concentration for a prolonged period without affecting the opioid receptor. Photolysis results in near instantaneous release of naloxone or [Leu]-enkephalin that then bind to MOR (Banghart and Sabatini, 2012). Naloxone competes with agonist at the receptor to inhibit the receptor-dependent activation of potassium conductance (Banghart et al., 2013). The rate of inhibition is dependent on agonist affinity and concentration, the concentration of CNV-NLX and the time and intensity of light used for photolysis. Thus at sub-saturating concentrations the decrease in opioid receptor dependent potassium conductance can be a reliable measure of the agonist off rate (Banghart et al., 2013). The off rate of agonists from MORs in locus coeruleus neurons was measured before and following desensitization using the photolysis of CNV-NLX. The results show that desensitization increased the off-rate of agonist from functional receptors. Using the same approach, the rate of MOR-induced increase in potassium conductance was measured using the caged agonist CYLE before and following desensitization (Banghart and Sabatini, 2012). Desensitization and prior treatment with β-CNA decreased the activation rate of potassium conductance suggesting that the kinetics of channel activation were dependent on receptor number. These results indicate that desensitization produced a population of functionally active receptors with apparent low affinity for agonist, which stands in contrast to the observations using fluorescent agonist (Birdsong et al., 2013). Thus desensitization results in at least two distinct pools of receptor: one functionally active with an apparent low affinity and one non-functional with high affinity.

MATERIALS AND METHODS

Slice preparation

Horizontal brain slices containing locus coeruleus (LC) neurons were prepared as described previously (Williams and North, 1984). Briefly, rats were killed and the
brain was removed, blocked and mounted in a vibratome chamber (Leica VT 1200S). Horizontal slices (240 μm) were prepared in cold cutting solution containing (in mM): 126 NaCl, 2.5 KCl, 1.2 MgCl₂, 2.6 CaCl₂, 1.2 Na₂HPO₄, 11 D-glucose and 21.4 NaHCO₃ and 0.01 MK801 (Abcam), equilibrated with 95% O₂/5% CO₂. Slices were stored at 34°C in glass vials with oxygenated (95% O₂/5% CO₂) artificial cerebrospinal fluid (ACSF) containing (in mM): 126 NaCl, 2.5 KCl, 1.2 MgCl₂, 2.6 CaCl₂, 1.2 Na₂HPO₄, 11 D-glucose and 21.4 NaHCO₃.

Recording

After an incubation period 30-60 min, slices were hemisected and transferred to the recording chamber and superfused with 34°C ACSF at a rate of 1.5 ml/min. Whole-cell recordings were made with an Axopatch 1D amplifier in voltage-clamp mode (V_hold = -60 mV). Recording pipettes (1.7–2.1 MΩ) were filled with internal solution containing (in mM): 115 potassium methanesulfonate or potassium methyl sulfate, 20 NaCl, 1.5 MgCl₂, 5 HEPES(K), 10 BAPTA or 0.1 EGTA, 2 Mg-ATP, 0.2 Na-GTP, pH 7.4, 275-280 mOsM. Series resistance was monitored without compensation and was <15 MΩ. Current was continuously recorded at 200 Hz with PowerLab (Chart version 5.4.2; AD Instruments, Colorado Springs, CO). Episodic currents were recorded at 10 kHz for 1 min using AuoGraphX (1.4.3). Drugs were applied by bath superfusion.

Uncaging of CNV-NLX was carried out with full field illumination using a 405 nm LED (Thorlabs) coupled through a 60x objective (Olympus - 0.9 NA). Light power was 10 mW at the back aperture of the objective. A solution containing CNV-NLX (5 μM, in 6 ml) was re-circulated for at least 5 min before an uncaging experiment. Opioid agonists were added to the solution containing CNV-NLX prior to photolysis. In most experiments slices were exposed to only a single light flash. In all experiments a saturating concentration of the alpha-2-adrenoceptor agonist UK14304 (3 μM) was applied and the resulting current was used as a postsynaptic control. In many experiments the amplitude of opioid induced current was presented as a fraction of the current induced by UK14304. Uncaging of CYLE (20 μM) was carried out in a recycled solution with full field illumination
using a 365 nm LED (Thorlabs) coupled through a 60x objective (Olympus 0.9 NA, 1.5 mW at objective). Morphine was obtained from NIDA. ME, DAMGO, endomorphin-2 and β-CNA were obtained from Sigma. Dermorphin was obtained from Phoenix Peptides. Somatostatin was obtained from Bachem. Nociceptin (OFQ), UK14304 and idazoxan and caged NA were obtained from Tocris. CNV-NLX prepared by Shanghai Medicilon Inc. using the procedure described in Banghart et al, 2013. CYLE was obtained from Peptech Inc. and further purified by HPLC as described in Banghart et al., 2012.

Data analysis

Summary data are presented as mean±SEM. The time constant of decay was fit to a single exponential using AxographX. The time courses covered the area from at least 5-95% of the total time course of the decline in agonist induced current. Statistical analysis was carried out using paired or unpaired Mann-Whitney Rank Sum Test where a p<0.05 was considered significant.

RESULTS

Increased rate of current decay induced by naloxone after desensitization

Caged-naloxone (CNV-NLX, 5 µM) was recirculated for a minimum of 5 min prior to the application of [Met5]enkephalin (ME). ME induced an outward current that was completely blocked following a flash of light to release naloxone (photolysis of CNV-NLX, 405 nm, 5 s, Figure 1). The time constant of inhibition was dependent on the concentration of ME. At a saturating concentration (30 µM) the time constant of decay was about 3.5 s and at a sub-saturating concentration (1 µM) was about 1 s (Figure 1, Banghart et al., 2013). The rate of naloxone block of the ME current was expected to be concentration dependent based on the fact that naloxone and ME compete for the same binding site on the receptor. With a prolonged application of ME (30 µM) the current peaked and desensitized over 10 min (Figure 1). The time constant of current decay induced
by photolysis of CNV-NLX (5 µM, 5s) after 10 min of ME decreased to about 1.5 s, roughly the same as the rate of decline measured with a sub-saturating concentration of ME (1 µM, Figure 1). Thus following desensitization, naloxone competes more effectively with a saturating concentration of ME.

The high affinity agonist DERM was used previously to characterize the rate of CNV-NLX induced decay over a wide range of concentrations (Banghart et al., 2013). At low concentrations (<100 nM) the time constant of decay reached a limiting level of about 4 s (Banghart et al., 2013). That limiting level was suggested to represent the off-rate of DERM from the receptor. Photolysis of CNV-NLX (5 µM, 5s) at the peak of the outward current induced by DERM (1 µM) blocked the current with a time constant of about 6.5 s (Figure 2). After 10 min the current induced by DERM (1 µM) declined to about 50% of the peak. Photolysis of CNV-NLX blocked the remaining current with a time constant of about 3 s (Figure 2). Although there is considerable spread in the results, the mean value of the time constant of decline was faster than that observed using much lower concentrations of DERM (10-100 nM, Banghart et al., 2013). The decay however remained slower than the maximum rate of decline (1-2 s) observed with multiple low affinity agonists (Banghart et al., 2013).

The increase in the rate of current decay induced by CNV-NLX was not dependent on the continued presence of opioid agonists. Slices were incubated in a saturating concentration of ME (30 µM, 1-2 hr) in order to induce desensitization. Following the incubation, the ME was washed (5-15 min) and recordings were made. During the recording CNV-NLX (5 µM, 5 min) was equilibrated followed by application of DERM (1 µM). Once the DERM induced current reached a peak, photolysis of CNV-NLX blocked the outward current with a time constant of about 4 s, the same as that found following acute desensitization (Figure 2B). Thus the induction of desensitization with ME resulted in an increase in the rate of current decline that outlasted the presence of agonist.
The increase in the rate of naloxone-induced decline in current suggested that the agonist affinity decreased following desensitization, however following photolysis of CNV-NLX in the high concentration of DERM (1 µM) the outward current began to recover. This recovery was presumably the result of naloxone dissociation and rebinding of DERM. In order to obtain a more quantitative assessment of a change in agonist affinity a low concentration of DERM (30 nM) was used. This concentration of DERM has been previously shown to be low enough that there was no detectable rebinding of agonist within the time course of the experiment. Thus the decline in current induced by CNV-NLX under these conditions is thought to be dependent on the K-off of DERM. The CNV-NLX-induced decline in the current in the presence of DERM (30 nM) was measured in untreated slices and slices that were incubated in ME (30 µM, 30-120 min) and washed for 10-20 min before the application of DERM (30 nM). In previous experiments the time constant of decline induced by CNV-NLX in the presence of DERM (30 nM) was 4.8±0.43 s (n=5, Banghart et al., 2013). This value was reproduced in additional current control experiments (4.9±0.23 s, n=7), so the two sets of data were combined (4.8±0.21 s, n=12). In slices that were incubated in ME (30 µM, desensitized) the CNV-NLX induced decline in the DERM-induced (30 nM) current was significantly faster (2.38±0.18 s, n=12, p<0.05, Figure 2B). These values along with the determination of the K-on for a fluorescent DERM (DERM-A594, 6.12*10^5 M^-1s^-1, Birdsong et al., 2013) were used to estimate the increase in Kd that resulted from desensitization (control 340 nM, desensitized 687 nM). Thus the results suggest that desensitization reduced the affinity of DERM for the receptor by about 50%.

**No change in rate of naloxone block following reduction of functional MORs with β-CNA**

It is possible that the decline in the amplitude of the current induced by desensitization with DERM could affect the rate of blockade by CNV-NLX. This possibility was examined using the partial irreversible inactivation of MORs with
β-CNA (100 nM, 2 min). This treatment decreased the current induced by DERM (1 µM, normalized to the current induced by a saturating concentration of UK14304, 3 µM) to a similar extent as that observed following desensitization (DERM/UK14304; peak 1.27±0.05, n=11; desensitized 0.66±0.4, n=10; post β-CNA 0.63±0.06, n=16). The time constant of inactivation was however not significantly changed from that found at the peak of the DERM current (Figure 2). The results indicate the simple removal of receptors using β-CNA did not affect the rate of receptor blockade following photolysis of CNV-NLX, unlike what was observed following acute desensitization. Thus the increase in the rate of current decay by photolysis of CNV-NLX following desensitization resulted from a decrease in agonist dependent signaling. The increase in deactivation rate observed with both ME and DERM is most readily interpreted as an increase in agonist off rate, which suggests that desensitization decreased agonist affinity.

**Increased rate of naloxone current decay is receptor selective**

It is possible that the increase in rate of decline caused by naloxone results from a change that is independent of MOR. Experiments were carried out with the use of saturating concentrations of somatostatin (SST, 3 µM) and nociceptin (OFQ, 3 µM) applied for 10 min, both of which also activate GIRK channels in LC neurons. Both agonists resulted in a peak and decline in the outward current that was similar to that seen with a saturating concentration of DERM (Virk et al., 2009; Llorente et al., 2012). During the application of either SST (3 µM) or OFQ (3 µM), CNV-NLX (5 µM, 5 min) was equilibrated in the recycling solution and DERM (1 µM) was applied in the continued presence of SST or OFQ. DERM increased the outward current and photolysis of the CNV-NLX resulted in an inhibition of the current. In the presence of SST, the time course of the inhibition was not different from the acute application of DERM alone (Figure 3). Treatment with OFQ resulted in a small increase in the rate of decay induced by CNV-NLX (Figure 3). To further investigate the potential interaction between OFQ and DERM, slices were incubated in OFQ (3 µM) for periods of 30-90 min,
OFQ was washed and the OFQ antagonist, UFP101 (3 µM), was applied before the addition of DERM (1 µM). In the absence of a current induced by OFQ, the rate of naloxone block of the current induced by DERM was not different from control (Figure 3). The small effect of OFQ on the inactivation of the current induced by DERM when applied simultaneously may suggest a common component of downstream signaling. However this component was small and reversed rapidly upon blockade of the OFQ receptor. Thus the increase in the rate of current decay was homologous resulting from the selective activation of MOR.

**The increase in rate of naloxone block depends on agonist affinity**

The relative role of agonist affinity on the increased rate of receptor blockade by CNV-NLX was examined using a series of agonists having different affinity for the receptor (Banghart et al., 2013). With the exception of morphine, application of each agonist for 10 min resulted in a decline from the peak current. Previous work found that the most reliable way to induce desensitization with morphine was to treat animals for 6-7 days with morphine (Levitt and Williams, 2012). Desensitization (and tolerance) to morphine was induced by the chronic treatment of animals with morphine using osmotic mini-pumps, slices were cut and maintained in morphine (1 µM, Levitt and Williams 2012). The current induced by CNV-NLX from morphine treated animals was compared with that induced in slices taken from untreated animals where the slices were cut and maintained in morphine.

The amplitude of the current and the rate of current decline induced by CNV-NLX were measured for a series of agonists before and following desensitization. In order to induce desensitization it was necessary to use a saturating concentration of each agonist. The amplitude of the opioid-induced current in each experiment was normalized to the current induced by a saturating concentration of the alpha-2-adrenoceptor agonist, UK14304 (3 µM, Figure 4B). Following desensitization with each agonist (DERM, endomorphin2, DAMGO, ME and morphine) there was a significant decrease in the amplitude of the opioid
The decrease in the steady-state amplitude of the opioid-induced current following desensitization is well established, however the kinetics of current activation have not been tested. Photolysis of the caged opioid agonist, CYLE was used to examine the amplitude and rising phase of agonist-induced current before and following acute desensitization (Figure 5). CYLE (20 µM) was recycled in the presence of bestatin (10 µM) and thiorphan (1 µM), to block peptidase activity, and photolysis was induced using an LED light source (100 ms, 365 nm). Flashes were applied at 3 min intervals and the amplitude (150-400 pA) and kinetics (rise time 160 ms; time to peak = 0.6-1.2 s) of the current remained
constant for at least 30 min using this protocol. After 2-3 light flashes, the CYLE-containing solution was washed out, ME (30 µM) was applied for 10 min and washed for 5 min before the recycling CYLE again. The initial amplitude of current induced by photolysis following desensitization was dramatically reduced and partially recovered over a period of 15-20 min (Figure 5). The rate of rise and time to reach the peak current were consistently slowed following desensitization (Figure 5). The decrease in the amplitude of current amplitude following desensitization was expected although the recovery from desensitization was less than that observed in previous experiments using steady state application of agonist (Osborne and Williams, 1995; Fiorillo and Williams, 1996; Quillinan et al., 2011).

The rate of current rise to a steady state was examined using a prolonged flash (5 s, CYLE 20 µM +bestatin 1 µM +thiorphan 10 µM). The first flash resulted in the activation of current with a time constant of 241±26 ms (n=6). In every case the current peaked and started to decline during the 5 s exposure to light (Figure 6). The current declined to the baseline after 2-3 min. A second flash resulted in a smaller current and the rate of rise was slowed (426±68 ms) with the early component of the current being the most affected. A third flash resulted in a smaller and more slowly rising current (473±85 ms). The decrease in amplitude and rate of rise following the first flash was taken as a sign of acute desensitization. Finally, slices were incubated in ME (30 µM, 30-90 min) washed for 10-15 min before applying a 5s flash (CYLE 20 µM). In these experiments the time constant of current rise was increased to 651±76 ms (n=6, Figure 6C). Thus, similar to short flashes, prior desensitization decreased the rate of current rise induced by a flash long enough to reach allow the current reach a steady state.

Treatment with β-CNA decreased the rate of agonist-induced current
The small decrease in the rate of current activation following desensitization could potentially result from a change in affinity of LE for the receptor or a change in downstream signaling. Given the change in the amplitude of current induced by CYLE experiments were done following treatment of the slice with β-CNA to remove receptor reserve. The rising phase of the current induced by CYLE was examined before and following treatment of slices with β-CNA (100 nM, 2 min, followed by a 10-15 min wash). The amplitude of the current induced by CYLE following treatment of the slice with β-CNA was decreased by about 50%, the same as that following desensitization (CYLE control 232±25 pA, desensitized 103±13 pA, n=14; β-CNA control 214±25 pA, post-β-CNA 96±14, n=11). Likewise the rate of rise of the current decreased after treatment with β-CNA (Figure 5D). Thus removal of functional receptors with β-CNA resulted in the same change in the rising phase of the CYLE-induced current as that following desensitization with a saturating concentration of ME. This suggests that there are two components involved in desensitization. One is a decrease in the apparent affinity of functional receptors for agonists measured as an increase in deactivation rate with photolysis of CNV-NLX. The second is a reduction in the number of functional receptors measured as a change in activation rate with CYLE.

**A heterologous mechanism does not alter MOR activation kinetics**

Both somatostatin and OFQ receptors have been shown to desensitize readily (Fiorillo and Williams, 1996; Virk et al., 2009). Activation of MORs with CYLE was examined before and during the application of saturating concentrations of OFQ (3 µM, 10 min). Although the amplitude of the current induced by CYLE was decreased by occlusion, the time constant of the rise in current induced by CYLE was not changed (Figure 7). Thus desensitization of non-opioid receptors did not affect the rate at which the activation of MORs activated potassium current.

The amplitude and rate of current activation induced by CYLE was also examined before and during the application of morphine (1 µM, Figure 7). This concentration results in a current that is about 75% of the peak such that the
amplitude of the current induced by CYLE was reduced by competition for MOR (Levitt and Williams, 2012). In these experiments the rate of rise of the current induced by CYLE was also decreased (Figure 7). Thus the occupation of receptors with morphine, a partial agonist, reduced the rate of current activation similar to that observed following desensitization or treatment with β-CNA.

In order to determine if the increase in rise time of the current induced by CYLE resulted from a change in downstream signaling, the rise time of the current induced by photolysis of caged noradrenaline was examined before and after desensitization induced by ME (30 µM, 10 min). Caged-noradrenaline (100 µM) was recycled and repeated flashes (365 nm, 100 ms, 1 min intervals) were applied to activate alpha-2-adrenoceptors (Figure 8). This protocol resulted in reproducible outward currents with a time to reach the peak of approximately 500 ms. The current was increased by cocaine (10 µM) and was blocked by the alpha-2-adrenoceptor antagonist, idazoxan (1 µM). The current induced by photolysis of caged-NA was initially occluded following the addition of DERM (10 µM, 10 min). As the DERM induced current declined the current induced by caged-NA increased (Figure 8). Following the addition of naloxone (1 µM) the current induced by caged-NA increased to 90% of the initial amplitude. The time it took to reach the peak current induced by noradrenaline was not changed during or after the application of DERM. Thus desensitization of MOR did not affect the signaling induced by activation of the alpha-2-adrenoceptor.

DISCUSSION

With the use of caged opioids, two distinct changes in opioid receptor signaling were observed following desensitization of MORs in locus coeruleus neurons. First there was an increase in the rate at which rapid application of naloxone decreased MOR dependent potassium current. Second, the rate of potassium current activation induced by photolysis of caged agonist was decreased following desensitization. There is a distinct mechanistic difference between the two observations. The increase in the rate of current inactivation induced by CNV-
NLX was not dependent on the receptor number. Reducing receptor number with an irreversible antagonist did not change the rate of current inhibition induced by CNV-NLX. The decrease in the rate of current activation induced by CYLE was however dependent on the number of receptors. Thus opioid receptor desensitization results in two distinct changes in receptor dependent kinetics: a reduction in the number of active receptors measured with the caged-agonist, and a decrease in agonist/receptor affinity of the remaining functional receptors measured with CNV-NLX. These functional receptors are distinct from the high affinity, most likely non-functional, receptors that were observed in HEK cells following desensitization using a fluorescent binding assay (Birdsong et al., 2013).

**Measuring Agonist off rate with a functional assay**

The decline in potassium current induced by the photolysis of CNV-NLX is dependent on the concentration of agonist, the concentration of naloxone released by photolysis and the off rate of the agonist from the receptor (Banghart et al., 2013). In order to induce desensitization, it was necessary to apply a saturating concentration of each agonist such that upon photolysis naloxone competed with the agonist at the receptor. Although the on rate of naloxone for the receptor is rapid, competition between naloxone and the saturating agonist concentration slowed the rate of current decline (Banghart et al., 2013). The increase in the rate of current decline induced by photolysis of CNV-NLX following desensitization therefore results from a more effective competition of naloxone for MOR. The most obvious explanation for this result is a reduction in the affinity of agonist for MOR. This conclusion is supported by results obtained with a subsaturating concentration of DERM (30 nM). Desensitization decreased the affinity of DERM for the receptor by about 50%.
The limiting rate of decline induced by photolysis of CNV-NLX was 1-2 s, a value that is thought to represent the maximum rate of GIRK inactivation (Ingram et al., 1997; Banghart et al., 2013). Following desensitization the rate of decline was never less than this limiting value suggesting that desensitization resulted in a decrease in agonist/receptor affinity rather than an increase in the rate of downstream mechanisms that terminate G-protein dependent signaling.

Pharmacological studies have indicated that acute desensitization reduces the number of functional receptors by about 90% (Osborne and Williams, 1995). The current that remained following desensitization is therefore dependent on a small population (10%) of receptors that have a lower apparent affinity for agonists. Binding assays on living cells following a desensitization protocol report that the majority of receptors were in a high affinity state (Birdsong et al., 2013). Although the G-protein coupled receptor is considered to have high affinity for agonists, the high affinity state that was induced by desensitization in the HEK cells is unlike the canonical model of GPCR/G-protein interaction (Werling, Puttfarcken and Cox, 1988). The desensitized receptor was insensitive to prior treatment with pertussis toxin (Birdsong et al., 2013). This result was interpreted to indicate that the desensitized (presumed uncoupled) receptor adopted a high affinity state that was independent of G-proteins. There is also precedent that agonist affinity increased with prolonged agonist incubation in hippocampal membranes (Scheibe et al., 1984). Had these high affinity receptors been functional, the agonist off rate seen in the present study would be expected to slow several fold. Previous studies looking at changes in affinity after desensitization and/or tolerance have not differentiated functional from non-functional receptors. Therefore, by implementing a functional assay the results provide evidence for at least two distinct pools of receptors following homologous desensitization: one functionally active with an apparent low affinity and one non-functional with high affinity.
Potential Mechanisms

The decrease in functional receptors found following desensitization is largely homologous, although the degree of heterologous desensitization is dependent on the age of the animal (Llorente et al., 2012). The activation of the same potassium conductance through the OFQ and somatostatin receptors had little or no effect on the naloxone induced inactivation of the current induced by DERM. The decay in the potassium current induced by naloxone was insensitive to the continued presence of a saturating concentration of somatostatin even after the current induced by somatostatin had declined to about 50% of the peak. Thus a global increase in the presence of molecules that may increase the inactivation of GIRK such as Regulators of G-protein Signaling (RGS) proteins may not account for the results. This does not however rule out the potential role of RGS proteins that are selectively associated with MORs.

Potential receptor selective mechanisms include phosphorylation. In spite of considerable effort there is no consensus on the role of phosphorylation in acute MOR desensitization, particularly the in brain (Williams et al., 2013). In HEK cells expressing MORs multiple sites on the C-terminal tail were phosphorylated by morphine and DAMGO (Lau et al., 2011; Just et al., 2013; reviewed Williams et al., 2013). Mutation of a sequence of phosphorylation sites beginning at S375, to alanines eliminated receptor trafficking and arrestin binding (Lau et al., 2011). Another sequence of C-terminal residues, TSST, starting at T354 were also identified as agonist-dependent phosphorylation sites (Lau et al., 2011). Mutation of those residues to alanine did not change the trafficking of receptors induced by DAMGO. The role of phosphorylation of these amino acids, both STANT (375-380) and TSST (354-357) in acute desensitization particularly in neurons is not known (but see Wang et al., 2002).

Receptor number regulates the rate of current activation
A surprising observation was that the decrease in the rate of CYLE induced current following MOR desensitization was dependent on receptor number. Treatment of slices with the irreversible antagonist, β-CNA decreased the rate of potassium current activation induced by CYLE to the same extent as that found after desensitization. The rate of current activation induced by opioids in acutely isolated LC neurons had a time constant of about 700 ms (Ingram et al., 1997). This value is significantly slower than that found in the present study, however the receptor reserve in acutely isolated neurons was reduced to the point that morphine was ineffective at activating a current. In fact, in the acutely dissociated cells morphine effectively blocked the current induced by efficacious agonists (Ingram et al., 1997). Taken together it appears that the number of active receptors plays a key role in the kinetics of MOR activated conductance.

There is building evidence based on various fluorescence assays that GPCRs including MORs exist in different states distinguished by the association with G-proteins. Receptors pre-associated with G-proteins (precoupled receptors) are thought to signal more rapidly than receptors that are dependent on collision coupling (Nobles et al., 2005; Riven et al., 2006; Philip et al., 2007; reviewed, Lohse et al., 2008; Vilardaga 2010). The stability and thus the relative number of the precoupled receptors is reportedly very low for some receptors (alpha-2-adrenoceptor, Qui et al., 2008) and substantially greater for others (muscarinic M3 receptor, Qin et al., 2011). Where functional studies have been done using the activation of GIRK dependent currents, precoupling has been suggested (Lober et al., 2006; Zhang et al., 2002; Zhou et al., 2012). Those experiments examined receptors (including MOR) that were immobilized and the mobility of G-proteins, GIRK channels or RGS proteins were assayed (Lober et al., 2006; Zhang et al., 2002; Zhou et al., 2012).

One possible explanation for the change in rate of rise following MOR desensitization is that there is a small percentage of MORs are in a state that favors rapid activation (precoupled). Photolysis of CYLE results in the rapid
application of a high agonist concentration such that a large percentage of receptors are activated near simultaneously. This method is ideal for the detection of precoupled receptors. Even if the population of precoupled receptors is less than 20% of the total (Qin et al., 2008), these efficiently coupled receptors could be sufficient to effectively activate downstream effectors. Elimination of receptors through desensitization, antagonism with β-CNA or acute dissociation of the cells, may not change in the relative number of ‘precoupled’ receptors but the absolute number of these receptors may decrease to a level below the limit of detection. Thus the rate of GIRK activation would be more dependent on the relatively large proportion of more slowly activated collision coupled receptors.
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Authorship Contributions

Participated in research design: Williams

Conducted experiments: Williams

Performed data analysis: Williams

Wrote or contributed to the writing of the manuscript: Williams
REFERENCES


MOL #92098


Footnotes

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Figure Legends

Figure 1. The rate of current decline induced by photolysis of CNV-NLX (5 µM) increased following desensitization induced by ME (30 µM). A, shows two experiments, one where photolysis of CNV-NLX was applied at the peak of the ME-induced current and the second 10 min after the application of ME. Below are examples of the change in current induced by CNV-NLX at the peak (left) and 10 min after the application of ME (right). B, is summarized results showing the time constant of decay at the peak (blue), after ME (10 min, green) and at the peak of a non-saturating concentration of ME (1 µM, black).

Figure 2. The rate of current decline increased following desensitization with the high affinity agonist DERM (1 µM). A, two experiments showing the inhibition of the DERM current at the peak of the current (left) and 10 min after the application of DERM (right). Below are example traces of the current decline induced by CNV-NLX at the peak (peak), 10 min after DERM (DERM 10 min) and after partial blockade of MORs with β-CNA (after β-CNA). B, dot plot of the time constant of CNV-NLX induced inactivation of the DERM current at the peak (peak closed circles), 10 min after DERM (10 min, open circles) and 5-15 min after slices were desensitized with ME (30 µM, 1-2 h, after-ME, open circles) and after treatment with β-CNA (post β-CNA, closed circles). Shaded areas illustrate the standard area of the time constant of decay measured at the peak (top) and after 10 min (bottom). Closed squares indicate the time constant of current decay induced by CNV-NLX following the application of DERM (30 nM). Larger squares are from the current experiments, smaller squares are from published work. Open squares are the time constant of current decay induced by CNV-NLX in slices that were pre-desensitized by incubation with ME (30 µM, 30-90 min and washed before application of CNV-NLX, after-ME). *p<0.05 by unpaired Wilcoxon-Mann-Whitney Rank Sum Test.
Figure 3. Application of DERM (1 µM) following desensitization with somatostatin and OFQ does not change the rate of current decline induced by CNV-NLX. A, example trace of an experiment in which the current induced by somatostatin (1 µM, 10 min) peaked and decayed, followed by the application of DERM and photolysis of CNV-NLX. B, dot plot of experiments measuring the time constant of current decay induced by CNV-NLX at the peak of the current induced by DERM (1 µM) in the presence of somatostatin (DERM+SST), OFQ (DERM+OFQ) and following the washout of OFQ (DERM wash OFQ). Shaded areas are the same as in figure 2, illustrating the standard error of experiments with DERM alone at the peak (top) and following 10 min (bottom). *p<0.05 by one way ANOVA compared to DERM alone, Dunnett post hoc.

Figure 4. Summary of the results obtained for 5 agonists showing the increase in the rate of decline following desensitization. A, histogram of the time constant of current decay induced photolysis of CNV-NLX (5 µM), for DERM (1 µM, same data as in figure 2), Endomorphin2 (1 µM), DAMGO (1 µM), ME (30 µM, same data as in figure 1) and morphine (1 µM). Open bars – measured at the peak current, solid bars are 10 min after application of the agonist, with the exception of morphine, which was following treatment of the animal for a week. In each case the increase in rate of decline after prolonged treatment (black bars) was significantly different from that measured at the peak. Horizontal line, dark and light gray boxes are the mean, SEM and SD of the time constant of decline in the current induced by EC50 concentrations of low affinity agonists (codiene, DAMGO, ME, DSLET, oxycodone and morphine, taken from Banghart et al 2013). B, histogram of current amplitude before and following desensitization with the indicated agonist (normalized to the current induced by UK14304, 3 µM). *p<0.05 by unpaired Wilcoxon-Mann-Whitney Rank Sum Test.

Figure 5. Desensitization decreased the amplitude and rate of rise of the current induced by photolysis of CYLE. A, example trace of an experiment where the
current induced by CYLE is shown before and following desensitization with ME (30 µ). Arrows indicate the light flash. B, summarized results showing the inhibition and recovery of the amplitude of the current induced by CYLE. C, two superimposed traces of the current induced by CYLE before and following the recovery from desensitization (10-20 min). D, summarized results showing the increase in the rise (time constant) before (C) and following recovery from desensitization (des) and before (C) or after application of β-CNA (CNA, 100 nM, 2 min). *p<0.05 by paired Wilcoxon-Mann-Whitney Rank Sum Test.

Figure 6. Repeated long flashes decreased amplitude and rate of current rise induced by photolysis of CYLE. A. Example trace of an experiment showing the current induced by two 5 s flashes of CYLE. B. Two superimposed traces showing the change in rate of rise of current induced by two flashes of CYLE. C. Summarized results showing the time constant of current activation induced by CYLE on three separate flashes applied at 3-5 min intervals (closed circles). The open circles summarize experiments where slices were incubated in ME (30 µM, 30-90 min) to induce desensitization and washed before photolysis of CYLE (20 µM, 5 s, after-ME).

Figure 7. CYLE induced current is decreased by a receptor selective mechanism. A, example traces of the current induced by CYLE before (black) and after desensitization with OFQ (1 µM, 8-10 min, red). B, example traces of the current induced by CYLE (black) and in the presence of morphine (1 µM, 8-10 min, blue). C, summarized results measuring the time constant of the rate of rise. Experiments with OFQ and morphine were paired and the control tau-On were combined for illustration. Statistics were done with a paired Wilcoxon-Mann-Whitney Rank Sum Test (*p<0.05).
Figure 8. MOR desensitization does not change the noradrenaline activation of GIRK. A, an example trace showing the current induced by photolysis of noradrenaline (arrows) during the application of DERM (1 µM) and DERM+naloxone (1 µM). B summarized results showing the NA current decreased in DERM through occlusion, the reversal with naloxone (1 µM), the increase induced by cocaine (10 µM) followed by the inhibition with idazoxan (1 µM). C, superimposed traces of the current induced by caged-NA before, 7-10 min in DERM, and after reversal with naloxone (1 µM). Below, summarized rate of rise of the NA-induced current. Statistics tested with an ANOVA, Dunnett post hoc.
Figure 2

A

DERM (1 μM)

CNV-NLX (405 nm, 5s)

CNV-NLX (405 nm, 5s)

DERM (peak)
tau - 7.4 s

DERM (10 min)
tau - 4.3 s

DERM (after β-CNA)
tau - 8.0 s

0 current

B

Tαu - CNV-NLX (s)

DERM (1 μM) DERM (30 nM)

peak 10 min after-ME β-CNA peak after-ME

ns * *
Figure 3

A. In CNV-NLX (5 μM) SST (1 μM)

DERM (1 μM)

B. 

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Figure 4

A Time Constant

B Current Amplitude

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Current (agonist/UK14304)
Figure 5

A  CYLE (20 μM)  

B  ME (30 μM)  

CYLE (365 nm, 100 ms)

100 pA

3 min

Figure 5

A  CYLE (20 μM)  

B  ME (30 μM)  

CYLE (365 nm, 100 ms)

100 pA

3 min

Figure 5

A  CYLE (20 μM)  

B  ME (30 μM)  

CYLE (365 nm, 100 ms)

100 pA

3 min

Figure 5

A  CYLE (20 μM)  

B  ME (30 μM)  

CYLE (365 nm, 100 ms)

100 pA

3 min

Figure 5

A  CYLE (20 μM)  

B  ME (30 μM)  

CYLE (365 nm, 100 ms)

100 pA

3 min

Figure 5

A  CYLE (20 μM)  

B  ME (30 μM)  

CYLE (365 nm, 100 ms)

100 pA

3 min

Figure 5

A  CYLE (20 μM)  

B  ME (30 μM)  

CYLE (365 nm, 100 ms)

100 pA

3 min

Figure 5

A  CYLE (20 μM)  

B  ME (30 μM)  

CYLE (365 nm, 100 ms)

100 pA

3 min

Figure 5

A  CYLE (20 μM)  

B  ME (30 μM)  

CYLE (365 nm, 100 ms)

100 pA

3 min

Figure 5

A  CYLE (20 μM)  

B  ME (30 μM)  

CYLE (365 nm, 100 ms)

100 pA

3 min
Figure 6

A

CYLE (20 μM)

flash (5s, 365 nm)

3 min

100 pA

B

1st

2nd

200 pA

5s flash (365 nm)

C

tau rise (s)

1st

2nd

3rd

after-ME

flash #
Figure 7

A. Control

B. Control

C. Control + OFQ + Morphine

[Graph showing current traces and statistical analysis]

Control: 7

NS

*