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Constitutively Active μ-Opioid Receptors

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Abstract

The µ-opioid receptor is the G protein coupled receptor (GPCR) responsible for the analgesic, rewarding and unwanted effects of morphine and similar drugs. Constitutive activity of GPCRs is a phenomenon that likely reflects receptors spontaneous adopting conformations that can activate G proteins, and is likely to be common to most if not all GPCRs. Basal constitutive activity has been observed in some systems with µ-opioid receptors, and constitutive activity is expressed by mutant µ-opioid receptors with amino acid substitutions in regions known to be important for signalling. However, µ-opioid receptors are unique in that a putative constitutively active state of the receptor, the µ*-state, has been suggested to be induced by prolonged agonist treatment. The µ*-state is thought to contribute to processes underlying adaptation to and withdrawal from opioid treatment, and may have a ligand sensitivity distinct from basal constitutive activity of the µ-opioid receptor or that exhibited by µ-opioid receptor mutants. In this Chapter we outline methods for measuring constitutively active µ-opioid receptors, including some that take advantage of the fairly direct coupling of the receptor to ion channels. We also briefly summarize the pharmacology of the different constitutively active µ-opioid receptor states, and highlight the areas where we need to know more. We hope that a better understanding of constitutive activity at the µ-opioid receptor may provide information useful in developing ligands that access subsets of receptor conformations, offering the potential to fine tune opioid pharmacotherapy.
Introduction

The constitutive activity of G protein coupled receptors (GPCR) has been recognized for some time, and is not a surprising phenomenon given the stochastic principles that underlie signalling in biology. Antagonist-sensitive, agonist-independent GPCR activity was noted from the earliest studies of purified β-adrenergic receptors (Cerione et al., 1984), however, it was a comprehensive study of native δ-opioid receptors that showed G-protein activity that could be stimulated by agonists and inhibited by “inverse agonists”, with both effects being blocked by neutral antagonists (Costa and Herz, 1989). The constitutive activity of GPCR is an important idea in drug action and some diseases result from mutations in GPCR that enhance basal receptor activity (Smit et al., 2007). While there are varying degrees of constitutive activity measurable at different GPCR, the presumption that it arises as an inevitable consequence of continual movement of receptors through a series of different conformational states means that constitutive activity per se will be common to all receptors.

The µ-opioid receptor is the GPCR responsible for the analgesic and rewarding properties of morphine and similar drugs, as well as for their unwanted effects. Constitutive activity has been convincingly demonstrated for the µ-opioid receptor (e.g. Burford et al., 2000; Liu and Prather, 2001; Mahmoud et al., 2010), but a great deal of additional experimental interest has been generated by the proposition that chronic treatment with µ-opioid agonists induces a constitutively active form of the receptor known as the µ*-state (Wang et al., 1994; Sadee et al., 2005). This privileged state of the µ-opioid receptor is reported to be induced by agonist treatment in vivo or in vitro and is thought to reflect persistent phosphorylation of the receptor. The consequences of prolonged use of morphine for pain relief or other purposes are a major medical and societal challenge, and a significant research effort continues to be devoted to understanding the mechanisms underlying tolerance to the analgesic affects of
morphine and the development of dependence on opioids. The suggestion that persistent constitutive activity of the μ-receptor is responsible for some or all of the adaptations that lead to tolerance to and dependence on morphine means that it is critically important to be able to measure μ-opioid receptor activity as directly as possible, and in as wide a variety of native tissues as possible. In this chapter we will describe biochemical and electrophysiological techniques for detecting and quantifying the constitutive activity of μ-opioid receptors in vitro, and endeavour to outline the strengths and potential shortcomings of each assay.

Experimental Questions

The hypothesis that the constitutively active μ*-state of the μ-opioid receptor plays a pivotal role in driving cellular adaptation to prolonged opioid agonist treatment raises a number of important experimental questions specific to the μ-opioid receptor. These include whether the basal constitutive activity of μ-opioid receptors is operationally equivalent to that of the μ*-state and what functional consequences constitutive μ-opioid receptor activity has for neuronal excitability and synaptic transmission. It remains unresolved if constitutively active μ-receptors and μ*-state receptors activate the same suite of G-proteins, whether their basal activity is sensitive to the same inverse agonists or even whether the constitutive activity observed with agonist naïve μ-opioid receptors simply reflects a low level of the phosphorylation event suggested to produce the μ*-state. It has also been extremely difficult to demonstrate μ-opioid receptor constitutive activity or any antagonist effects consistent with the reversal of constitutive μ-opioid receptor activity in isolated neurons or neurons in brain slices. It remains an open question whether techniques currently used in experiments on relatively intact neurons lack sufficient sensitivity to detect constitutively active μ-opioid receptors (μ* or otherwise) or whether there is simply nothing to see.
Methods for Measuring Constitutive Activity

General Considerations

The activity of GPCR can be quantified quite directly by measuring agonist-stimulated GTPase activity or the binding of stable GTP analogs to G proteins. One of the earliest events after receptor activation is the exchange of GDP on the $G_{\alpha}$ subunit for GTP (Figure 1). Consequently, the $[^{35}S]GTP_{\gamma}S$ assay, which measures binding of the radiolabelled, slowly hydrolysable, GTP analog $[^{35}S]GTP_{\gamma}S$ to activated $G_{\alpha}$ proteins has been widely employed.

The subsequent hydrolysis of GTP bound to the activated $G_{\alpha}$ proteins can also be measured, as can modulation of adenylyl cyclase (AC) downstream of GTP-bound $G_{\alpha}$ protein or ion channels downstream of released $G_{\beta\gamma}$ subunits. It is, of course, important to define the receptor that is responsible for the activity, where possible to distinguish constitutively active receptors from constitutively active G proteins, and to ensure that the measured signal does not come from the activity of endogenous agonists remaining in the preparation. This latter consideration is less problematic for $\mu$-opioid receptors, as the endogenous peptide agonists are likely to be scarce in the reduced preparations used for these studies, although lipophilic synthetic agonists used for pretreatment may be harder to remove.

The difficulty in measuring constitutive activity is of course that by its nature it is unstimulated. While it is possible to carefully measure basal GTPase activity while changing levels of receptor or G protein (Cerione et al., 1984), this approach is not practical for addressing most experimental questions. The voltage-dependent inhibition of calcium channels ($I_{Ca}$) by G protein $\beta\gamma$ subunits provides an instantaneous and repeatable measurement of constitutive activity in single cells simply by varying membrane voltage (see below), but it
is only good for isolated neurons. In general, constitutive activity is defined by the effects of ligands that stabilize poorly coupled state(s) of the µ-receptor. These compounds are called “inverse agonists” or described as ligands with “negative intrinsic efficacy” (Kenakin 2004). A list of readily available µ-opioid receptor ligands with reported negative intrinsic activity is found in Table 1. The degree to which these ligands inhibit the basal level of the output being measured is considered to reflect the amount of constitutive activity present in the system, although it is difficult to prove that any ligand completely inhibits constitutive activity in the absence of a non-pharmacological method of defining this activity. Evidence that ligands with negative intrinsic activity are acting to change signalling via their cognate receptor is provided by reversal of the inhibition of signalling by a neutral antagonist (Costa and Herz, 1989). Putative neutral opioid antagonists are also listed in Table 1. An advantage of using ligands to define constitutive activity is that the receptor specificity of the drugs is usually well defined. However, different ligands have been reported to have different amounts of negative efficacy, which presumably reflects differences in their propensity to stabilize the G-protein uncoupled states of the µ-receptor. Some laboratories report virtually all antagonists of µ-opioid receptor function have negative intrinsic activity (Sally et al., 2010), with essentially no compounds being completely devoid of positive or negative efficacy. Although somewhat at odds with the observed lack of effect of µ-opioid receptor antagonists in most assays, the notion that binding to a receptor stabilizes a subset of conformations suggests that it would be difficult for a high affinity ligand not to affect the distribution of receptor-coupled conformations one way or the other (Kenakin and Onaran, 2002).

\textit{G protein measures: the [\textsuperscript{35}S]GTP\_γS/Eu-GTP assays}

Two methods are employed for measuring agonist activity at the level of the G protein. They respectively take advantage of the binding of GTP to the G\_α subunit following agonist-
induced dissociation of GDP and the subsequent hydrolysis of GTP bound to the Gα subunit to terminate the signal (Figure 1). Both responses show an increase following agonist occupation of a GPCR and therefore should show a response to a receptor that constitutively activates Gα in the absence of agonist. In practice, the measure of GTP binding to the Gα subunit using labelled GTP analogues is the easiest and has been much more frequently employed in the study of constitutive activity.

The most common way to measure constitutive activity of µ-opioid receptors is to assess the non-agonist-stimulated binding of labelled GTP analogues to membranes from cells or tissues expressing the receptor. The analogues used are either 35S labelled GTP ([35S]GTPγS) or europium labelled GTP (Eu-GTP), although the commercial availability of the latter ligand is now uncertain. Assays for the two ligands are very similar except that the amount of [35S]GTPγS bound is determined by scintillation counting and the binding of Eu-GTP by time-resolved fluorescence. In assays of ligand-stimulated receptor activity µ-opioid agonists increase the binding of the GTP analogs to appropriate Gα proteins. However, in the absence of agonist and even in the presence of a high concentration of unlabelled GTPγS to define non-specific binding, there is some degree of “basal” binding of the labelled nucleotide. This basal binding of comprises binding of [35S]GTPγS (or Eu-GTP) to unoccupied or constitutively active heterotrimeric G proteins, binding to small G proteins and binding caused by receptors activating Gα proteins in the absence of agonist, i.e. binding reflecting constitutive activity.

To measure labelled GTP binding a low concentration of the labelled nucleotide (usually 0.1 nM for [35S]GTPγS or 1 nM for Eu-GTP, which has lower affinity for Gα subunits) is incubated in membranes from cells or tissues expressing µ-opioid receptors for sufficient time
to reach steady state (usually 60-120 mins). Specific binding of the labelled nucleotide is
defined in the presence of a saturating concentration of unlabelled GTPγS. GTP-conjugate
labelled Gα subunits remain bound to the cell membranes and the assay is terminated by
filtration or immunoprecipitation, with signal from the retained proteins measured by
traditional scintillation counting (Traynor and Nahorski, 1995) or Scintillation Proximity
Assay (DeLapp, 2004) for [35S]GTPγS or time-resolved fluorescence for Eu-GTP (Labrecque
et al., 2009)

To observe receptor driven increases in binding it is necessary to include GDP, Na and Mg in
the incubation medium. GDP fills empty guanine nucleotide binding sites on the Gα protein
which reduces the basal level of GTP conjugate binding and supplies GDP-bound Gα
substrate for agonist-driven activation. The concentration of GDP needed to give an optimal
signal-to-noise ratio must be determined experimentally for each system; it is usually between
3 and 30 µM for cells expressing cloned receptors, but may be higher for brain tissue.
However, increasing the concentration of GDP increases the efficacy requirements of the
system, conversely lowering GDP will raise basal binding and has been used to identify
inverse agonists in other systems (Roberts and Strange 2005). Mg is necessary to promote
agonist-stimulated binding of [35S]GTPγS, and the concentration can be critical for the
identification of inverse agonists (Wang et al. 2001). Higher concentrations of Mg can inhibit
binding, so again, the concentration should be optimised for each system. Assays are usually
performed in the presence of 100 mM Na because Na selectively promotes agonist-stimulated
binding over basal binding, improving the agonist signal. However, such a high
concentration of Na tends to uncouple the receptor from G proteins and so reduces
spontaneous coupling. Thus, the chances of observing constitutive activity can be improved
by replacement of Na with K (Szekeres and Traynor, 1997; Liu et al. 2001) or with N-methyl-
D-glucamine (Lin et al. 2006) to increase receptor-effector coupling and promote spontaneous activation of $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding. This increased constitutive signal obviously allows for the easier determination of inverse agonist efficacy. Finally, endogenous adenosine can mask the signal arising from other GPCR, particularly in brain tissue, so membranes should be incubated with adenosine deaminase prior to experiments (Breivogel et al. 2004) or an adenosine receptor antagonist can be included in the assay (Horswill et al. 2007). A detailed review of the assay protocol can be found in (Harrison and Traynor, 2003), and specific experimental details in (Kara and Strange, 2010).

Several considerations need to be born in mind when using GTP analog binding assay to identify constitutive activity at $\mu$-opioid receptors. Firstly, the assay measures an event very close to the receptor itself and therefore the response is not amplified as seen with downstream assays. Consequently it can be difficult to observe a small constitutively active signal unless receptors are over expressed or conditions such as Na concentration are altered. Further, because of the impermeable nature of the guanine nucleotides the assay is performed in isolated membranes, and this raises the possibility of disrupting the receptor-G protein coupling or scaffolding mechanisms potentially necessary to maintain constitutive activity. Finally, it is necessary to show that the constitutive effects derive from the receptor taking up active conformations and not some other process, such as changes in the spontaneous activity of the G-proteins themselves. For $\text{G}\alpha\text{i/o}$ coupled receptors such as the $\mu$-opioid receptor this can be achieved with pertussis toxin (PTX) pretreatment (usually 100 ng ml$^{-1}$ overnight). PTX ADP ribosylates a Cys in the C-terminus of the $\text{G}\alpha$ protein and prevents its coupling to receptor, thus inhibiting both agonist and constitutive activation of $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding. Importantly, PTX does not alter other properties of the G protein, such as ability to bind guanine nucleotides. However, use of PTX does not identify which receptor in a system in
responsible for the observed constitutive activity. For example, PTX reduces basal 
\[^{35}\text{S}]\text{GTP}\gamma\text{S}\) binding in SH-SY5Y cells (Traynor and Nahorski, 1995), although there is no specific evidence for \(\mu\)-opioid constitutive activity in these cells. The observed effect could be due spontaneous activity of one of any number of \(G\alpha\text{i/o}\)-coupled GPCRs expressed in these cells, including 5HT1A, ORL1 and CB1 receptors. Nonetheless, PTX is an important tool to confirm the role of receptor G protein coupling, especially in heterologous systems over-expressing one receptor.

**Native \(\mu\)-opioid receptors**

Unlike many other GPCRs that couple to \(G\alpha\text{i/o}\) proteins such as the \(\delta\)-opioid receptor (Costa and Herz, 1989) and the CB1 receptor (Howlett, 1994), it has proved difficult to show basal, agonist-independent, activation of \[^{35}\text{S}]\text{GTP}\gamma\text{S}\) binding G protein by \(\mu\)-opioid receptors in naïve native tissues (Wang et al., 2004; Raehal et al., 2005) or often even in heterologous expression systems (Neilan et al., 1999). This suggests that under normal physiological conditions there is insufficient receptor in active conformations to give a stimulation of \[^{35}\text{S}]\text{GTP}\gamma\text{S}\) binding that can be inhibited by ligands with negative efficacy. However, it has been demonstrated that the non-selective opioid antagonists 7-benzylidenenaltrexone (BNTX) and \(\beta\)-chloraltrexamine (\(\beta\)-CNA) cause a small reduction in basal \[^{35}\text{S}]\text{GTP}\gamma\text{S}\) binding in homogenates of whole brain from wild-type mice but not their \(\mu\)-opioid receptor knockout counterparts, suggesting constitutive activity of the native receptor Figure 2A (Wang et al., 2004). Moreover, changes in the levels of basal, non-agonist stimulated \[^{35}\text{S}]\text{GTP}\gamma\text{S}\) binding have been reported in several cell lines in which the \(\mu\)-opioid receptor has been over expressed. For example, an approximately 25 % increase in the basal \[^{35}\text{S}]\text{GTP}\gamma\text{S}\) signal is seen in HEK cells expressing a \(\mu\)-opioid receptor (HEK\(\mu\)) (Burford et al., 2000) and a 67 % increase was reported in \(\mu\)-opioid receptor expressing GH3 cells (GH3\(\mu\), (Liu et al., 2001). By
contrast, no change was reported in µ-receptor expressing C6 glioma cells (C6µ; (Neilan et al., 1999; Divin et al., 2009). In general, the higher the µ-opioid receptor expression the higher the level of constitutive coupling to Gα proteins that should be observed due to increased chances of fruitful collisions occurring. However, GH₃µ cells show higher than basal [³⁵S]GTPγS binding at receptor expression levels similar to those found endogenously (0.4pmol/mg protein) and agonist-independent increases in [³⁵S]GTPγS binding have been reported in HEKµ cells expressing 1 pmol mg⁻¹ receptor (Wang et al., 2001). As mentioned above, the constitutive increase in basal [³⁵S]GTPγS binding is increased by removal of Na ions (Liu et al., 2001) although in C6µ cells this still did not generate any constitutive activity that could be attributed to the µ-opioid receptor. The alkylating agent β-CNA inhibited the constitutive activation of [³⁵S]GTPγS binding in HEKµ cells, possibly by locking the receptor in an inactive conformation (Burford et al., 2000), although this was not seen at the µ-opioid receptor in naïve GH₃ cells (Liu and Prather, 2001).

Insert Figure 2 about here

GTPγS binding after chronic agonist treatment

Chronic opioid agonist treatment produces the putative constitutively activated µ*-state of the µ-opioid receptor both in heterologous systems and following in vivo administration of opioid ligands (Connor, 2009). Studies in SH-SY5Y cells suggest this process involves receptor phosphorylation (Wang et al., 1994). Thus, chronic treatment with agonists provides a strategy to increase the level of µ-opioid receptor constitutive activity from the rather low levels seen in naïve systems, or even to induce activity in silent systems. This makes it easier to identify compounds with inverse agonist activity. Many of these experiments have been
performed by assaying the activity of adenylyl cyclase (see below), but there are several examples that have used the $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding assay.

Using brain membranes from mice, several studies found that 3 day treatment with morphine converts naltrexone and naloxone from ligands with a small positive efficacy to ones with modest negative efficacy, and this occurred in a region specific manner (Wang et al., 2004; Raehal et al. 2005). The results suggest a induction of $\mu^*$-state receptors, however the measured responses were small, with a less than 10 % decrease in basal GTP$\gamma$S binding produced by the inverse agonists. It should be noted that other studies have failed to see any increases in basal GTP$\gamma$S activity after chronic morphine treatment of animals (e.g. Selley et al., 1997). By contrast, basal binding of $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ in GH3$\mu$ cells, CHO and HEK 293 cells expressing $\mu$-opiod receptors is increased considerably following chronic opioid treatment (Liu et al., 2001; Wang et al., 2007; Xu et al., 2007) and there is a concomitant increase in the apparent negative efficacy of most tested antagonists (Figure 2B). Interestingly, chronic treatment with the peptide agonist [D-Ala$^2$,N-MePhe$^4$,Gly-ol]-enkephalin (DAMGO) did not produce a constitutively active $\mu$-opioid receptor in C6 glioma cells as defined by increases in $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding (Divin et al., 2009), again highlighting the system dependence of $\mu$ opioid receptor constitutive activity. The induction of constitutive activity is ligand dependent in some studies but not others, for example Liu and Prather (2001) found that both DAMGO and morphine treatment induced constitutive activity in GH3$\mu$ cells (although only DAMGO was reported to change basal $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding), while Xu et al., (2007) found that heroinin but not DAMGO pretreatment changed basal $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding in CHO cells.
Of course, prolonged treatment with opioids can cause a number of changes in the cell including changes in receptor, Gα protein and accessory protein expression, and these should be born in mind when interpreting results (e.g. Liu and Prather, 2001; Xu et al., 2007). Also, it is vital to demonstrate that the opioid used for chronic treatment is washed out of the preparation since residual drug will stimulate [35S]GTPγS, thus giving a false impression that constitutive activity is present.

**Constitutively active mutants**

Experimental mutagenesis of the μ-opioid receptor has helped define regions that are important for stabilization of inactive receptor conformations as well as those that contribute to the active conformations responsible for coupling to G proteins. Some mutations appear to produce constitutively active receptors and as consequence it has been possible to use such mutations in the search for ligands that stabilize inactive conformations.

*The DRY (Asp-Lys-Tyr) motif:* This amino-acid sequence at the interface of TM3 and the second intracellular loop is highly conserved across GPCRs and is thought to be important for stabilization of inactive states of the receptor as well as to be playing a role in receptor activation (Rosenbaum et al., 2009). Mutation of amino acids in this region leads to constitutive activity in many GPCRs. In the μ-opioid receptor the DRY motif is amino acids 164-166. Replacement of Asp\(^{164}\) with His produces a μ-opioid receptor that readily promotes a high degree of [35S]GTPγS binding in the absence of agonist in both transiently transfected HEK293 cells and stably transfected CHO cells (Li et al., 2001b). Indeed, addition of the efficacious agonist DAMGO to the mutant receptor produces little further increase in [35S]GTPγS binding. Qualitatively similar activity is obtained with when Asp\(^{164}\) is replaced by Tyr, Glu, or Met (Li et al., 2001b). The constitutive activity of these mutants is PTX-
sensitive and is seen at receptor levels close to physiological expression levels. Similar to the results in some studies of prolonged agonist treatment of wild-type µ-opioid receptors, naltrexone and naloxone acted as inverse agonists at the constitutively active mutants, but CTAP (D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH$_2$) and diprenorphine were neutral (Li et al., 2001b). It is very important to note that expression of the constitutively active µ-opioid receptors was only detectable following prolonged incubation with high levels of naloxone (20 µM for 4 days). Naloxone stabilized the µ-opioid receptor protein and at the same time prevented constitutive internalization of the mutant receptor (Li et al., 2001a).

 Junction of intracellular loop 3 and transmembrane domain 6: Another site implicated in the stabilization of inactive conformations of GPCRs is a conserved XBBXXB sequence (where B is a basic amino-acid and X is non-basic) at the junction of intracellular loop 3 and transmembrane domain 6. In the µ-opioid receptor this sequence is Leu$^{275}$-Arg-Arg-Iso-Thr-Arg$^{280}$. The exchange of Thr$^{279}$ for Lys in this sequence produces a µ-opioid receptor that shows increase basal activation of [$^{35}$S]GTP$_{\gamma}$S binding when stably expressed in CHO cells, although in this case there is also an additional DAMGO-induced increase (Huang et al., 2001). This constitutively active µ-opioid receptor mutant behaves very similarly to the Asp$^{164}$ mutants in that prolonged incubation with naloxone is needed for detectable protein expression and the constitutive activation of G protein is PTX sensitive.

 C-terminal tail mutations: Replacement of both Cys$^{348}$ and Cys$^{353}$ in the C-terminal tail of the µ-opioid receptor also produces a receptor that shows increased basal activation of [$^{35}$S]GTP$_{\gamma}$S binding, 1.5-fold higher than wild-type receptors (Brillet et al., 2003). DAMGO further increased [$^{35}$S]GTP$_{\gamma}$S binding, in a PTX sensitive manner. However, unlike the mutations at the junctions between transmembrane domains and intracellular loops,
spontaneous activity of the C-terminal mutant was not completely reversed by PTX, suggesting coupling to non-Gαi/o receptors or other means of promoting [35S]GTPγS binding. The C-terminal mutant receptor is stable and expressed at high levels even without prolonged naloxone treatment, presumably because the C-terminus is not critical for maintaining a stability of the transmembrane domain helices. In the Cys348Cys353 double mutant naltrexone and naloxone were neutral agonists, but cyprodime and the CTAP analogue CTOP (d-Phe-Cys-Tyr-d-Trp-Orn-Thr-Pen-Thr-NH₂) showed inverse agonist activity (Brillet et al., 2003).

The advantage of working with chronically µ-opioid treated systems or constitutively active mutant receptors is that they can express a high degree of constitutive activity with a large signal-to-noise ratio. On the other hand, the natural constitutively activated state(s) of the wild-type receptor may not be accurately or fully represented in these systems and so they may not identify compounds that might be inverse agonists at wild-type receptors. This problem is highlighted by the different inverse agonists identified using the different systems (Table 1). Although the molecular basis for these differences is unknown, a likely explanation for the distinct ligand efficacy profiles is that different receptor conformations are responsible for constitutive activity in the different systems, for example the constitutively active mutants do not mimic the chronic morphine-dependent µ*-state, and the DAMGO-treated system may be different from the herkinorin-treated system. Moreover, photoaffinity labelling studies have suggested that there may be differences in the profile of agonist-stimulated versus constitutively-stimulated Gα subunits (Liu et al., 2001), implying that the agonist stimulated and constitutively active states may not be equivalent. This idea is reinforced by the mutant µ-opioid receptors, where constitutive activity is obtained with very different mutations - some that involve amino acids that play a role in movement of the transmembrane helices and others that causes changes in the C terminal tail downstream of helix movement.
Adenylyl cyclase assays

Adenylyl cyclase enzymes are a family of nine members (Sadana and Dessaur, 2009) that convert ATP to the second messenger cAMP. AC activity is stimulated by GTP-bound G\(\alpha_s\) and inhibited by GTP-bound G\(\alpha_i/o\) proteins. The AC assay is useful for studying constitutive activity and inverse agonism acutely, but since alterations are seen in the activity of the enzyme following chronic \(\mu\)-opioid exposure changes in AC have been widely used in the characterization of the \(\mu^*\)-opioid receptor state. Resting levels of AC in a cell are generally low so to show effects of \(\mu\)-opioids the direct AC activator forskolin is often included at a concentration between 10 \(\mu\)M and 30 \(\mu\)M (an approximate EC\(_{50}\) concentration) to stimulate AC activity. Alternatively, native G\(\alpha_s\)-coupled receptors (e.g. PGE\(_2\) or \(\beta\)-adrenergic receptors) can be activated by a suitable agonist to increase cAMP levels. Assays are run on adherent cells in culture medium (in the absence of serum) for between 5 and 15 mins in the presence of a phosphodiesterase inhibitor such as 3-isobutyl-1-methylxanthine (IBMX, 1 mM). Accumulation of cAMP is measured using a variety of methods including ligand binding, ELIZA, or time-resolved fluorescence resonance energy transfer. An example of a detailed assay can be found in (Clark and Traynor, 2004). Additionally, there are newer methods of analysis of cAMP, such as cAMP-mediated activation of cyclic nucleotide gated ion channels (Reinscheid et al., 2003) and monitoring of cAMP signaling in neurons using genetically-encoded FRET probes that allow for real-time monitoring (Vincent et al., 2008).

An important advantage of cAMP measurements is that unlike G protein assays they can be made in intact cells, thereby avoiding alterations in membrane architecture and protein-protein interactions that might compromise constitutive activity. Moreover, activity at AC is downstream of G protein activation and thus benefits from amplification of the response.
In a system that expresses constitutively active Gαi/o-coupled receptors AC activity should be reduced. Although measurements of cAMP levels would appear to be an ideal assay for the study of constitutive activity it has seldom been employed to study basal constitutive activity or the constitutive activity of μ-opioid receptor mutants. In cells where the \[^{35}\text{S}]\text{GTP}_\gamma\text{S} \) assay showed constitutive μ-opioid receptor signaling the basal (unstimulated by forskolin) level of cAMP was about half that seen with wild-type cells (Liu and Prather, 2001). The reduction in cAMP was inhibited by alkylation of the receptor with β-flunaltrexamine, this also reduced constitutive stimulation of \[^{35}\text{S}]\text{GTP}_\gamma\text{S} \) binding (Liu and Prather, 2001). In striatal homogenates of the mouse basal cAMP levels were increased by β-CNA and BNTX (Wang et al. 2004). Similarly, in HEKμ cells forskolin-stimulated cAMP accumulation was increased by the irreversible antagonist β-CNA, the non-equilibrium antagonist clocinnamox, as well as BNTX, ligands that all showed negative efficacy in the \[^{35}\text{S}]\text{GTP}_\gamma\text{S} \) assay in the same cells (Wang et al., 2001).

More use has been made of assays for AC activity to define changes occurring as a result of chronic opioid exposure. Prolonged agonist treatment of μ-opioid receptor expressing cells produces cellular homeostatic changes that result in a sensitization of AC. Following challenge with an antagonist there is a rebound increase in cAMP levels (cAMP overshoot) that is absent in naïve cells (Watts and Neve 2005). This cAMP overshoot response is also seen in brain tissue from animals following chronic opioid treatment (Williams et al., 2001) and has been linked to specific effectors mediating the opioid withdrawal syndrome (Bagley et al., 2005b). Measures of the cAMP overshoot response in chronic morphine-dependent systems have been used to describe the μ*-state and to differentiate neutral antagonists from inverse agonists. Indeed, the first description of the μ*-state was provided by Wang and colleagues (Wang et al., 1994) using this methodology. To obtain a cAMP overshoot
response cells are treated chronically with µ-opioid agonist (usually 10 µM for 24-48h), then the agonist is removed and/or the antagonist added together with an agent to stimulate AC activity (either a Gαs receptor coupled agonist or forskolin). In SH-SY5Y cells washout of chronic morphine resulted in an increase in cAMP levels compared to naive cells, but addition of naloxone resulted in a greater overshoot than simply washing off the morphine. The difference was attributed to the ability of naloxone to reverse the µ*-state form of the receptor back to the basal µ-state (Wang et al., 1994). A similar degree of overshoot was seen with naltrexone and diprenorphine. In contrast the peptidic antagonists CTAP and CTOP did not increase overshoot to any greater degree than simply removing the morphine, and they blocked the effect of naloxone. Thus, as defined by this experimental protocol, naloxone, naltrexone and diprenorphine are inverse agonists that drive the µ*-state back to the resting receptor state whereas CTAP and CTOP are neutral antagonists. Similar findings have been shown in GH3 immortalized rat pituitary cells (Liu and Prather, 2001), and in HEKµ cells (Wang et al., 2001). However, this effect of chronic agonists to produce a µ*-state of the receptor that is differentially sensitive to antagonists does not appear to be universal. In C6µ cells chronically exposed to morphine, all antagonists tested produced a degree of cAMP overshoot corresponding to that expected due to displacement of agonist from the receptor (Divin et al., 2009) while in herkinorin or DAMGO-treated CHOµ cells CTAP and naloxone produced an equivalent degree of cAMP overshoot (Xu et al., 2007).

While these experimental protocols are straightforward and appear in certain systems to provide a constitutive form of the µ-receptor, care has to be taken with the interpretation of results. In particular, complete washout of agonist has to be confirmed to ensure that findings are not the result of antagonism of residual agonist. In addition, if neutral antagonists and inverse agonists are identified then they should compete. Further, cAMP overshoot is seen
with prolonged activation of any Gαi/o coupled receptor (Watts and Neve, 2005), and can be induced after only minutes of treatment (Levitt et al., 2009) so there is no guarantee that a rebound increase in cAMP levels actually represents a specific change in opioid receptor activity-dissociating these changes from those reflecting changes in the enzyme itself or other modulators of GPCR signaling is a challenge. Finally, as with the [35S]GTPγS studies, whilst these experiments can inform about changes induced by chronic treatment of cells/tissues expressing µ-opioid receptors, they may not provide information on constitutively active receptors formed under other conditions.

Measuring constitutive activity using ion channels

The actions of Gα or Gβγ subunits on ion channels provide another readily measurable cellular effect that is one step removed from µ-opioid receptor activation of G-protein heterotrimers. For Gi/Go-coupled receptors, including µ-opioid receptors, the most easily measured of these interactions are the inhibition of voltage dependent calcium channels (I_{Ca}) and activation of G protein gated inwardly rectifying potassium channels (GIRK). Both processes involve the direct binding of Gi/Go-derived βγ subunits to the channel proteins, and both processes have been used as sensitive probes for changes in µ-opioid receptor activity. The excess of Gi/Go-proteins over µ-opioid receptors (Sternweis and Robishaw, 1984; Selley et al., 1998) and the relatively low abundance of ion channels in neurons means that the amplification of receptor coupling to I_{Ca} and GIRK is significant, as demonstrated by the large receptor reserve for these processes (Christie et al., 1987; Connor et al., 1999).

Calcium channels

Although there are several pathways by which Gi/Go coupled receptors can inhibit I_{Ca}, most attention has focussed on the rapid, reversible and voltage dependent inhibition of N-type
(Ca_{2.2}) and P/Q-type (Ca_{2.1}) \( I_{Ca} \) by G protein \( \beta\gamma \) subunits (Ikeda, 1996; Herlitze et al., 1996). This pathway is defined by a characteristic voltage-dependence of the interaction between the G\( \beta\gamma \) subunits and the channels—when the cell membrane is strongly depolarised, the G\( \beta\gamma \) inhibition of \( I_{Ca} \) is transiently removed. This means that an index of channel inhibition can be obtained by comparing current amplitude before and after a strong depolarisation (see Figure 3). The ratio (S2:S1) of the current amplitude evoked by the control step (S1) and the step after the conditioning depolarization (S2) is often used a measure of the amount of voltage-dependent inhibition, when inhibition is present the \( I_{Ca} \) amplitude after the depolarisation is larger, and the ratio correspondingly greater. Importantly, the double pulse protocol (Figure 3) can detect constitutive activation of the voltage-dependent inhibitory pathway, in this case the basal ratio is greater than 1. Another feature of G\( \beta\gamma \) inhibition of N- and P/Q-type \( I_{Ca} \) is an apparent slowing of channel activation when \( \beta\gamma \) subunits are bound (Ikeda, 1996; Herlitze et al., 1996). Although the mechanisms underlying this slowing of whole cell P/Q- and N-type channels are distinct (Colecraft et al., 2001), strong depolarisation reverses the slowing, providing another measure of relief from G\( \beta\gamma \) inhibition. The facilitation of N-type currents by a depolarising conditioning step is usually more profound than that of P/Q-type channels, reflecting the larger inhibition of these channels by the G\( \beta\gamma \) pathway.

*Insert Figure 3 about here*

The constitutive activity of several receptors has been studied using facilitation of \( I_{Ca} \) as the key assay (Pan et al., 1998; Beedle et al., 2004; Guo and Ikeda, 2004; Mahmoud et al., 2010). In these experiments, which have largely been performed in sympathetic neurons, microinjection of appropriate receptor mRNA increases the basal facilitation ratio and this
increase is reversed by superfusion of appropriate antagonists, which also increase the absolute amplitude of the $I_{Ca}$. The constitutive activity of the opioid-related ORL1 receptor (NOP) has been explored in some detail in both tsA-201 HEK-293 cells and sympathetic neurons (Beedle et al., 2004, Mahmoud et al., 2010), with the latter study also including a few experiments on neurons injected with µ-opioid receptor mRNA (Mahmoud et al., 2010; see Figure 8). In these experiments, the µ-opioid receptor ligand CTAP increased the amplitude of the N-type $I_{Ca}$ and reduced the facilitation ratio, while DAMGO produced a further inhibition of the $I_{Ca}$. This shows that high levels of receptor expression can induce constitutive activity detectable at a single cell level, without use of ligands.

The detection of the constitutive activity of native receptors using $I_{Ca}$ assays is much more unusual (Pan et al., 1998). In general, little or no facilitation of baseline $I_{Ca}$ by a positive conditioning step is observed in native cells (e.g. Connor et al., 1999; Borgland et al, 2001; Bagley et al., 2005a), although a careful study reported modest facilitation in a subpopulation of small dorsal root ganglion neurons (Beedle et al., 2004). In a small population of rat pelvic ganglion neurons expressing native cannabinoid CB1 receptors, the CB1 ligand SR 141716A was shown to inhibit tonic activation of the G$\beta$γ pathway, probably by inhibiting constitutively active CB1 receptors (Pan et al, 1998). The only example of constitutive inhibition of $I_{Ca}$ by native µ-opioid receptors was demonstrated in cultured dorsal root ganglion neurons from arrestin3 knockout mice (Walwyn et al., 2007). In these neurons the basal facilitation ratio of $I_{Ca}$ was around 1, but this ratio was reduced to about 0.8 by naltrexone. The effects of naltrexone were blocked by CTAP, indicating that they were mediated via the µ receptor. In neurons from wild type mice, naltrexone had no effect on basal $I_{Ca}$. This study demonstrates the utility of the electrophysiological approach, even in cells with native levels of receptor expression.
The putative constitutively active $\mu^*$-state of the $\mu$-opioid receptor should also produce a tonic inhibition of $I_{Ca}$ in appropriate neurons, assuming that it activates a similar subset of $G$ proteins as agonist-activated receptors. However, no differences in facilitation ratio were found between locus coeruleus neurons from chronically morphine treated (CMT) and vehicle rats (Connor et al., 1999), or in periaqueductal gray neurons from CMT and vehicle mice (Bagley et al., 2005a). Both cell types exhibit an increased facilitation ratio and $I_{Ca}$ activation when opioid agonists are superfused, indicating that the molecular machinery appropriate for expression of constitutive activity is present. Similarly, superfusion of naloxone and simply washing off morphine produced an equivalent increase in $I_{Ca}$ in chronically morphine treated SH-SY5Y cells, indicating that $\mu^*$-state receptors could not be detected using electrophysiology in this preparation (Kennedy and Henderson, 1992).

The $G\beta\gamma$ subunit modulation of $I_{Ca}$ is ubiquitous and easy to measure using standard whole cell patch clamp techniques. There are, however, a few possible confounds that need to be kept in mind. Firstly, many studies have shown that depolarising steps can facilitate $I_{Ca}$ independently of the $G\beta\gamma$ pathway, although the $I_{Ca}$ affected is usually L- ($Ca_{v}1.x$) or P/Q-type rather than N-type (Dolphin 1996). The facilitation can be associated with channel phosphorylation (Dolphin 1996) or be mediated by a direct effect of Ca (Chaudhuri et al., 2007). Ca-dependent facilitation can be minimized without interfering with $G\beta\gamma$-mediated channel inhibition by using Ba as the charge carrying cation (Chaudhuri et al., 2007). Conversely, it can be difficult to obtain a basal facilitation ratio of around 1, owing to the propensity of $I_{Ca}$ to desensitise. Ca-dependent desensitization can again be reduced by using Ba as a charge carrier while voltage-dependent processes can be attenuated by limiting the duration and amplitude of the test steps and the conditioning depolarisation, and by allowing
sufficient time between pulse sets to minimize the accumulation of channel desensitization. We and others have found that a conditioning depolarisation to +80 mV for 50-80 ms is sufficient to produce a robust facilitation of µ-receptor inhibited \( I_{ca} \) in a variety of central and peripheral neurons, without any significant channel desensitization (Connor *et al.*, 1999; Bagley *et al.*, 2005a; Walwyn *et al.*, 2007; Mahmoud *et al.*, 2010). Of course, it is also possible that a G\( \beta \gamma \)-mediated inhibition of \( I_{ca} \) results from constitutive activity of G-proteins in a cell, quite independent of any altered G-protein/receptor coupling. Given that Go-type G proteins are much more abundant than either µ-opioid receptors or \( I_{ca} \), small changes in basal G-protein activity could produce significant effects on \( I_{ca} \), although in this situation the facilitation would not be sensitive to receptor antagonists.

Another possible pitfall is a change in the mix of \( I_{ca} \) produced by a treatment. For example, in trigeminal ganglion neurons from morphine-treated mice there is a significant reduction in the amount of P/Q-type \( I_{ca} \) (Johnson *et al.*, 2006). If there were constitutively active receptors present in these cells, the basal facilitation ratio may actually increase, because the contribution of N-type \( I_{ca} \) to the total current is greater, and N-type \( I_{ca} \) are inhibited more effectively by the G\( \beta \gamma \) pathway (Bourinet *et al.*, 1996; Connor and Christie, 1998).

*Potassium channels*

GIRK (Kir 3.x) channels are directly gated by G\( \beta \gamma \) subunits, and they also provide an easily measurable output very closely tied to G protein activation. Unlike measuring \( I_{ca} \), which is episodic and rarely sustainable for more than about 30 minutes, continuous recordings of current or membrane potential can be used to monitor GIRK activity for periods of hours. GIRK currents are also most readily measured in brain slices, preparation of which generally requires less tissue handling or exposure to unphysiological solutions than dissociation (and
culturin) of neurons for recording $I_{Ca}$. There are a few reports of constitutively active GIRK in mammalian cells (Chen and Johnston, 2005; Dobrev et al., 2005), but we are not aware that constitutive activity associated with a specific GPCR has been detected in a mammalian cell using measurements of GIRK function. Several studies have examined coupling of GPCR to GIRK in sympathetic neurons and HEK 293 cells transfected with both receptors and channels, and constitutive activation of heterologously expressed GIRK was small and infrequently observed (Ruiz-Velasco and Ikeda, 1998; Guo and Ikeda, 2004; Johnson et al., 2006).

There are several reasons why constitutively active GIRK may be rarely reported, and activation of GIRK mediated by constitutively active receptors not at all. Firstly, maximal $G_{\beta\gamma}$ activation of the GIRK channels requires binding of multiple $G_{\beta\gamma}$ subunits (Sadja et al., 2002), which is in contrast to the single $\beta\gamma$ subunit required for inhibition of $I_{Ca}$ (Dascal, 2001). Thus, more constitutively active receptors may be required to produce a detectable $G_{\beta\gamma}$ signal at GIRK than at $I_{Ca}$. Secondly, there is no obvious ligand-free strategy for detecting basally active GIRK currents; there are no specific GIRK channel blockers and $G_{\beta\gamma}$ modulation of the currents is not intrinsically voltage-dependent (Doupnik et al., 1995).

Despite the necessity of using ligands to detect constitutive receptor activation of GIRK, $\mu^*$-state stimulation of GIRK should be apparent when opioids which inhibit $\mu^*$-state signalling are superfused onto neurons from chronically morphine treated animals. Reversal of $\mu^*$-state activity would be seen as ligand-precipitated closing of a K conductance or a membrane depolarization. This has not been observed in studies where naloxone has been washed onto brain slices from morphine treated animals (Christie et al., 1987), and studies that have reported opioid antagonist-mediated depolarization in PAG have attributed this to non-GIRK
conductances (Chieng et al., 1996; Bagley et al., 2005b). Opioid receptor modulation of cAMP-regulated conductances or neurotransmitter release onto neurons in brain slices are a potential source of significant confounds in studies of constitutive activity in these preparations.

Conclusions
Constitutive activity reflects fundamental properties of GPCR. µ-opioid ligands are extremely important therapeutic drugs, with significant unwanted effects and a better understanding of constitutive activity at the µ-opioid receptor may provide information useful in developing ligands that access subsets of receptor conformations, offering the potential to fine tune opioid pharmacotherapy. The unique role suggested for the putative µ*-state of the receptor also provides an intriguing possibility for therapeutic exploitation. While readily amenable to standard methods of measuring constitutive activity, µ-opioid receptors also couple to G proteins that modulate ion channels, providing the opportunity for studying receptor states in single neurons in real time. Together with emerging techniques for relatively direct studies of conformational states within GPCR (Yao et al. 2009), and access to purified µ-opioid receptors (Kuszak et al. 2009), the methods outlined in this Chapter should provide the tools for many fruitful investigations of this intriguing property of the µ-opioid receptor.
Acknowledgements

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References


Figure Legends

Figure 1

Common (and potential) assay points for constitutively active µ-opioid receptors. A diagram of where µ-opioid receptor activity can be assayed relatively straightforwardly, with respect to the G protein cycle. Brief comments about the nature of each assay are included.

Figure 2

Detection of constitutively active µ-opioid receptors using the $[^{35}\text{S}]{\text{GTP}}_{\gamma}S$ assay.

A) Basal $[^{35}\text{S}]{\text{GTP}}_{\gamma}S$ binding in homogenates (10µg) of whole brains from wild-type (MOR +/+ ) or µ-opioid receptor knockout (MOR−/−) mice in the absence or presence of the opioid antagonists β-CNA (1 µM) or BNTX (10µM). β-CNA and BNTX reduced basal binding only in brain homogenates from the wild-type animals (**p < 0.01). The assays were performed using 0.1nM $[^{35}\text{S}]{\text{GTP}}_{\gamma}S$ in the presence of 10µM GDP, 100mM NaCl and 4mM MgCl$_2$ for 30 mins at 30°C as described in (Wang et al. 2004). B) In homogenates of untreated HEKµ cells (5µg), BNTX (1µM) acts as an inverse agonist to reduce basal $[^{35}\text{S}]{\text{GTP}}_{\gamma}S$ binding; naloxone (10µM) and 6β-naltrexol (10µM) reverse the effect of BNTX. In DAMGO-treated HEKµ cells (5µg) (1µM, 24h) naloxone is converted to an inverse agonist, but 6β-naltrexol is still a neutral antagonist (**p < 0.01; *p < 0.0). Assays were performed as above but in the presence of 150mM KCL and 1 mM MgCl$_2$ (Wang et al. 2007). It should be noted from a practical standpoint that the differences observed are very small and the assay components critical, emphasizing the low level of basal µ-opioid receptor constitutive activity and the difficulty of studying this phenomenon. The figure was redrawn from data in Wang et al., (J. Pharmacol. Exp. Ther. 308, 512-520, 2004) 2004 (A) and Wang et al., (J. Pharmacol. Exp. Ther. 321, 544-552, 2007), http://www.jpet.aspetjournals.org, with permission.
Detection of constitutively active µ-opioid receptors using voltage-gated calcium channels as a reporter. These patch clamp recordings were made from cultured sympathetic neurons injected with µ-opioid receptor mRNA. $I_{Ca}$ were elicited using a double pulse protocol, neurons were stepped from -80mV to +10 mV to elicit control currents (prepulse) and then βγ subunit-mediated inhibition of the channels was transiently relieved by a long step to +80 mV, the current amplitude was retested by another step to +10 mV following a brief rest at -80 mV (postpulse). **A)** A representative time course of a typical experiment showing plotting pre- and postpulse amplitudes. **B)** Representative current traces illustrating the effects of superfusion of the µ-opioid antagonist CTAP and the agonist DAMGO. Note that even before drug application the amplitude of the postpulse is greater than that of the prepulse, indicating constitutive inhibition of $I_{Ca}$, which is reversed by the highly selective µ-opioid ligand CTAP and mimicked by a subsequent application of the agonist DAMGO. The Figure is based on Figure 8 in Mahmoud et al., *(Mol. Pharmacol. 77, 840-817, 2010, used with permission)*, with the traces kindly provided by Dr Victor Ruiz-Velasco.
**Table 1:** The activity of ligands for the µ-opioid receptor as modulators of \[^{35}S\]-GTPγS binding in basal and agonist pretreated conditions, and to constitutively active mutants of the µ-opioid receptor. Ligand efficacy is reported as positive (stimulates binding), negative (inhibits binding) or neutral (no significant effect on binding) according to the results presented in the cited studies. Because of differing experimental conditions and methods of presenting data, we have assigned just direction to efficacy, not a strength. Not all ligands tested in these studies are reported here, full references can be found at the end of the manuscript.

<table>
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<td>negative D164Q</td>
<td>neutral C348/353A</td>
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<td>CTAP had negative efficacy in both conditions in Hi K buffer in</td>
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a) Brillett et al., 2003  
b) Burford et al., 2000  
c) Divin et al., 2009  
d) Li et al., 2001a  
e) Liu and Prather 2001  
f) Sally et al., 2010  
g) Traynor and Nahorski, 1995  
h) Wang et al., 2001  
i) Wang et al., 2007  
j) Wang et al., 1994
Figure 1

- Stimulation of GTPγS binding
  - cumulative single point assay
  - ligand dependent

- Stimulation of GTPase activity
  - cumulative single point assay
  - ligand dependent

- Inhibition of adenylyl cyclase
  - cumulative single point assay
  - ligand dependent

- Inhibition of Ca channels
  - rapid, continuous, ligand independent

- Activation of K channels
  - rapid, continuous, ligand dependent

μ-opioid receptor

Gα

Gβ

Gγ

[35S]-GTPγS

GDP

GTP

GDP + P_i