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Buprenorphine is a weak partial agonist that inhibits opioid receptor desensitization

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Abstract

Buprenorphine is a weak partial agonist at mu-opioid receptors that is used for treatment of pain and addiction. Intracellular and whole cell recordings were made from locus coeruleus (LC) neurons in rat brain slices to characterize the actions of buprenorphine. Acute application of buprenorphine caused a hyperpolarization that was prevented by previous treatment of slices with the irreversible opioid antagonist, β-chlornaltrexamine (β-CNA), but was not reversed by a saturating concentration of naloxone. As expected for a partial agonist, sub-saturating concentrations of buprenorphine decreased the [Met]^5 enkephalin (ME) induced hyperpolarization or outward current. When the ME induced current was decreased below a critical value, desensitization and internalization of μ-opioid receptors (MOR) was eliminated. The inhibition of desensitization by buprenorphine was not the result of prior desensitization, slow dissociation from the receptor, or elimination of receptor reserve. Treatment of slices with sub-saturating concentrations of etorphine, methadone, oxymorphone or β-CNA also reduced the current induced by ME but did not block ME-induced desensitization. Treatment of animals with buprenorphine for a week resulted in the inhibition of the current induced by ME and a block of desensitization that was not different from the acute application of buprenorphine to brain slices. These observations show the unique characteristics of buprenorphine and further demonstrate the range of agonist selective actions that are possible through G-protein coupled receptors.

Keywords

desensitization; tolerance; locus coeruleus; morphine; methadone; oxycodone; fentanyl; chronic treatment

INTRODUCTION

Buprenorphine is a thebaine derivative used as an analgesic and as an effective alternative to methadone in the treatment of opiate dependence (West et al., 2000; Gerra et al., 2004; Vigezzi et al., 2006; Connock et al., 2007; Soyka et al., 2008). Two properties distinguish buprenorphine from other opioids. It has a bell shaped analgesic dose-response curve (Lutfy et al., 2003; Yamamoto et al., 2006) and a ceiling effect for respiratory depression (Dahan et al., 2005; Dahan et al., 2006). Thus buprenorphine is an attractive compound for use in clinical settings because of reduced potential for toxicity and overdose (Kakko et al., 2007, 2008;Pergolizzi et al., 2007; Hayes et al., 2008).

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Buprenorphine has slow receptor association/dissociation kinetics and a half-life of 2–5 hours. These properties contribute to both a low abuse liability and minimal withdrawal symptoms upon cessation (Tzschentke, 2002). Buprenorphine is a partial agonist at MOR (Selley et al., 1997; Yu et al., 1997; Lutfy et al., 2003) and ORL-1 receptors (Wnendt et al., 1999; Bloms-Funke et al., 2000; Lutfy et al., 2003). It has mixed but primarily antagonistic actions on κ- (KOR) and δ-opioid receptors (DOR, Sadee et al., 1982; Richards and Sadee, 1985; Kajiwara et al., 1986; Leander, 1987; Zhu et al., 1997; Huang et al., 2001). The bell shaped dose-response curve for buprenorphine has been attributed to activation of MORs at low doses and ORL-1 at higher concentrations (Lutfy et al., 2003; Yamamoto et al., 2006). Though the in vivo properties of buprenorphine have been characterized, the underlying pharmacology and signaling, particularly in neurons, remains poorly understood.

LC neurons express both ORL-1 and MOR, but not KOR or DOR. These neurons are well suited to examine MOR signaling and receptor regulation following buprenorphine binding. This study shows that buprenorphine is a partial agonist at MOR. The partial agonist activity of buprenorphine decreased the current induced by more potent agonists including ME and etorphine. Furthermore, pre-treatment with buprenorphine eliminated the desensitization induced by each of these agonists. Treatment with buprenorphine also inhibited subsequent ME induced MOR internalization, which makes it different from the effects of an irreversible antagonist, β-CNA. These results indicate that buprenorphine is unique among opiates and illustrates the diversity of MOR signaling and regulation.

MATERIALS AND METHODS

Tissue Preparation and Recording

Adult (150–250 g) male Sprague Dawley rats (Charles River Laboratories, Wilmington, MA) were used for all experiments. Details of the method of slice preparation and recording have been published previously (Virk and Williams, 2008). Briefly, rats were anesthetized with halothane and killed. The brain was dissected, blocked and mounted in a vibratome chamber in order to cut horizontal slices (260 μm thick) containing the locus coeruleus (LC). Slices were stored at 35°C in an artificial cerebro-spinal fluid (aCSF) containing (in mM) 126 NaCl, 2.5 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 21.4 $NaHCO_3$, and 11 D-glucose while being continuously equilibrated with 95% O_2/5% CO_2. Slices were incubated for a minimum of 1 hr to remove residual drug from the tissue. Slices were hemisected, transferred to the recording chamber (0.5 ml) and superfused with aCSF (35°C at 1.5 ml/min). Whole-cell recordings were made from LC neurons with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) in the voltage-clamp mode (−55 mV). Pipettes (1.7–2.1 MΩ) were filled with an internal solution containing the following (in mM): 115 Methyl potassium sulfate, 20 NaCl, 1.5 $MgCl_2$, 10 HEPES, 10 BAPTA, 2 Mg-ATP, 0.5 Na-GTP, and 10 phosphocreatine, pH 7.3. Data was collected with PowerLab (Chart version 4.2.3) and sampled at 100 Hz. Analysis was performed with Prism and Kaleidagraph software. Values are presented as arithmetic mean ±SEM. One-way ANOVA followed by Dunnett's or Tukey's Multiple Comparison Test were performed. Results where $p<0.05$ were considered significant.

Two photon microscopy and Flag MOR-transgenic mice

A transgenic mouse that expressed an extracellular Flag epitope on MOR in LC cells was used as described previously (Arttamangkul et al., 2008). All data were collected from hemizygous FlagMOR-Tg/+ mice. Brain slices (200–220 μM) from the transgenic mouse were prepared as those described for electrophysiological experiments. Slices were incubated in a solution containing M1 antibody (Sigma, St Louis MO) conjugated with Alexa594 (Molecular Probes, Eugene, OR, 10 μg/ml, 45–60 min). The tissue was visualized with an upright microscope (Olympus, Center Valley, PA.) equipped with a custom-built two-photon apparatus. Data were

acquired and collected using Scan Image Software (Pologruto et al., 2003). A z-series was collected at 1 μm intervals for 15 μm. Drugs were applied by perfusion.

Drugs

All drugs were applied by bath superfusion. Drugs included: [Met^5]enkephalin (Sigma), bestatin (Sigma), thiorphan (Sigma), buprenorphine (NIDA–Neuroscience Center), orphanin FQ/nociceptin (Sigma), β-chlornaltrexamine (Sigma), UK14304 (Sigma), and yohimbine (Sigma). UK14304 was dissolved in dimethyl sulfoxide. Thiorphan was dissolved in ethanol. The maximum concentration used in the superfusion solution was 0.01% (DMSO) and 0.0001% ethanol. All other compounds were dissolved in water.

Protocols

Desensitization and recovery from desensitization were measured as described previously (Virk and Williams, 2008). Desensitization was defined by two measurements: (1) the depression in GIRK current during a continuous 10 min treatment with a saturating concentration of ME (30 μM) and (2) the depression of the current induced by ME (300 nM, EC50) 5 min following treatment with the saturating concentration. Recovery from desensitization was measured by repeated applications of ME (300 nM, EC50) at 10 min intervals for 45 minutes.

Drug Treatment

Rats were implanted with osmotic minipumps (Alzet, 2ML1) in order to deliver buprenorphine (NIDA – Neuroscience Center) or carrier (control). The minipumps have a 2 ml reservoir and deliver their contents for 7 days at the rate of 10 μl/hour. Pumps were filled with the required concentration of drug, dissolved in water, based on the weight of the rat and the desired dosing parameter (buprenorphine: 1, 5, 10 mg/kg/day). Buprenorphine was dissolved in 40% dimethylsulfoxide and water constituted the balance. Rats were anesthetized with isoflurane and an incision was made in the mid-scapular region to insert the pump subcutaneously. Rats were returned to their housing facility upon recovery. Experiments were performed on day 6 or 7 following minipump implantation. Control animals consisted of naive animals and those implanted with vehicle-filled pumps.

Drug Concentration Analysis

All brain and plasma samples were analyzed at the University of Utah, Center for Human Toxicology under the supervision of Dr. Roger Foltz in conjunction with NIDA. Plasma and whole brain samples were obtained for drug (buprenorphine) concentration analysis at the time of brain slice preparation. Following halothane anesthesia, 3 ml whole blood was obtained via cardiac puncture with a heparinized syringe. Blood was centrifuged and plasma was collected. Brain tissue removed after blocking the LC was collected and homogenized in water. Samples were frozen at −20°C and shipped to University of Utah, Center for Human Toxicology for analysis. Samples were analyzed by liquid chromatography/tandem mass spectrometry using electrospray ionization and selected reaction monitoring. The limit of detection was 0.2–20 ng/ml. Plasma samples contained 5.2, 27.7 and 58.1 ng/ml for the three doses of buprenorphine (1,5,10 mg/kg/day) and brain samples contained 12.2, 29.1 and 64.5 ng/ml for the three doses. Thus the osmotic mini pump delivered buprenorphine efficiently and predictably.

RESULTS

Buprenorphine is a partial agonist

Intracellular recordings were used to determine the acute action of buprenorphine. This recording technique provides a stable and sensitive assay necessary to identify the slow actions

of buprenorphine. When applied at a saturating concentration, buprenorphine (1 μM, 15 min) caused a hyperpolarization (14.5±1.7 mV; n=8) and blocked the transient hyperpolarization induced by pressure ejection applied ME (arrows, Fig 1). Application of a lower concentration of buprenorphine (200 nM) induced a hyperpolarization of 6.4±1.4 mV after 35 min (Fig 1B, n=4). The hyperpolarization induced by superfusion of ME (1 μM) was decreased to 91.5±3.2% of control within 5 min of buprenorphine (200 nM) application, and to 12.3±4.2% of control after 25 min. The buprenorphine-induced hyperpolarization was not reversed by naloxone (10 μM) even after 30 min application and did not affect the hyperpolarization induced by the alpha-2-adrenoceptor agonist, UK14304 (3 μM, Fig 1B) or the ORL-1 agonist, orphanin FQ/nociceptin (2 μM, Fig 1A). Both these receptors are coupled to the same GIRK conductance suggesting that buprenorphine (1 μM) did not block the GIRK conductance directly. These results are consistent with occupation of receptors by a partial agonist that binds with high affinity.

Examination of the acute action of buprenorphine by superfusion of concentrations less than 200 nM was not possible because of the slow onset of action. In order to determine whether buprenorphine was occupying receptors or capable of signaling at lower concentrations, slices were pre-incubated in buprenorphine (5 nM, 1 hr) before the beginning of the experiment. A sustained hyperpolarization was observed in recordings made from slices pre-incubated in buprenorphine (5 nM, 1 hr, Fig S1C). Although it was not possible to determine the amplitude of the hyperpolarization induced by this treatment protocol, the spontaneous firing that is characteristic of LC neurons was eliminated and the membrane potential was approximately −65 mV, which is 10 mV more hyperpolarized than cells in untreated slices. This observation demonstrated that pre-incubation with buprenorphine (5 nM, 1 hr) resulted in a sustained hyperpolarization that was smaller than that induced by buprenorphine (1 μM). In slices pre-incubated with buprenorphine (5 nM, 1 hr), application of ME (30 μM) caused a further hyperpolarization (14±1.1 mV, n=7, Fig S1) that was less than that induced by the acute application of ME (34.5 ± 1.5 mV, n=5) in control slices. Thus pre-treatment with buprenorphine (5 nM, 1hr) occupied a fraction of MORs without complete saturation.

The slow dissociation rate of buprenorphine from receptors was indicated by the inability of naloxone (10 μM, 30 min) to reverse the hyperpolarization induced by buprenorphine (Fig 1B). Although naloxone did not reverse the buprenorphine-induced hyperpolarization, pretreatment of slices with the irreversible opioid antagonist, β-CNA (1 μM, 10 min) completely blocked the hyperpolarization induced by high concentration buprenorphine (1 μM, 15 min, 0.8 mV, n=4). Taken together, the results suggest that the hyperpolarization induced by buprenorphine (≤1 μM) was mediated by the activation of MORs. The slow activation of the buprenorphine-induced hyperpolarization likely resulted from the slow binding kinetics, low efficacy, and the necessity for a high degree of receptor occupancy in order to cause a change in membrane potential.

Buprenorphine eliminated MOR desensitization

With the use of whole cell recording, buprenorphine did not cause a detectable outward current, even when applied at a saturating concentration (1 μM). It did, however, decrease the amplitude of the outward current induced by ME. Buprenorphine (100 nM) applied for a period of 20–30 min resulted in complete inhibition of the current induced by ME (300 nM, 2 min, n=3), and the current induced by ME (30 μM) was reduced by more than 70%. In addition, the current induced by ME (30 μM) immediately following pre-incubation with buprenorphine (5 nM, 1 hr) was reduced to 43±5% of the current induced by the alpha-2-adrenoceptor agonist, UK14304 (3 μM, n=6) compared to 135±6% (n=20) in untreated slices. When slices were washed for 4 hr following buprenorphine treatment, the current induced by ME remained at 48±3% of that caused by UK14304 (3 μM, n=6). Thus preincubation with buprenorphine

resulted in a stable, long lasting inhibition of the current induced by ME that was consistent with slow dissociation kinetics.

In control slices, ME (30 μM) caused a peak current of 461±28 pA that desensitized by 35±1% over a 10 min application (Fig 2A). The peak current induced by ME (30 μM) varied among individual cells (276–698 pA), but the amount of desensitization after application of ME (30 μM, 10 min) was 35±1% and did not vary with the amplitude of the initial current (Fig 2D, vertical line, 95% confidence limits 23–47%). The decline in the ME-induced current was the same after a 5 and 15 min application, indicating that after about 5 min the rate of desensitization and recovery from desensitization had reached equilibrium (Dang and Williams, 2004). Following pre-incubation with buprenorphine (5 nM, 1 h), the peak ME (30 μM) current was reduced to 175±13 pA (n=18, Fig 2B). In slices pre-incubated in buprenorphine (5 nM, 1 hr), ME (30 μM, 10 min) desensitization was considerably attenuated, but varied as a function of the peak current (fraction desensitization −25% to 30%). When the initial current induced by ME was less than 150 pA, the current increased in amplitude during the 10 min application (Fig 2D, circles, positively sloping line). Thus as more receptors were occupied by buprenorphine, the amount of ME induced desensitization decreased.

It was possible that buprenorphine blocked desensitization by decreasing the number of receptors as a result of the high affinity and slow dissociation rate. To test this, receptors were eliminated by pre-incubation of slices with the irreversible MOR antagonist β-chlornaltrexamine (β-CNA, 5–40 nM, 1 hr). Multiple concentrations of β-CNA were tested in order to obtain a range of currents induced by ME that could be compared with the results obtained with buprenorphine. Regardless of the β-CNA concentration used, the peak current induced by ME (30 μM, 10 min) desensitized (Fig 2C). Furthermore, when the peak current induced by ME was 150 pA or less, the amount of desensitization was greater (Fig 2D, triangles, negatively sloping line) than in untreated controls. Thus, removal of receptors with the irreversible antagonist, β-CNA, increased the extent of ME-induced desensitization, whereas incubation with buprenorphine had the opposite effect.

The action of buprenorphine to block desensitization was further tested by using etorphine. Etorphine was chosen because it is a high affinity agonist that is structurally similar to buprenorphine. In control experiments, etorphine caused an outward current that declined by 35±1.7% of the peak after 10 min, which was similar to that caused by ME (Fig 3A, n=6). After incubation of slices in buprenorphine (5 nM, 1 h), the peak current induced by etorphine was decreased from 117±6.0% (n=6) to 57±7% (n=9) of the current induced by the alpha-2-adrenoceptor agonist, UK14304 (3 μM). Acute desensitization induced by etorphine (1 μM, 10 min) was also eliminated by incubating slices with buprenorphine (Fig 3B). After 10 min, the etorphine-induced current was 100±4% of the peak value (n=9). The results indicate that the desensitization induced by both ME and etorphine was blocked by pre-treatment with buprenorphine.

The elimination of MOR desensitization is unique to buprenorphine

Previous experiments demonstrated that desensitization was not blocked by pre-treating slices with the irreversible antagonist β-CNA (Fig 2). In order to determine whether this effect was specific to buprenorphine, ME induced desensitization was measured following pre-incubation with four other agonists: etorphine, methadone, oxymorphone and oxycodone.

Etorphine—It was possible that the high affinity and slow binding kinetics of buprenorphine alone could result in signaling that resulted in the block of desensitization and that any agonist with similar properties would have the same effect. To test this possibility slices were pre-incubated with the high affinity agonist etorphine. Slices were incubated in a low concentration of etorphine (2 nM, 1hr) prior to testing for desensitization induced by ME (Fig 4A). After

incubation with etorphine, desensitization induced by ME (30 μM, 10 min) was examined. The peak current induced by ME (30 μM) measured 149±14 pA (n=7) and declined to 79±14 pA after 10 min (desensitized by 49±9%). After washing ME, the opioid antagonist, naloxone (1 μM) was applied to determine if etorphine remained in the slice. Naloxone caused an inward current of −151±42 pA (n=7). Thus, etorphine did not wash out of the brain slice as has been previously demonstrated (Virk and Williams, 2008). Etorphine did not eliminate ME-induced desensitization and is therefore distinct from buprenorphine.

Methadone—Methadone is a partial agonist that causes both desensitization (Virk and Williams, 2008) and internalization (Arttamangkul et al., 2008). Incubation of slices with methadone (1 μM, 1 hr) resulted in a sustained outward current that was detected by the application of naloxone (1 μM) at the end of the experiment (Fig 4B, −158±22 pA, n=3). Application of ME (30 μM) resulted in a peak current that desensitized (by 47±11%, n=3). Thus treatment of slices with methadone had no obvious effect on acute MOR desensitization.

Oxymorphone—Pretreatment with oxymorphone (1 μM) resulted in a sustained outward current (85±23 pA, n=5) that was reversed by naloxone (1 μM) and a small decrease in the amplitude of the current induced by a saturating concentration of ME (30 μM, 100.7±10.0% of the current induced by UK14304, 3 μM, n=7). In slices that were incubated in oxymorphone (1 μM, 1 hr, Fig. 4C, n=9), application of ME (30 μM, 10 min) caused an outward current that desensitized by 35.8±5.4 %. Thus ME induced desensitization following pre-incubation with oxymorphone remained intact.

Oxycodone—Oxycodone applied at a saturating concentration (15 μM) does not induce desensitization (Virk and Williams, 2008). Pre-incubation of slices in oxycodone (1 μM, 1 hr) had no affect on ME (30 μM) induced desensitization. The current induced by ME decreased by 35±4% during a 10 min application of ME (Fig 4D).

Thus unlike the results obtained with buprenorphine, desensitization induced by ME remained completely intact after treatment of slices with each of these other agonists.

Buprenorphine treatment did not block desensitization of ORL-1 receptors

To determine if buprenorphine blocked desensitization of other G protein coupled receptors that are expressed on LC neurons, slices were incubated with buprenorphine (5 nM, 1 hr) before the ORL-1 receptor agonist, OFQ/N was tested. Application of OFQ/N (2 μM, 10 min) caused a peak outward current of 257±22 pA (n=11) that declined by 34±2.9% (not shown). Thus ORL-1 receptors, which activate the same GIRK conductance as MORs, desensitized in buprenorphine-incubated slices. This demonstrates that the block of opioid receptor dependent desensitization did not generalize to other receptors.

Buprenorphine eliminated MOR internalization

It has been established that buprenorphine does not induce receptor internalization in several preparations (Zaki et al., 2000). A transgenic mouse that expressed an epitope (Flag) tagged MOR in tyrosine hydroxylase containing neurons was used to examine the change in receptor trafficking induced by buprenorphine. In these experiments, slices were incubated in a solution containing the antiFlag antibody (M1) that was conjugated with Alexa594 for 30 min before imaging and then treated with ME (30 μM), buprenorphine (15 μM) or untreated for 15 min and imaged before treatment of the slice with a calcium-free solution (+EGTA 0.5 mM). The calcium-free solution resulted in the displacement of the antibody from the receptor in the extracellular space such that the remaining fluorescence resulted only from internalized MOR. Experiments where no agonist was applied prior to treatment with the calcium-free solution were used to control for autofluorescence (Fig 5B, calcium-free alone). When a supersaturating

concentration of buprenorphine (15 μM) was applied to the slice, internalization was not induced (Fig 5A). In contrast, ME (30 μM) resulted in robust internalization, as reported previously (Arttamangkul et al., 2008).

Slices were then pre-incubated with buprenorphine (5 nM, 1 hr) prior to the application of a saturating concentration of ME (30 μM, 15 min). In these experiments ME did not induce any internalization above baseline (Fig 5B). It was possible that the sensitivity of the assay was too low to detect a small number of internalized receptors. In order to address this possibility, slices were pre-treated with β-CNA (10 nM, 1 hr), to reduce the receptor number before measuring ME induced internalization. In slices pre-incubated in β-CNA, ME (30 μM, 15 min) caused a detectable amount of internalized receptors that, as expected, was less than that found in control (Fig 5A,B). Thus it was possible to observe receptor internalization under conditions where the number of receptors was depleted. These results indicate that buprenorphine alone did not induce internalization at a high concentration and a low concentration blocked both desensitization and internalization induced by ME.

Chronic Buprenorphine Treatment

Animals were treated with 3 different doses of buprenorphine (1, 5, and 10 mg/kg/day) for 6–7 days before slices were prepared. All experiments used a saturating concentration of ME (30 μM, 10 min) to induce desensitization followed by the application of a lower concentration to measure the extent of recovery from desensitization. The results of these experiments show that there were two effects of buprenorphine that were dose dependent: the peak current induced by ME (30 μM) was reduced (Fig 6A,B,C) and ME (30 μM/10 min) induced desensitization was inhibited (Fig 6B,D,E).

The amplitude of the ME induced current was reduced as the dose of buprenorphine increased (Fig 6). The current induced by ME (30 μM), expressed as a percentage of current induced by UK-14304 (3 μM), was 136±5% (n=20) in control. Peak ME (30 μM) induced currents decreased to 96±4% (n=6), 74±7% (n=6), and 47±4% (n=8) as buprenorphine treatment increased from 1, 5, and 10 mg/kg/day (Fig 6C). This dose-dependent decrease in current amplitude suggests that the percentage of buprenorphine occupied MORs increased as the treatment dose increased.

In untreated animals, the ME (30 μM) current desensitized by 35±2% during a 10 min application. Desensitization after 10 min was 45±3%, 24±5%, and 10±6% in slices taken from animals that received 1, 5, and 10 mg/kg/day buprenorphine, respectively (Fig 6D). Thus, desensitization was inhibited in animals treated with buprenorphine in a dose dependent manner. The decline in current induced by a low concentration of ME following desensitization was also dependent on the dose. In slices taken from animals treated with 1 mg/kg/day the desensitization was not different from slices taken from untreated animals. With higher doses, the amount of desensitization was reduced and recovery from the reduced desensitization was more complete (fig 6E).

DISCUSSION

The results indicate that buprenorphine is a weak partial agonist that has extremely slow dissociation kinetics at MORs. The greater sensitivity and stability of intracellular recording allowed the measurement of an acute hyperpolarization induced by buprenorphine. Once bound, buprenorphine did not dissociate during the time course of the recordings. While the outward current induced by high affinity agonists such as etorphine and fentanyl was reversed by naloxone, it was not possible to reverse the buprenorphine-induced hyperpolarization. Following incubation with buprenorphine (5 nM, 1 hr), a large fraction of the receptor population was occupied as determined by the reduction in the peak current caused by ME. In

addition, receptor desensitization and internalization induced by ME were eliminated. Incubation with a series of opioid agonists, partial agonists and antagonists also decreased the amplitude of the current induced by ME, but did not block desensitization. Thus buprenorphine is unique among opioids in that it is a weak, partial agonist that blocked the desensitization and internalization induced by ME.

Mechanisms of buprenorphine action

The cellular actions of buprenorphine have been examined by measuring multiple effectors. In virtually every assay it has been shown to be a partial MOR agonist. It binds with nanomolar affinity to MORs, stimulates GTP-gamma-S binding, inhibits adenylyl cyclase, activates MAP kinase (Zaki et al., 2000; Lutfy et al., 2003; Clark et al., 2008) and, as shown in the present study, activates GIRK conductance. The low intrinsic efficacy of buprenorphine results in the slow turnover of activated G proteins. Rapid GTP hydrolysis of the Gi-alpha subunit due to the presence of Regulator of G Protein Signaling (RGS) proteins is thought to further decrease the efficacy of buprenorphine. These characteristics are presumed to contribute to the limited ability of buprenorphine to activate various effectors (Clark et al., 2008). Buprenorphine did not induce internalization and competitively blocked etorphine induced internalization in cell lines (Zaki et al., 2000). Finally, a G protein independent, pertussis toxin insensitive, increase in cell surface expression was induced by buprenorphine, similar to that caused by naloxone, has been demonstrated in cell lines (Zaki et al., 2000). Buprenorphine therefore has pharmacological properties that are a mix of a weak partial agonist and an antagonist.

The block of desensitization shown here was only observed under conditions where the number of available receptors was dramatically depressed. Desensitization with a saturating concentration of ME (30 μM, 5 min) has been shown to decrease the MOR reserve by approximately 90% (Osborne and Williams, 1995). Buprenorphine (5 nM, 1 hr) pre-treatment decreased the peak current induced by ME (30 μM) to a greater extent than desensitizing the receptors with a 10 min application of ME (30 μM). It follows that buprenorphine caused a more dramatic decrease in receptor reserve than ME induced desensitization. In buprenorphine treated slices, the block of ME induced desensitization was variable, but occurred as a function of the initial peak ME current. When the peak current was >150 pA, some desensitization occurred. However, when the peak current was <150 pA, the ME induced current increased over the course of a 10 min application. Pretreatment with other high affinity agonists also decreased the peak ME induced current, but had no effect on desensitization. Moreover, in contrast to the results obtained with buprenorphine, when β-CNA pre-treatment was used to reduce receptor reserve, ME-induced desensitization was facilitated (Fig 2D). This result is consistent with experiments in HEK293 cells showing that reducing MOR reserve with the irreversible antagonist, β-FNA, increased etorphine-induced desensitization (Law et al., 2000). Thus, unlike all other ligands tested, buprenorphine reduced the ability of ME to activate the potassium conductance, induce internalization, and cause desensitization.

It is unlikely that buprenorphine caused desensitization of MORs. Using intracellular recordings, a sustained hyperpolarization was observed following application of high (1 μM) and low concentrations (200 nM and 5 nM). As was found with other low efficacy agonists, buprenorphine did not result in receptor phosphorylation at saturating concentrations (Yu et al., 1997). Although buprenorphine is known to activate MAP kinase, the minimum effective concentration was greater than 30 nM (Lutfy et al., 2003). In the present study, buprenorphine (5 nM) resulted in a sustained and stable hyperpolarization.

Buprenorphine acted on opioid receptors

Buprenorphine activates the ORL1 receptor to limit antinociception, motor stimulation and conditioned place preference mediated by MORs (Lutfy et al., 2003; Marquez et al., 2007;

Marquez et al., 2008). The ORL1 receptor knockout animal was more sensitive to buprenorphine, whereas the MOR knockout animal was insensitive to the rewarding and antinociceptive actions of buprenorphine (Lutfy et al., 2003; Marquez et al., 2007; Marquez et al., 2008). The role of the ORL1 receptor in mediating the actions of buprenorphine in vivo remains to be completely characterized (Yamamoto et al., 2006; Spagnolo et al., 2008). The affinity of buprenorphine for the ORL1 receptor is 50 fold lower than that for MOR (Spagnolo et al., 2008; Marquez et al., 2008). Experiments presented here show that the hyperpolarization induced by saturating concentrations of buprenorphine (1 μM) was completely blocked by the irreversible MOR antagonist, β-CNA, and this concentration far exceeded the primary experimental concentration of 5 nM. Additionally, buprenorphine had no effect on the peak hyperpolarization induced by OFQ/N. Taken together, the results of the present study do not appear to involve the activation of the ORL1 receptor.

Chronic Treatment

Chronic treatment with buprenorphine resulted in a concentration dependent inhibition of the current induced by ME that was identical to that observed with acute application to brain slices. Both peak ME current and desensitization were decreased in a dose-dependent fashion. Thus the results indicate that there were few if any adaptive changes measured at the single cell level that resulted from the chronic treatment and may be a therapeutic advantage.

The doses used in this study (1, 5, 10 mg/kg/day) are in the range of those used to achieve maximal analgesia. A single subcutaneous doses of 2.5, 10 and 40 mg/kg induced maximal analgesia within 60 min, using the tail immersion assay (Meert and Vermeirsch, 2005). The high affinity of buprenorphine for MOR has been viewed as a double-edged sword. While the slow pharmacokinetics may diminish the ability to experience opiate mediated euphoria in patients on maintenance therapy, it may also be a liability in the same population if opiate analgesia is required for pain management. The results presented here indicate that slices prepared from animals maintained on effective doses still signal acutely. There is also concern over the safety of buprenorphine because of the slow dissociation kinetics and the possibility that naloxone does not displace buprenorphine from receptors. Although naloxone was unable to displace buprenorphine over a period of 10–30 min in the present experiment, in vivo studies examining the ability of naloxone to reverse buprenorphine-induced analgesia have found that this is possible, particularly with high or repetitive doses of naloxone (Kogel et al., 2005).

Conclusion

Buprenorphine is a reasonable analgesic as well as a safe and effective alternative to methadone for opiate maintenance therapy (Johnson et al., 2000; Raisch et al., 2002; Kakko et al., 2003; 2008). Chronic buprenorphine treatment almost completely eliminated the ability of opioid abusers to subjectively detect an acute injection of morphine (Teoh et al., 1994). Thus treatment with buprenorphine can limit relapse to opioid abuse, though it may not be effective in all individuals (Teoh et al., 1994). Moreover, there was little or no sign of adverse cardiovascular, respiratory or temperature reactions associated with acute administration of morphine or cocaine in patients maintained on daily buprenorphine treatment (Teoh et al., 1993). This suggests that there is no increase in physiological risk for patients maintained on buprenorphine, an important consideration for a population often engaged in polydrug abuse. Treatment of animals with buprenorphine results in tolerance to opioids and supports the idea that there is an inverse correlation between efficacy and the development of tolerance (Walker and Young, 2001; Koch et al., 2005; Grecksch et al., 2006). The present study offers a cellular explanation that supports the observation that effective analgesia can be obtained in patients receiving low dose buprenorphine maintenance therapy (Alford et al., 2006). Buprenorphine has agonist activity, yet exhibits pharmacological properties that are more characteristic of an

antagonist. The combined pharmacological actions distinguish it from other opioids and may have important implications for clinical utility.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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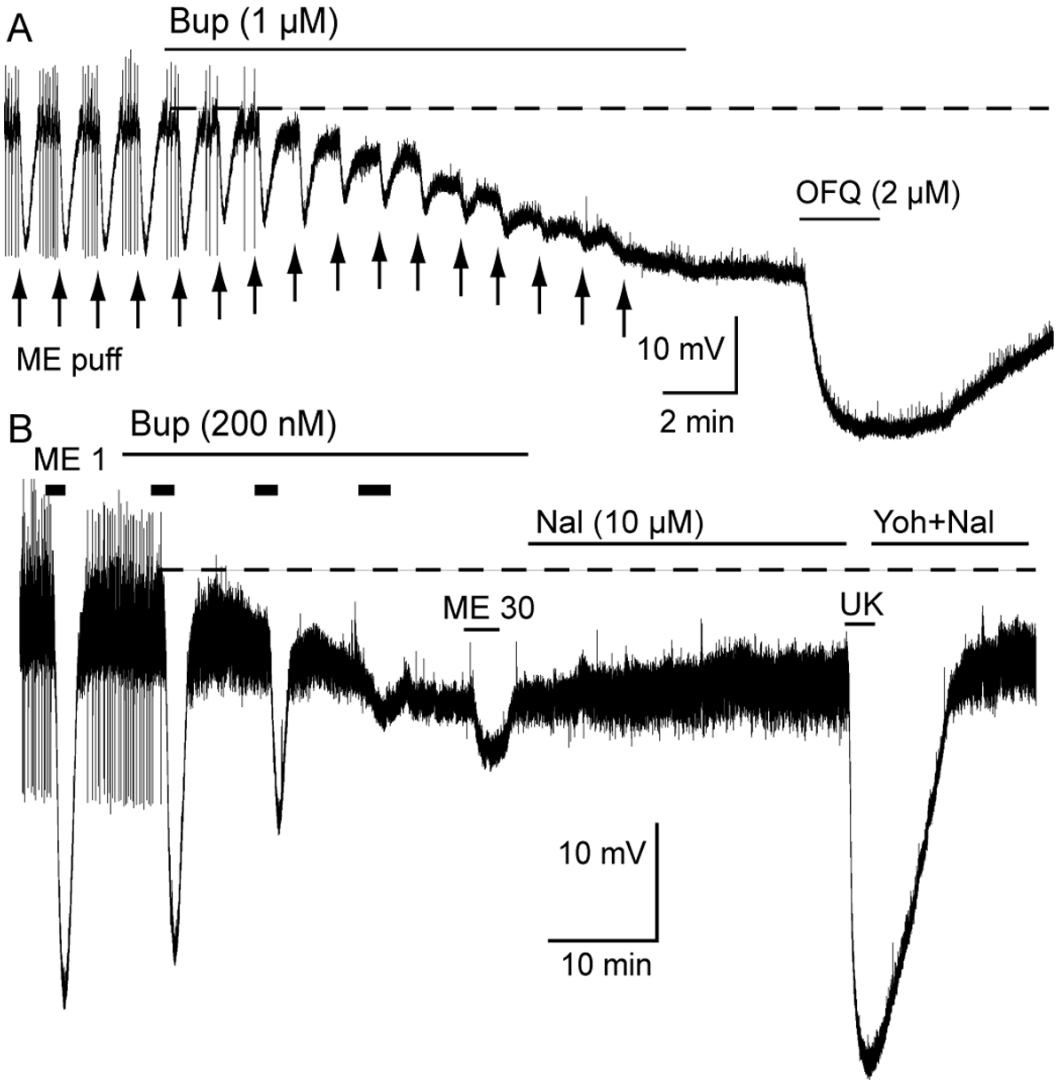

Figure 1.

Buprenorphine hyperpolarized LC neurons and blocked the ME-induced hyperpolarization. Voltage recording made with intracellular electrodes. A. Buprenorphine (1 μM) applied for 15 min and caused a sustained hyperpolarization. Pressure ejection of ME (ME puff, arrows) caused a transient reproducible inhibition in spontaneous firing and hyperpolarization that was blocked by the application of buprenorphine. Application of orphanin FQ/nociceptin (OFQ) resulted in a further hyperpolarization. B. ME (1 μM, 2 min) caused an inhibition of spontaneous firing and a hyperpolarization of about 25 mV. Buprenorphine (200 nM) caused a hyperpolarization over a period of 25 min. The hyperpolarization induced by ME (1 μM) was decreased by buprenorphine and after 25 min, application of ME (30 μM) caused only a small hyperpolarization. Naloxone (10 μM for 25 min) had little effect on the membrane potential. UK14304 (UK, 3 μM) caused a hyperpolarization of about 35 mV.

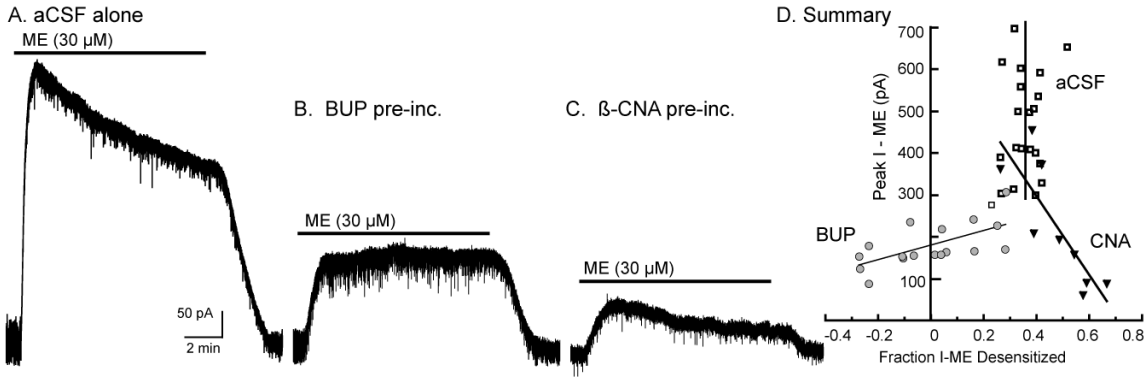

Figure 2.

Buprenorphine limits ME induced desensitization. Voltage clamp recordings made with whole cell electrodes. A) a control experiment using an untreated slice. ME (30 μM) caused a large outward current that declined during the 10 min application period. B) an experiment taken from a slice that was pre-incubated with buprenorphine (5 nM, 1 hr). ME (30 μM) caused a small outward current that did not desensitize during the 10 min application. C) an experiment using a slice that was pre-incubated with β-CNA (20 nM, 1 hr). ME (30 μM) caused a small outward current that desensitized during the 10 min application period. D) summary of results, plotting the peak amplitude of the current induced by ME (30 μM) against the amount of desensitization (the change in current from the peak to the end of the 10 min application – divided by the peak current). The open boxes (vertical line at 35%) indicate experiments done in control slices (part A). The amount of desensitization was independent of the initial amplitude of current induced by ME. The gray circles (positive sloping line) are experiments done after buprenorphine (part B). In this case, when the current induced by ME was larger, the amount of desensitization was greater. When the ME currents were smaller than 150 pA, the desensitization was eliminated. Solid triangles (negative sloping line) are experiments done after β-CNA (part C). In this case as the current induced by ME decreased, the amount of desensitization was increased.

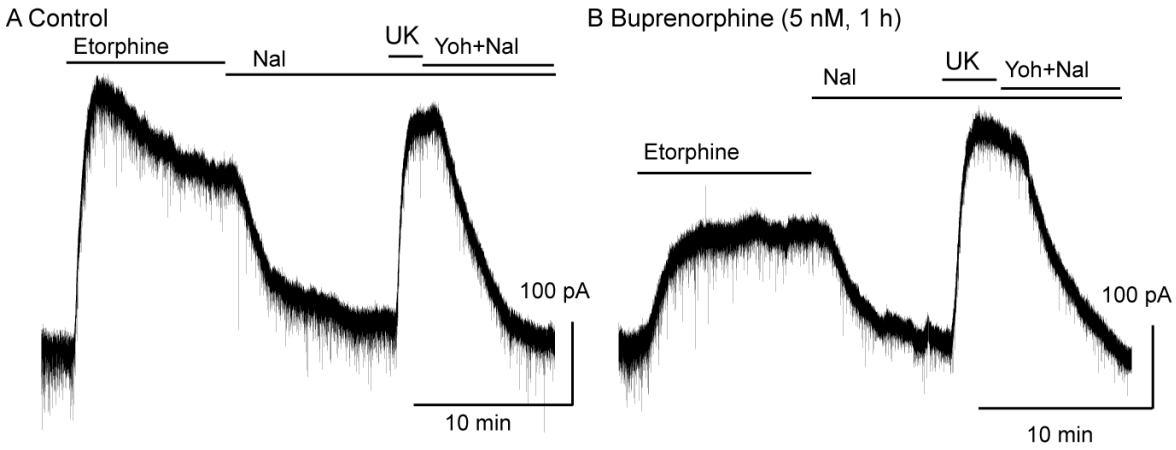

Figure 3.

Buprenorphine blocked desensitization induced by etorphine. A. a control experiment showing the desensitization induced by etorphine (1 μM), the reversal of the current induced by naloxone (1 μM) and the current induced by UK14304 (UK, 3 μM). B. an experiment done in a slice that was incubated in buprenorphine (5 nM 1 hr). The current induced by etorphine (1 μM) is significantly smaller and did not desensitize during the 10 min application period.

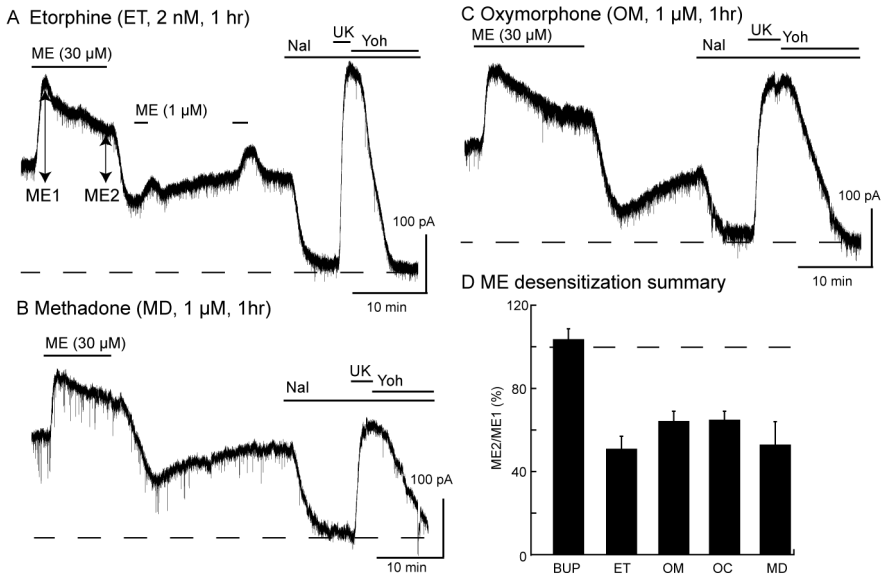

Figure 4.

Incubation of slices with etorphine, oxymorphone, methadone or oxycodone did not block ME induced desensitization. A. sample experiment from a slice that was incubated with etorphine (2 nM, 1 hr) before recording the outward current induced by ME (30 μM, 10 min). The ME current peaked (ME1) and declined (ME2) during the 10 min application period. Superfusion with naloxone (1 μM) caused an inward current indicating the presence of etorphine in the slice. B. the same experiment done with slices that were incubated with methadone (1 μM) C. the same experiment done with oxymorphone (1 μM) for 1 hr. D. summarized results plotting the ratio of ME2/ME1 in experiments using slices incubated in buprenorphine (BUP, 5 nM), etorphine (ET, 2 nM), oxymorphone (OM, 1 μM), oxycodone (OC, 1 μM), or methadone (MD, 1 μM) for 1 hr before the experiment. There was a marked decrease in the ME current in all experiments except in slices incubated in buprenorphine.

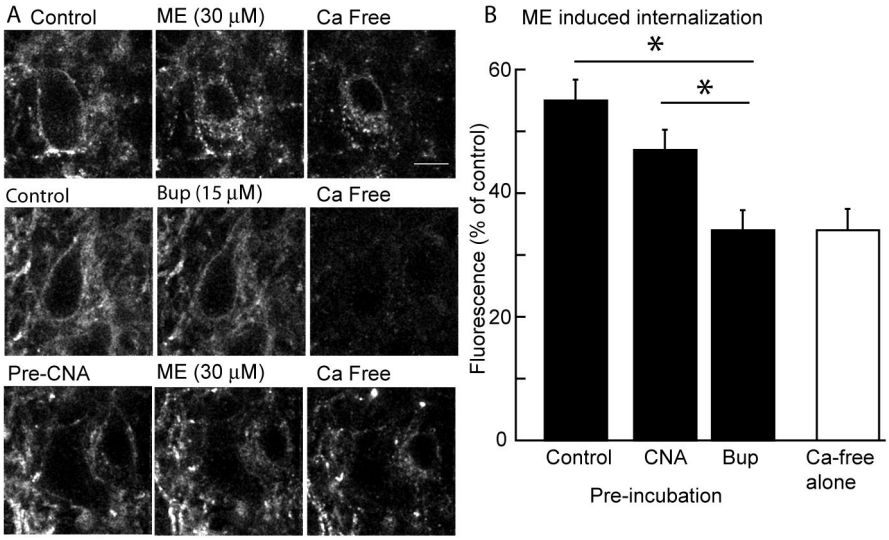

Figure 5.

Buprenorphine does not cause receptor internalization and blocked the internalization induced by ME. A. images of cells in three experiments. Left side, images taken at the beginning of the experiment after incubating slices with M1-anti-Flag antibody without drugs (top two images) or β-CNA (10 nM, 1 hr, bottom image). The top three images are a control experiment demonstrating the internalization of receptor evoked by ME (30 μM, 15 min). The fluorescence image on the right is only internalized receptors following treatment of the slice with a calcium-free solution to strip antibody bound to extracellular surface. Middle images show that buprenorphine (15 μM) did not induce any internalization. The bottom images show that treatment of slices with a low concentration of β-CNA reduced but did not abolish the ME-induced receptor internalization. Scale bar is 10 μm. B. summarized results from several experiments showing the amount of internalization (fluorescence as a % of the control) induced by ME in control, after treatment of slices with β-CNA (10 nM, 1 hr), buprenorphine (5 nM, 1 hr). The open bar is the background fluorescence measured after treatment of the slices with calcium-free solution without application of any drug. * indicates $p<0.05$

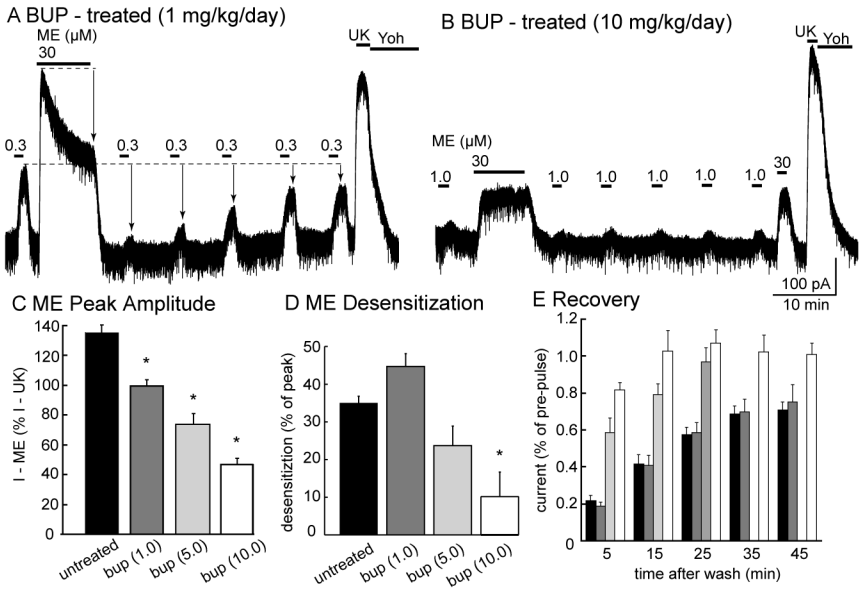

Figure 6.

Chronic treatment of animals with buprenorphine. A and B. Representative experiments in slices taken from animals treated with a low (1 mg/kg/day, A) or high (10 mg/kg/day, B) dose of buprenorphine. A) in a slice taken from an animal treated with a low dose of buprenorphine (1 mg/kg/day) desensitization and the recovery from desensitization is the same as that observed in untreated animals. B) desensitization was completely blocked in slices taken from animals treated with a high dose. C) summary of the peak current induced by ME (30 μM) in slices from control and buprenorphine treated animals. D) summary of the decline in the current induced by ME (30 μM) during a 10 min application. E) summary of the recovery from desensitization induced by ME (30 μM, 10 min). The amount of recovery and the speed at which recovery occurred was increased in slices from buprenorphine treated animals. The current induced by UK14304 (3 μM, UK) was reversed by the application of yohimbine (1 μM, YOH).