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**μ** Opioid Receptor Activation Inhibits GABAergic Inputs to Basolateral Amygdala Neurons Through Kv1.1/1.2 Channels

Thomas F. Finnegan, Shao-Rui Chen, and Hui-Lin Pan

Department of Anesthesiology, Pennsylvania State University College of Medicine, The Milton S. Hershey Medical Center, Hershey, Pennsylvania

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**Introduction**

The amygdala consists of a heterogeneous collection of discrete nuclei working together to integrate both sensory and emotional information (Swanson and Petrovich 1998). The amygdala is an important link between environmental stimuli and pain modulation (Fox and Sorenson 1994; Helmstetter et al. 1998). It remains uncertain how μ opioid receptor stimulation in the BLA influences the vPAG to produce the analgesic effect. The CeA is not only the major output nuclei of the amygdala but also receives afferent inputs from the BLA (Finnegan et al. 2005; Pitkanen et al. 1997; Smith and Millhouse 1985; Swanson and Petrovich 1998). While the BLA projects directly to the PAG, its projection to the CeA is more important for opioid analgesia (Rizvi et al. 1991). Synaptic plasticity occurs in afferents from the BLA (Finnegan et al. 2005; Pitkanen et al. 1997; Smith and Millhouse 1985; Swanson and Petrovich 1998).

Behavioral and anatomical studies suggest that the BLA is a region involved in opioid analgesia through a projection to the CeA, which in turn activates the descending modulatory pathway (Helmstetter et al. 1998). It remains uncertain how μ opioid receptor stimulation in the BLA influences the vPAG to produce the analgesic effect. The CeA is not only the major output nuclei of the amygdala but also receives afferent inputs from the BLA (Finnegan et al. 2005; Pitkanen et al. 1997; Smith and Millhouse 1985; Swanson and Petrovich 1998). While the BLA projects directly to the PAG, its projection to the CeA is more important for opioid analgesia (Rizvi et al. 1991). Synaptic plasticity occurs in afferents from the BLA to CeA in animal models of chronic pain (Neugebauer et al. 2003). Although nociceptor stimuli alter the activity of CeA-

and cingulated cortices with the BLA support the important involvement of this amygdaloidal region in the emotional aspect of pain (Swanson and Petrovich 1998). The BLA is also essential for the expression of fear conditioning and plays an important role in stress-induced analgesia (Koo et al. 2004).

Stimulation of μ opioid receptors within the BLA results in analgesia (Helmstetter et al. 1998; McGarvaughy and Heinricher 2002; Rodgers 1977). However, the cellular mechanisms of opioid action in the BLA are unclear. The BLA contains μ opioid receptors in greater abundance than the CeA (Daunais et al. 2001; Ding et al. 1996; Paden et al. 1987). Direct microinjection of morphine or the specific μ opioid receptor agonist, [d-Ala², N-Me-Phe⁵, Gly⁵-ol] enkephalin (DAMGO, 1 μM), significantly reduced the frequency of miniature inhibitory postsynaptic currents (mIPSCs) in 77% of cells tested. DAMGO also significantly decreased the peak amplitude of evoked IPSCs in 75% of cells examined. However, DAMGO did not significantly alter the frequency of mEPSCs or the peak amplitude of evoked EPSCs in 90% and 75% of labeled cells, respectively. Bath application of the Kv channel blockers, 4-AP (Kv1.1, 1.2, 1.3, 1.5, 1.6, 3.1, 3.2), α-dendrotoxin (Kv1.1, 1.2, 1.6), dendrotoxin-K (Kv1.1), or tityustoxin-K (Kv1.2) each blocked the inhibitory effect of DAMGO on mIPSCs. Double immunofluorescence labeling showed that some of the immunoreactivities of Kv1.1 and Kv1.2 were colocalized with synaptophysin in the BLA. This study provides new information that activation of presynaptic μ opioid receptors primarily attenuates GABAergic synaptic inputs to CeA-projecting neurons in the BLA through a signaling mechanism involving Kv1.1 and Kv1.2 channels.

Address for reprint requests and other correspondence: Hui-Lin Pan, Department of Anesthesiology and Pain Medicine, University of Texas, M.D. Anderson Cancer Center, 1400 Holcombe Blvd., Unit 409, Houston, TX 77030.

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projecting BLA neurons, it is not clear if and how μ opioid receptor stimulation affects synaptic transmission in the BLA. In this study, we combined retrograde tract tracing and whole cell patch-clamp recordings to determine the effect of μ opioid receptor activation on both excitatory and inhibitory synaptic inputs to BLA neurons that project to the CeA. We also defined the specific Kv channels involved in the inhibitory effect of the μ opioid receptor agonist on GABAergic inputs to CeA-projecting BLA neurons.

**METHODS**

Retrograde labeling of CeA-projecting BLA neurons

Male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing between 80–120 g were used in this study. The surgical preparations and experimental protocols were approved by the Animal Care and Use Committee of the Pennsylvania State University College of Medicine and conformed to the National Institutes of Health guidelines on the ethical use of animals. Bilateral microinjection of the fluorescent tracer, 1,1'-dioctadecyl-3,3',3',3'tetramethylindocarbocyanine perchlorate (DiI; 7 mg/100 μL; Molecular Probes, Eugene, OR), was performed to label CeA-projecting BLA neurons. The animals were initially anesthetized with 2% halothane and received an ip injection of a ketamine/xylazine (90 and 5 mg/kg, respectively) mixture. A borosilicate micropipette (~30 μm tip diameter) was lowered into the CeA (coordinates from bregma: AP, −25.0 mm; ML, ±40 mm; and DL, −67.0 mm from lambda), and DiI was pressure ejected (~10 nl/side; Nanoject II; Drummond Scientific; Broomall, PA) into each side of the CeA (Fig. 1A). After injections, the wounds were sutured and rats were returned to their cage for ≥2 days to allow the tracer to be transported to the BLA.

Slice preparations

The rats were rapidly decapitated under halothane anesthesia 2–5 days after fluorescent dye injection. The brain was quickly removed and placed in ice-cold and oxygenated (95% O₂, 5% CO₂) artificial cerebral spinal fluid (ACSF) for ~2 min. A tissue block containing the amygdala was cut and glued onto the stage of the vibratome (Technical Product International, St. Louis, MO). Coronal slices containing the BLA (300 μm in thickness) were cut from the tissue block in ice-cold, oxygenated ACSF. The slices were incubated in oxygenated ACSF at 36°C for ≥15 min before being transferred into the recording chamber. The ACSF contained (in mM) 126 NaCl, 2.5 KCl, 1.3 MgSO₄, 2.4 CaCl₂, 1.2 NaH₂PO₄, 11.0 glucose, and 25.0 NaHCO₃ (pH 7.4, osmolarity 295–300 mOsm).

Recordings of postsynaptic currents of BLA neurons

Recordings of excitatory and inhibitory postsynaptic currents (EPSCs and IPSCs) were performed using whole cell voltage-clamp methods as previously described (Finnegan et al. 2004, 2005; Li et al. 2003; Pan et al. 2002). The electrode for the whole cell recordings was pulled with a puller (P-97, Sutter Instrument, Novato, CA) using borosilicate glass capillaries (OD, 1.2 mm; ID, 0.86 mm; World Precision Instruments, Sarasota, FL). The resistance of the pipette was about 5 MΩ when filled with an internal solution containing (in mM) 110 CsSO₄, 0.5 CaCl₂, 2.4 MgCl₂, 5.0 BAPTA, 10.0 HEPES, 5.0 Na₂ATP, 0.33 GTP-Tris salt, 10.0 QX314, and 5.0 TEA-Cl, adjusted to pH 7.2 and osmolarity 280–290 mOsm. The G protein inhibitor, guanosine 5’-O-(2-thiodiphosphate) (GDP-β-S, 1 mM), was added to the internal solution in the evoked protocol to block the potential postsynaptic effect of opioids (Finnegan et al. 2005; Pan et al. 2002).

The brain slice was placed in a glass-bottomed chamber (Warner Instruments, Hamden, CT) and fixed with a grid or parallel nylon threads supported by a U-shaped stainless steel weight. The slice was perfused at 3.0 ml/min at 36°C maintained by an inline solution heater and a temperature controller (TC-324, Warner Instruments). BLA neurons were visualized in a transparent triangular region adjacent to the CeA (Fig. 1B). Fluorescence-labeled BLA cells were briefly identified in the slice with epifluorescence (rhodamine filter) on a fixed-stage microscope (Fig. 1B; BX50WI, Olympus). The neurons were viewed with Nomarski optics through a water immersion objective (Fig. 1C). The tissue image was captured and enhanced through a CCD camera and displayed on a video monitor. The BLA and CeA can be easily discerned under the microscope (Finnegan et al. 2005). We did not specifically determine the morphology of the labeled neurons recorded in the BLA. After the labeled neuron was identified, positive pressure was applied to the pipette, which was advanced toward the identified neuron. Once the pipette touched the membrane of the neuron, the pressure was immediately released and slight negative pressure was applied to establish a gigahm seal. The cell membrane was ruptured by further suction to establish the whole cell configuration. Recordings of postsynaptic currents began about 5 min after whole cell access was established and the current reached steady state.

The evoked postsynaptic currents in the labeled BLA neurons were induced by electrical stimulation (0.1 ms, 0.1–0.5 mA, and 0.2 Hz) through a bipolar tungsten electrode connected to a stimulator (S48, Grass Instruments, W. Warwick, RI). The stimulating electrode was placed either in the dorsal or ventral regions of the BLA. Evoked inhibitory postsynaptic currents (eIPSCs) and evoked excitatory postsynaptic currents (eEPSCs) were recorded at a holding potential of 0 and −70 mV, respectively (Finnegan et al. 2004; Pan et al. 2002). The electrical stimulus strength was adjusted to a minimal level at the beginning of the recording so that the amplitude of eIPSCs and eEPSCs was between 300–800 and 200–600 pA, respectively. To determine the effect of DAMGO on paired-pulse facilitation, two synaptic responses were evoked by a pair of stimuli given at short intervals (50 ms). Paired-pulse facilitation was expressed as the amplitude ratio of the second synaptic response to the first synaptic response. Miniature inhibitory postsynaptic currents (mIPSCs) and miniature excitatory postsynaptic currents (mEPSCs) were recorded at a holding potential of 0 and −70 mV, respectively (Finnegan et al. 2004; Pan et al. 2002). All mIPSCs were recorded in the presence of TTX (1 μM) and 6-cyano-7-nitroquinolxaline-2,3-dione (CNQX, 20 μM). The mEPSCs were recorded in the presence of 1 μM TTX and 20 μM bicusculine. Currents were measured using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). Signals were filtered at 1–2 kHz, digitized at 10 kHz (DigiData 1320A, Axon Instruments, Foster City, CA), and recorded into a Pentium computer using the pClamp 8.01 program.

**Experimental protocols**

The input resistance was continuously monitored throughout the recording period. Recordings were abandoned if the input resistance changed >15%. To determine the effect of DAMGO on the mIPSCs and mEPSCs in labeled BLA neurons, 1 μM DAMGO was perfused
into the slice for ~2 min after recording the IPSCs and EPSCs for 3 min as the baseline control. To ensure the specific effect of DAMGO, the effect of 1 μM DAMGO was examined in the presence of the specific μ opioid antagonist, H-D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH2 (CTAP, 1 μM) (Vaughan and Christie 1997). Also, eIPSCs and eEPSCs were measured before and during perfusion of 1 μM DAMGO, as described above. CNQX and bicuculline were obtained from Sigma (St. Louis, MO). DAMGO and CTAP were purchased from Bachem (King of Prussia, PA), whereas TTX was obtained from Alomone Labs (Jerusalem, Israel).

Additional protocols were used to identify the role of specific voltage-gated potassium (Kv) channels in the inhibitory effect of DAMGO on IPSCs. In this set of experiments, we first applied DAMGO to ensure the labeled cell being examined was a responder. After washout of the effect of DAMGO, a selective Kv channel blocker was perfused for 5 min. DAMGO was perfused for ~2 min in the presence of the Kv channel blocker. The following Kv channel blockers were used: 4-aminopyridine (4-AP; 2 mM; Sigma, St. Louis, MO) (Bergevin et al. 2002; Ishikawa et al. 2003; Vaughan et al. 1997), α-dendrotoxin (α-DTX; 10 nM; Alomone Labs) (Cunningham and Jones 2001; Mo et al. 2002; Vaughan et al. 1997), dendrotoxin-K (DTX-K, 10 nM, Alomone Labs) (Beekwilder et al. 2003; Speake and Brown 2004; Speake et al. 2004), and tityustoxin-Kα (TTY; 100 nM; Alomone Labs) (Dodson et al. 2003; Werkman et al. 1993).

Double immunofluorescence labeling of Kv1.1/1.2 and synaptophysin in the BLA

Male adult rats weighing ~200 g were perfused intracardially with 50 ml of ice cold 0.02 M PBS containing 500 units of heparin followed by 250 ml of 4.0% paraformaldehyde and 4.0% sucrose in 0.1 M PBS (pH 7.4). The brains were harvested and sectioned at a thickness of 35 μm. Brain sections were stored in cryoprotectant solution at −20°C until the immunolabeling experiment. Sections were double immunolabeled for Kv1.1 or Kv1.2 and synaptophysin, as described previously (Finnegan et al. 2005; Vydyanathan et al. 2005). Briefly, the sections were rinsed in 0.02 M PBS, blocked with 4.0% normal goat serum and 0.3% Triton X-100. The sections were incubated with either rabbit anti-Kv1.1 (dilution, 1:100; Chemicon International, Temecula, CA) and mouse anti-synaptophysin (dilution, 1:100; Chemicon International) or rabbit anti-Kv1.2 (dilution, 1:100; Chemicon International) and mouse anti-synaptophysin for 2 h at room temperature and for 48 h at 4°C. Sections were subsequently rinsed in 0.1 M PBS and incubated with goat anti-rabbit secondary antibody conjugated to Alexa Fluor 488 (1:200; Molecular Probes, Eugene, OR) and biotin-SP-conjugated goat anti-mouse secondary antibody (1:200; Jackson ImmunoResearch Laboratories) for 2 h at room temperature. The sections were rinsed again with 0.1 M PBS. The sections were incubated with streptavidin conjugated to Alexa 594 (1:200; Molecular Probes, Eugene, OR) for 1.5 h at room temperature. After labeling, sections were mounted on slides, dried, and coverslipped. All sections were examined on a laser scanning confocal microscope (Leica, Wetzlar, Germany), and areas of interest were photodocumented and digitally merged. Because the Kv1.1 and Kv1.2 antigens are less abundant and because a monoclonal synaptophysin antibody was used, higher concentrations of these primary antibodies were required in the double immunofluorescence labeling experiments. In the higher magnification images, the colocalization was indicated by the color change and represents colocalization because the optical section thickness (0.3 μm) is thin enough to minimize the possibility of superimposition (Finnegan et al. 2004, 2005; Pan et al. 2002).

Data analysis

Data are presented as means ± SE. The mIPSCs and mEPSCs of labeled BLA neurons were analyzed off-line with a peak detection program (MiniAnalysis, Synaptosoft, Leonia, NJ). The cumulative probability of the amplitude and interevent interval of mIPSCs and mEPSCs was compared using the Komogorov-Smirnov test, which estimates the probability that two distributions are similar. Analysis of the effects of drugs on the amplitude of eIPSCs and eEPSCs were performed using Clampfit (Axon Instruments, Foster City, CA). Cells were considered as responders if the frequency of mIPSCs/mEPSCs or the amplitude of evoked IPSCs/EPSCs changed >20% by DAMGO. The effects of drug treatment on postsynaptic currents were determined by either a paired t-test or repeated measures ANOVA with Dunnett’s post hoc test. P < 0.05 was considered to be statistically significant.

RESULTS

Effect of DAMGO on eIPSCs in labeled BLA neurons

Evoked IPSCs were elicited by placing a stimulating electrode in either the dorsal or ventral borders of the BLA. Because the results did not differ based on electrode placement, data were grouped together. The effect of 1 μM DAMGO was examined in a total of 12 labeled BLA neurons. In 9 of the 12 (75%) cells tested, DAMGO significantly

![Graph](image-url)
reduced the amplitude of eIPSCs (from 401.24 ± 64.29 to 273.20 ± 30.28 pA, *P* < 0.05; Fig. 2, A and B). This effect was evident about 1.5 min after DAMGO application and was washed out after ∼5 min. In two separate cells, 1 μM DAMGO had little effect on the eIPSC amplitude (759.10 vs. 893.04 and 846.31 vs. 737.32 pA). In the remaining labeled neuron, 1 μM DAMGO resulted in an increase in the amplitude of eIPSCs (from 643.22 to 965.12 pA during DAMGO application and 755.33 pA after washout of DAMGO). Bath application of 20 μM bicuculline abolished eIPSCs in all 10 labeled neurons tested (Fig. 2A).

To further determine the presynaptic effect of DAMGO on GABAergic inputs to labeled BLA neurons, we examined the effect of 1 μM DAMGO on the paired-pulse ratio (peak amplitude of the 2nd IPSC/the peak amplitude of the 1st IPSC) of evoked IPSCs. DAMGO significantly increased the paired-pulse ratio of eIPSCs in all five labeled BLA neurons tested (Fig. 2, C and D).

**Effect of DAMGO on mIPSCs in labeled BLA neurons**

In the presence of TTX, 1 μM DAMGO significantly reduced the mIPSC frequency in seven of nine (77%) labeled BLA neurons examined (from 3.96 ± 0.82 to 1.67 ± 0.35 Hz, *P* < 0.05; Fig. 3, A–C). DAMGO did not significantly alter the amplitude of mIPSCs in the recorded cells (24.50 ± 1.71 vs. 24.16 ± 2.79 pA; Fig. 3C). The mIPSC frequency of these cells returned to near baseline after ∼5 min of washout (Fig. 3C). The decay phase of mIPSCs in these cells was best fitted to a double exponential function. DAMGO did not significantly change the fast (8.31 ± 0.87 vs. 10.21 ± 1.55 ms) or the slow (15.19 ± 3.48 vs. 15.76 ± 3.29 ms) components of the decay time constant of mIPSCs. The cumulative probability analysis of mIPSCs revealed that the interevent interval distribution shifted to the right after DAMGO (Fig. 3B). However, the amplitude of mIPSCs was not changed after DAMGO application (Fig. 3B). In two of nine cells, DAMGO had little effect on the mIPSC frequency (3.80–4.29 and 1.21–1.33 Hz) or amplitude (35.03–35.50 and 30.46–24.62 pA). Bicuculline (20 μM) completely abolished mIPSCs in all seven labeled cells examined (Fig. 3A).

In another eight labeled BLA neurons, the μ opioid receptor antagonist, CTAP, was used to examine the specific effect of DAMGO. In all eight labeled cells, initial application of 1 μM DAMGO significantly reduced the mIPSC frequency (from 5.05 ± 0.58 to 2.53 ± 0.46 Hz, *P* < 0.05). Bath application of 1 μM CTAP alone had no significant effect on the mIPSC frequency. Application of 1 μM DAMGO in the presence of CTAP failed to alter significantly the mIPSC frequency (4.28 ± 0.95 vs. 4.11 ± 0.84 Hz).

**Effect of DAMGO on eEPSCs in labeled BLA neurons**

The effect of 1 μM DAMGO on eEPSCs was examined in a total of 12 labeled BLA neurons. In 9 of 12 (75%) labeled cells, DAMGO did not significantly change the amplitude of eEPSCs (368.04 ± 35.95 vs. 378.41 ± 38.44 pA; Fig. 4, A and B). However, DAMGO significantly decreased the eEPSC amplitude in the remaining three labeled cells (from 401.07 ± 69.06 to 251.71 ± 20.63 pA; *P* < 0.05, Fig. 4C). In 8 of the above 10 cells tested, 20 μM CNQX completely abolished eEPSCs (Fig. 4A). CNQX only partially reduced the eEPSC amplitude by 51% (from 279.10 to 137.06 pA) in one cell and 42% (from 277.20 to 160.04 pA) in the other. Among those three cells in which DAMGO decreased the amplitude of eEPSCs, one of the cells had CNQX-resistant EPSCs.

**Effect of DAMGO on mEPSCs in labeled BLA neurons**

In 9 of 10 (90%) labeled BLA neurons, 1 μM DAMGO had no significant effect on the mEPSC frequency (1.73 ± 0.35 vs. 2.47 ± 0.35 Hz; Fig. 5, A–C) and amplitude (19.53 ± 1.27 vs. 17.90 ± 1.21 pA; Fig. 5, A–C). In the remaining one labeled cell, DAMGO decreased the mEPSC frequency (from 2.27 to 1.61 Hz) but not the amplitude (22.02 vs. 22.60 pA). The decay phase of mEPSCs of these cells was best fitted with a single exponential function. DAMGO did not significantly change the decay time constant of mEPSCs (2.91 ± 0.35 vs. 3.09 ± 0.24 ms). In a total of six labeled BLA neurons examined, 20 μM CNQX abolished all mEPSCs (Fig. 5A).
Role of voltage-gated potassium (Kv) channels in the effect of DAMGO on IPSCs

It has been shown that Kv channels may be involved in opioid receptor modulation of synaptic GABA release. For example, in both the PAG and ventral tegmental area, the inhibitory effect of DAMGO on presynaptic GABA release is mediated by 4-AP–sensitive Kv channels (Bergevin et al. 2002; Vaughan and Christie 1997). We therefore further determined the specific Kv channels involved in the effect of DAMGO on IPSCs in labeled BLA neurons.

4-AP is a nonspecific Kv channel blocker affecting Kv channels containing Kv1.1, 1.2, 1.3, 1.5, 1.6, 3.1, and 3.2 subunits (Coetzee et al. 1999; Grissmer et al. 1994). Bath application of 2 mM 4-AP blocked the effect of 1 μM DAMGO on the mIPSC frequency in all eight labeled BLA cells examined (Fig. 6, A–C). Similarly, 2 mM 4-AP (n = 7) abolished the effect of 1 μM DAMGO on the eIPSC amplitude. The eIPSC amplitude during the control and initial application of DAMGO was 605.28 ± 85.56 and 298.00 ± 34.26 pA (P < 0.05), respectively. In the presence of 2 mM 4-AP, repeated application of 1 μM DAMGO failed to decrease the eIPSC amplitude (614.85 ± 76.37, P > 0.05 compared with the control). In another six labeled BLA neurons, repeated application of 1 μM DAMGO reproducibly decreased the mIPSC frequency (Fig. 6D). The effect of the second DAMGO application was washed out after ~5 min (Fig. 6D).

It has been shown that the Kv1 family may be responsible for the inhibitory effect of DAMGO on mIPSCs ( Vaughan et al. 1997). In 10 separate labeled BLA neurons, 100 nM α-dendrotoxin (α-DTX, a Kv1.1, 1.2, and 1.6 blocker) (Coetzee et al. 1999; Grissmer et al. 1994; Vaughan et al. 1997), completely blocked the effect of 1 μM DAMGO on mIPSCs (Fig. 6E).

Furthermore, bath application of dendrotoxin-K (DTX-K; 10 nM), the specific Kv1.1 channel blocker (Beekwilder et al. 2003), eliminated the inhibitory effect of 1 μM DAMGO on mIPSCs in a total of six labeled BLA neurons (Fig. 7, A–C). Additionally, bath application of tityustoxin-Kα (TTY; 100 nM), the Kv1.2 channel blocker (Werkman et al. 1993), also abolished the inhibitory effect of DAMGO on mIPSCs in another six labeled BLA neurons (Fig. 7D).

Immunoreactivities of Kv1.1/Kv1.2 and synaptophysin in the BLA

To confirm the presence of presynaptic Kv1.1 and Kv1.2 subunits in the BLA, we performed double immunofluorescent

FIG. 4. Effect of 1 μM DAMGO on electrically evoked EPSCs in labeled BLA neurons. A: representative tracing showing evoked EPSCs during control, application of 1 μM DAMGO, washout, and 20 μM CNQX in a labeled BLA neuron. B and C: summary data showing differential effects of DAMGO on the amplitude of evoked EPSCs in 12 labeled BLA neurons. Data presented as means ± SE. *P < 0.05 compared with control.

FIG. 5. Effect of DAMGO on mEPSCs in labeled BLA neurons. A: representative tracing showing mEPSCs during control, application of 1 μM DAMGO, washout, and 20 μM CNQX in a labeled BLA neuron. B and C: summary data showing the lack of effect of DAMGO on the frequency and amplitude of mIPSCs in 9 labeled BLA neurons. Data presented as means ± SE. *P < 0.05 compared with the control.

FIG. 6. Effect of 1 μM DAMGO on mIPSCs in labeled BLA neurons. A: representative tracing showing mIPSCs during control, application of 1 μM DAMGO, washout, and 20 μM CNQX in a labeled BLA neuron. B and C: summary data showing differential effects of DAMGO on the mIPSC frequency in all eight labeled BLA cells examined. Data presented as means ± SE. *P < 0.05 compared with the control.
labeling of Kv1.1/Kv1.2 and synaptophysin in the BLA. In all sections examined, there was extensive punctate labeling of both the Kv1.1 and Kv1.2 immunoreactivity in the BLA (Fig. 8, A and B). Figure 8 shows the colocalization of synaptophysin with either Kv1.1 (Fig. 8A) or Kv1.2 immunoreactivity (Fig. 8B). While both Kv1.1 and Kv1.2 immunoreactivities were colocalized with synaptophysin (yellow color; Fig. 8, A and B), there also existed Kv1.1 and Kv1.2 immunoreactivities that were not colocalized with synaptophysin (Fig. 8, A and B). Because the intact cell bodies were not present in the thin optical sections and synaptic contact can be made on dendrites, some of the synaptophysin immunoreactivity likely reflects nerve terminals apposing dendritic regions of the soma. Control experiments that omitted the Kv1.1, Kv1.2, or synapto-

FIG. 6. Effect of 2 mM 4-aminopyridine (4-AP) or 100 nM α-dendrotoxin on DAMGO-induced attenuation of the mIPSC frequency. A: representative tracing showing mIPSCs during control, application of 1 μM DAMGO, washout, 2 mM 4-AP, and 4-AP with DAMGO in a labeled BLA neuron. B: cumulative probability plots showing the distribution of interevent interval and peak amplitude of this neuron during control, DAMGO application, and the combination of 4-AP and DAMGO. C: summary data showing the inhibitory effect of DAMGO on the mIPSC frequency, and block of this effect by 4-AP in 8 labeled BLA neurons. D: summary data of 8 labeled BLA neurons showing that the repeated application of 1 μM DAMGO produced a similar reduction in the mIPSC frequency. E: summary data showing the inhibitory effect of DAMGO on the mIPSC frequency before and after application of 100 nM α-dendrotoxin (α-DTX) in 10 labeled BLA neurons. Data presented as means ± SE. *P < 0.05 compared with control.

FIG. 7. Effect of dendrotoxin-K or tityustoxin-Kα on DAMGO-induced reduction in the mIPSC frequency. A: representative tracing showing mIPSCs during control, application of 1 μM DAMGO, washout, 10 nM dendrotoxin-K, and DAMGO plus dendrotoxin-K in a labeled BLA neuron. B: cumulative probability plots showing the distribution of interevent interval and peak amplitude of this neuron during control, DAMGO application, and the combination of dendrotoxin-K and DAMGO. C: summary data showing inhibitory effect of DAMGO on the frequency of mIPSCs before and after application of 100 nM dendrotoxin-K in 6 labeled BLA neurons. D: summary data showing inhibitory effect of DAMGO on the mIPSC frequency before and after application of 100 nM tityustoxin-Kα in 10 labeled BLA neurons. Data presented as means ± SE. *P < 0.05 compared with control. Dendrotoxin-K, DTX-K; tityustoxin-Kα, TTY.
physin primary antibody did not reveal any immunopositive staining in the BLA.

**DISCUSSION**

This is the first study determining the role of presynaptic μ opioid receptors in the control of inhibitory and excitatory synaptic inputs to CeA-projecting BLA neurons. We found that the majority of mIPSCs and eIPSCs were attenuated after administration of the specific μ opioid receptor agonist, DAMGO (77% and 75%, respectively). DAMGO did not, however, have significant effects on mEPSCs or eEPSCs in most cells examined (90% and 75%, respectively). We also showed that the specific Kv channel blockers, dendrotoxin-K (Kv1.1) and tityustoxin-Kα (Kv1.2), abolished the effect of DAMGO on mIPSCs. Double immunofluorescence labeling showed that Kv1.1 and Kv1.2 immunoreactivities were present in the BLA and some were colocalized with synaptophysin. Collectively, these data suggest that stimulation of presynaptic μ opioid receptors predominantly decrease GABA release onto CeA-projecting BLA neurons through activation of both Kv1.1 and Kv1.2 channels.

The amygdala is a region within the temporal lobe that is comprised of many subnuclei with reciprocal projections to and from cortical and subcortical regions (Sah et al. 2003; Swanson and Petrovich 1998). This diversity of connections suggests a role for the amygdala in affective, memory, sensation, and motor activity (Pitkanen et al. 1997; Sah et al. 2003; Swanson and Petrovich 1998). The modulation of pain in the amygdala mainly involves two specific subnuclei, the CeA and BLA. The role of the CeA in both pain and opioid analgesia is well established (Bernard and Besson 1990; Finnegan et al. 2005; Manning 1998). The BLA has been shown to play a primary role in the affective component of pain in a conditioned place aversion paradigm (Tanimoto et al. 2003). However, little is known if and from where the BLA receives nociceptive inputs. All three major opioid receptors (μ, δ, and κ) have been localized to the BLA (Ding et al. 1996; Paden et al. 1987). Although it has been shown that opioid peptides can be released endogenously in the BLA (Rocha et al. 1994; Vankova et al. 1996), activation of opioid receptors by a synaptically release agonist is not clear. Behavioral studies have shown that μ opioid receptor stimulation in the BLA can elicit opioid analgesia that requires both the PAG and RVM (Helmstetter et al. 1998; McGaraughty et al. 2004). Furthermore, the analgesia produced by μ opioids in the BLA may require the release of endogenous opioids and neurotensin within the PAG (Tershner and Helmstetter 2000).

We found that the IPSCs were GABAergic in nature because bicuculline completely eliminated IPSCs in all cells tested. The EPSCs were largely glutamatergic because CNQX abolished EPSCs in the majority of cells examined. It is likely that glutamatergic input to the BLA is derived from reciprocal
connections with the prefrontal cortex and local circuitry (McDonald et al. 1996; Smith and Dudek 1996). GABA is also highly concentrated in the BLA, originating from interneurons and regions such as the lateral amygdala (Carlsen 1988; Li et al. 2002; Rainnie et al. 1991; Washburn and Moises 1992). It has been shown that GABAergic neurons make synaptic contact with projection and nonprojection neurons within the BLA (Carlsen 1988; Li et al. 2002; Rainnie et al. 1991; Washburn and Moises 1992). Similar to our findings, it has been shown that IPSCs and EPSCs in the BLA are GABAergic and glutamatergic, respectively (Smith and Dudek 1996). The GABAergic tone influences the excitability of neurons in the BLA, because bicuculline stimulates burst neuronal firing in the BLA (Rainnie et al. 1991). Notably, CNQX-resistant EPSCs were encountered in some labeled BLA neurons in this study. Because the occurrence of CNQX-resistant EPSCs was rare and unpredictable, we did not attempt to identify the nature of CNQX-resistant EPSCs in our study. We have shown previously that some CNQX-resistant EPSCs are mediated by nicotinic receptors in the brain stem (Finnegan et al. 2004). It has been shown that cholinergic nicotinic receptors are present in the BLA (Barros et al. 2005; Blozovsky and Dumery 1987). Thus it is possible that some of the CNQX-resistant eEPSCs in the BLA are mediated by nicotinic receptors.

The mechanism through which μ opioid receptors in the BLA activate the PAG to produce antinociception is little known. The CeA, which receives input from the BLA and sends efferents to the PAG, is the most likely intermediary (Smith and Millhouse 1985; Swanson and Petrovich 1998). No information is available on the effect of μ opioid receptor stimulation on GABA or glutamate synaptic inputs to BLA neurons that project to the CeA. We found that stimulation of μ opioid receptors with DAMGO decreases evoked GABAergic IPSCs in CeA-projecting BLA neurons. However, DAMGO had little effect on glutamatergic EPSCs in most labeled BLA cells examined. The selective μ opioid antagonist, CTAP, blocked the effect of DAMGO on IPSCs. Furthermore, DAMGO increased the paired-pulse ratio of evoked IPSCs and decreased the frequency but not the amplitude of mIPSCs. These data strongly suggest that μ opioid receptors are located on the presynaptic GABAergic terminals in the BLA. The opioid receptor activity in the BLA may be mainly presynaptic caused by the lack of localization of μ opioid receptors on cell bodies (Ding et al. 1996). However, the existence of postsynaptic μ opioid receptors on BLA neurons cannot be excluded. It should be noted that the potential postsynaptic effect of the μ opioid receptor was blocked in this study by the addition of the GDP-β-S to the recording pipette. The selective effect of DAMGO on the GABAergic input to labeled BLA neurons suggests that disinhibition may play a significant role in increased activity of CeA-projecting BLA neurons.

Activation of μ opioid receptors inhibits adenylate cyclase, increases membrane potassium conductance, and reduces voltage-gated calcium channel currents (Loh and Smith 1990; Wu et al. 2004). Voltage-gated potassium (Kv) channels are important for the effect of μ opioids on presynaptic GABA release. In the PAG, application of both 4-AP or α-dendrotoxin blocks the effect of metenkephalin on GABA release (Vaughan et al. 1997). DAMGO or morphine also decreases spike frequency through activation of Kv1.2 in the lateral amygdala (Faber and Sah 2004). Similarly, activation of the adenosine A1 receptor reduces the presynaptic release of GABA in the spinal cord in a 4-AP-sensitive manner (Yang et al. 2004). We found that application of the nonspecific Kv channel blockers, 4-AP and α-dendrotoxin, completely blocked the effect of DAMGO on mIPSCs in CeA-projecting BLA neurons (Faber and Sah 2003, 2004). Bath perfusion of the selective Kv1 blockers, dendrotoxin-K (Kv1.1) and tityustoxin-Kα (Kv1.2), also abolished the effect of DAMGO. The role of both Kv1.1 and Kv1.2 in DAMGO-mediated attenuation of GABA release is further supported by immunocytochemistry evidence showing the presence of presynaptic Kv1.1 and Kv1.2 in the BLA. It has been shown that both Kv1.1 and Kv1.2 subunits can form heteromultimeric Kv channels in vivo (Scott et al. 1994; Wang et al. 1993). It is possible that Kv1.1 and Kv1.2 subunits in the BLA may form a complex together, and individually blocking either Kv1.1 or Kv1.2 could impair the Kv channel function. Our data strongly suggest that presynaptic Kv1.1/Kv1.2 channels are critically involved in the effect of the μ opioid on GABAergic tone of the BLA output neurons.

The μ opioid receptor may modulate Kv channels through stimulation of phospholipase A2 (PLA2). Activation of the μ opioid receptor increases the release of arachidonic acid in a PLA2-dependent manner (Fukuda et al. 1996). Metabolites of arachidonic acid, particularly those generated through the 12-lipoxygenase pathway (i.e., 12-HETE and hepopxlysin A3), may be responsible for activation of Kv channels (Vaughan et al. 1997). Similarly, blockade of PLA2 abolishes the effects of morphine on spike activity in the lateral amygdala (Faber and Sah 2004). Furthermore, application of arachidonic acid mimics the effect of morphine on spike activity, while pretreatment with α-dendrotoxin significantly attenuates the effect of arachidonic acid (Faber and Sah 2004). Thus it is possible that the inhibitory effect of DAMGO on presynaptic GABA release onto BLA-CeA neurons is through a PLA2-arachidonic acid-Kv pathway.

In summary, this study provides new functional evidence that stimulation of presynaptic μ opioid receptors primarily attenuates GABAergic inputs onto BLA neurons that project to the CeA. We also showed that the presynaptic effect of the μ opioid agonist on GABAergic IPSCs in the BLA is mediated by a downstream signaling mechanism involving Kv1.1 and Kv1.2 channels at presynaptic terminals in the BLA. Behavioral studies have shown that activation of μ opioid receptors in the BLA and CeA, as well as the PAG and RVM, elicits analgesia (Llewelyn et al. 1986; Manning 1998; McGaraughty and Heinricher 2002; Morgan et al. 1997; Rodgers 1977). Opioids also inhibit the presynaptic release of GABA in the CeA, PAG, and RVM (Finnegan et al. 2004, 2005; Vaughan and Christie 1997). Thus stimulation of μ opioid receptors in the BLA may attenuate nociception and the affective behavior associated with pain through disinhibition of CeA-projecting BLA neurons.

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