Effect of the \( \mu \) Opioid on Excitatory and Inhibitory Synaptic Inputs to Periaqueductal Gray-Projecting Neurons in the Amygdala

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ABSTRACT

Opioids are potent analgesics, but the sites of their action and cellular mechanisms are not fully understood. The central nucleus of the amygdala (CeA) is important for opioid analgesia through the projection to the periaqueductal gray (PAG). In this study, we examined the effects of \( \mu \) opioid receptor stimulation on inhibitory and excitatory synaptic inputs to PAG-projecting CeA neurons retrogradely labeled with a fluorescent tracer injected into the ventrolateral PAG of rats. Whole-cell voltage-clamp recordings were performed on labeled CeA neurons in brain slices. The specific \( \mu \) opioid receptor agonist, [D-Ala\(^2\),N-Me-Phe\(^4\),Gly\(^5\)-ol]-enkephalin (DAMGO, 1 \( \mu \)M), significantly reduced the frequency of miniature inhibitory postsynaptic currents (mIPSCs) without altering the amplitude and decay constant of mIPSCs in 47.6% (10 of 21) of cells tested. DAMGO also significantly decreased the peak amplitude of evoked IPSCs in 69% (9 of 13) of cells examined. However, DAMGO did not significantly alter the frequency of miniature excitatory postsynaptic currents (EPSCs) and the amplitude of evoked EPSCs in 69% (9 of 13) and 83% (10 of 12) of labeled cells, respectively. The IPSCs were blocked by the GABA\(_A\) receptor antagonist bicuculline, whereas the EPSCs were largely abolished by the non-\( N \)-methyl-\( d \)-aspartate antagonist 6-cyano-7-nitroquinocxaline-2,3-dione. The immunoreactivity of \( \mu \) opioid receptors was colocalized with synaptophysin, a presynaptic marker, in close appositions to labeled CeA neurons. These results suggest that activation of \( \mu \) opioid receptors on presynaptic terminals primarily attenuates GABA\(_A\)-ergic synaptic inputs to PAG-projecting neurons in the CeA.

The amygdala plays a central role in the emotional interpretation of sensory information, especially pain- and fear-related behavior (Pitkanen et al., 1997; LeDoux, 2000). The endogenous \( \mu \) opioids involved in the stress-induced analgesia are probably produced within the amygdala complex, especially the central nucleus (CeA) and stria terminalis (Roberts et al., 1982; da Costa Gomez and Behbehani, 1995; Wiedenmayer et al., 2002). The CeA is an important site for pain perception and analgesia produced by environmental stress and opioids. For example, the CeA receives afferent inputs from the spinal cord dorsal horn and parabrachial nucleus (Bernard et al., 1993; Burstyn and Potrebic, 1993). The spinopontoamygdaloid pathway has been shown to specifically transmit nociceptive information (Bernard and Benson, 1990; Bester et al., 2000). Also, this amygdaloid nucleus contains all major opioid receptors, including the \( \mu \) opioid receptor (Paden et al., 1987). Furthermore, lesioning the CeA largely eliminates the antinociceptive effect of systemic morphine in both tail-flick and formalin tests in rats (Manning and Mayer, 1995a,b; Manning, 1998). This effect may be produced through the projection from the CeA to periaqueductal gray (PAG) (Rizvi et al., 1991; da Costa Gomez and Behbehani, 1995). Thus, CeA neurons not only respond to noxious stimuli but also play an important role in opioid-produced analgesia.

Because the PAG receives abundant, highly organized projections from the CeA (Rizvi et al., 1991), the CeA may represent the rostral extension of a serial connection of opioid receptors.
oid-sensitive neurons extending caudally to the spinal cord. The μ opioid receptors in the basolateral amygdala (BLA) are also important for opioid analgesia. In this regard, microinjection of the μ opioid agonist, [D-Ala²,N-Me-Phe⁴,Gly⁸-ol]-enkephalin (DAMGO), into the BLA increases the tail-flick latency, an action mediated by the PAG and rostral ventromedial medulla (RVM) (Helmstetter et al., 1998; Tershner and Helmstetter, 2000). Importantly, although both the CeA and BLA send projections to the PAG, the primary input from the BLA to the PAG includes a critical synapse within the CeA (Rizvi et al., 1991). Thus, some of the analgesic actions of μ opioids are likely due to modulation of the descending inhibitory pathway involving the CeA. This pathway typically includes the PAG and its projection to the RVM, which in turn sends terminals to modulate nociceptive inputs at the level of the spinal dorsal horn (Basbaum and Fields, 1984; Manning, 1998). Activation of this pathway, by glutamate, opioids, or electrical stimulation, results in profound antinociception (Basbaum and Fields, 1984; McGowan and Hammond, 1993). Although the CeA is important for opioid analgesia (Manning and Mayer, 1995b; Manning, 1998), the cellular mechanisms underlying the opioid action in the CeA are little known.

Understanding how opioids affect synaptic inputs to CeA-PAG neurons is critical to clarify the opioid action. Specifically, the effect of μ opioids on synaptic transmission in the amygdala has not been studied previously. In the present study, using a combination of retrograde tracing and brain slice whole-cell recording techniques, we determined the effect of μ opioid receptor activation on excitatory and inhibitory synaptic inputs to CeA neurons that project to the PAG. Furthermore, immunofluorescent labeling was utilized to determine the possible presynaptic location of μ opioid receptors relative to labeled CeA-PAG neurons.

**Materials and Methods**

**Retrograde Labeling of PAG-Projecting CeA Neurons.** Male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing between 80 and 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-tetramethylindocarbocyanine perchlorate (DiI; 7 mg/100 μl; Molecular Probes, Eugene, OR) was performed to label PAG-projecting CeA neurons. The animals were initially anesthetized with halothane and then received an i.p. injection of a ketamine/xylazine mixture (90 and 5 mg/kg, respectively). A borosilicate micropipette (~30-μm tip diameter) was lowered to the ventrolateral PAG (vPiPAG; coordinates from lambda: anteroposterior, −1.0 mm; mediolateral, ±6.0 mm; and dorsoventral, −5.2 mm), and DiI was injected (40 nl/side, Nanoject II; Drummond Scientific, Broomall, PA) into both sides of the vPiPAG (Fig. 1A). After injection, the wounds were sutured, and rats were returned to their cage for at least 2 days to allow enough tracers to be transported to the CeA.

**Slice Preparation.** The rats were rapidly decapitated under halothane anesthesia 2 to 5 days after the 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 approximately 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 CeA (300-μm thickness) were cut from the tissue block in ice-cold, oxygenated aCSF. The slices were then incubated in oxygenated aCSF at 36°C for at least 1 h before being transferred into the recording chamber. The aCSF contained: 126 mM NaCl, 2.5 mM KCl, 1.3 mM MgSO₄, 2.4 mM CaCl₂, 1.2 mM NaH₂PO₄, 11.0 mM glucose, and 25.0 mM NaHCO₃ (pH 7.4 and osmolality 295–300 mOsm). After sacrificing the rat, the brain tissue containing the PAG was sectioned and viewed using a fluorescent microscope to verify the injection and diffusion site of the tracer. Rats with a misplaced injection site or one that extended beyond the vPiPAG were not included in the study.

**Recordings of Postsynaptic Currents and Firing Activity of CeA Neurons.** Recordings of miniature and evoked postsynaptic currents (EPSCs) and inhibitory postsynaptic currents (IPSCs) were performed using whole-cell voltage-clamp methods as previously described (Pan et al., 2002, 2004; Finnegan et al., 2004). The electrode for the whole-cell recordings was pulled with a puller (P-97; Sutter Instrument Company, Novato, CA) using borosilicate glass capillaries (o.d., 1.2 mm; i.d., 0.86 mm; World Precision Instruments, Inc., Sarasota, FL). The resistance of the pipette was about 2 MΩ when filled with an internal solution containing: 110 mM CsSO₄, 0.5 mM CaCl₂, 5.0 mM MgCl₂, 1.2-hydroxy-5-nitro-2-aminophenoxylethyl-N,N,N',N'-tetraacetico acid, 10.0 mM HEPES, 5.0 mM Na₂ATP, 0.33 mM GTP-Tris salt, 10.0 mM QX314, and 5.0 mM TEA-Cl (pH 7.2 and osmolality 280–290 mOsm). The G-protein inhibitor, GDP-β-S (1 mM), was added to the internal solution in the evoked protocol to eliminate the potential postsynaptic effect of opioids (Pan et al., 2002, 2004). Also, the internal pipette solution contained 0.2% biocytin (Sigma-Aldrich, St. Louis, MO) to label the recorded neuron for later use in the immunofluorescence labeling (Finnegan et al., 2004).

The brain slice was placed in a glass-bottom chamber (Warner Instrument, 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 chamber. The aCSF contained: 126 mM NaCl, 2.5 mM KCl, 1.3 mM MgSO₄, 2.4 mM CaCl₂, 1.2 mM NaH₂PO₄, 11.0 mM glucose, and 25.0 mM NaHCO₃ (pH 7.4 and osmolality 295–300 mOsm). After sacrifice, the rat, the brain tissue containing the PAG was sectioned and viewed using a fluorescent microscope to verify the injection and diffusion site of the tracer. Rats with a misplaced injection site or one that extended beyond the vPiPAG were not included in the study.

**Fig. 1.** Retrograde labeling of PAG-projecting CeA neurons and identification of labeled CeA neurons in brain slices. A, a representative photomicrograph showing the DiI injection site in the ventrolateral PAG in one rat. B, identification of the CeA and BLA regions in a brain slice viewed with differential interference contrast (DIC) optics. C, DiI-labeled CeA neuron (+) viewed with epifluorescence illumination. D, the same cell in C viewed with DIC optics.
monitor. After the labeled neuron was identified, positive pressure was applied to the pipette, which was then 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 Gi seal. The cell membrane was then 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. Miniature inhibitory postsynaptic currents (mIPSCs) and miniature excitatory postsynaptic currents (mEPSCs) were recorded at a holding potential of 0 and −70 mV, respectively (Pan et al., 2002; Finnegang et al., 2004). All mIPSCs were recorded in the presence of tetrodotoxin (TTX; 1 μM) and 6-cyano-7-nitroquinolin-2-3-dione (CNQX; 20 μM). The mEPSCs were recorded in the presence of 1 μM TTX and 20 μM bicuculline. The evoked postsynaptic currents in the labeled CeA 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, Quincy, MA). The tip of the stimulating electrode was placed directly in the center 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 (Pan et al., 2002; Finnegang et al., 2004). Currents were measured using an Axopatch 200B amplifier (Axon Instruments Inc., Union City, CA). Signals were filtered at 1 to 2 kHz, digitized at 10 kHz (DigiData 1320A; Axon Instruments Inc.), and recorded into a Pentium computer using the pClamp 8.01 program.

The firing activity of labeled CeA neurons was recorded using the whole-cell current-clamp technique (Pan et al., 2002, 2004). The recording procedures were similar to those used for postsynaptic current recordings as described above except that TTX and QX-314 were not used. Because labeled CeA neurons showed no spontaneous discharges, action potentials were elicited by injection of a series of depolarizing currents (from 0–600 pA in 30- to 50-pA increments, 300-ms duration). Recordings of the evoked firing activity of labeled CeA neurons began about 5 min after the whole-cell access was established and the elicited firing rate was reproducible. Signals were processed, recorded, and analyzed as described above.

**Experimental Protocols**. The resting membrane potential and the input resistance were continuously monitored throughout the recording period. Recordings were abandoned if the input resistance changed more than 15% (Finnegang et al., 2004; Pan et al., 2004). To determine the effect of DAMGO on the firing rate, mIPSCs, and mEPSCs in labeled CeA neurons, 1 μM DAMGO was perfused into the slice for up to 2 min after recording the mIPSCs and mEPSCs 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 opioid antagonist, H-D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH2 (CTAP; 1 μM) (Vaughan and Christie, 1997; Finnegang et al., 2004). The effects of 1 μM DAMGO on eIPSCs and eEPSCs were also measured. CNQX and bicuculline were obtained from Sigma-Aldrich. DAMGO and CTAP were purchased from Bachem Biosciences (King of Prussia, PA), whereas TTX was obtained from Alomone Labs (Jerusalem, Israel).

**Immunocytochemical Labeling of μ Opioid Receptors and Synaptophysin in Recorded CeA Neurons**. After recording, the tissue slice was fixed in 4% paraformaldehyde in PBS (pH 7.4) and kept at 4°C for 7 to 10 days. The tissue was then placed in cryoprotectant solution at −20°C until the immunolabeling experiment. The tissues were cut to 35 μm and were free-floating in 0.1 M PBS. For μ opioid receptor and synaptophysin (a presynaptic marker) double immunofluorescence labeling, the first primary antibody (anti-synaptophysin) was labeled conventionally using an immunofluorescent-conjugated secondary antibody, whereas the labeling by the second primary antibody (anti-μ receptor) was enhanced with tyramide signal amplification in conjunction with the specific secondary antibody. Briefly, the sections were rinsed in 0.1 M PBS, blocked with 4.0% normal goat serum and 0.3% Triton X-100. The sections were then incubated with mouse anti-synaptophysin primary antibody (dilution, 1:200; Chemicon International, Temecula, CA) for 2 h at room temperature and 48 h at 4°C. Subsequently, sections were rinsed in 0.1 M PBS and incubated with goat anti-mouse secondary antibody conjugated to Alexa Fluor 594 (1:400; Jackson Immunoresearch Laboratories Inc., West Grove, PA) for 1.5 h at room temperature. The sections were rinsed in Tris-buffered saline (0.1 M Tris-HCl and 0.15 M NaCl, pH 7.8), and the peroxidase activity was quenched with 1% H2O2. The second primary antibody, rabbit anti-μ receptor (1:2000; Neuronics, Northfield, MN), was then applied for 2 h at room temperature and 24 h at 4°C. Sections were rinsed in Tris-buffered saline and 0.05% Tween 20 and exposed to the horse-radish peroxidase-conjugated goat anti-rabbit secondary antibody (1:100; Jackson Immunoresearch Laboratories Inc.) for 90 min at room temperature. After wash, sections were incubated in fluorescein isothiocyanate-typsidime (1:100; PerkinElmer Life and Analytical Sciences, Boston, MA) for 10 min at room temperature. Finally, to identify biocytin-labeled cells, the sections were incubated with streptavidin-conjugated Alexa Fluor-350 (dilution, 5 μg/ml; Molecular Probes) for 1.5 h at room temperature. After labeling, the sections were mounted on slides, dried, and coverslipped. All sections were examined on a confocal scanning microscope (Leica, Wetzlar, Germany), and areas of interest were photodocumented and digitally merged. In the higher magnification photographs, the colocalization was indicated by the color change and represents colocalization because the optical section thickness (0.3 μm) of a confocal image is thin enough to minimize the possibility of superposition of stained neurons (Pan et al., 2002, 2004; Finnegang et al., 2004).

**Data Analysis**. Data are presented as means ± S.E.M. The mIPSCs, mEPSCs, and firing activity of labeled CeA neurons were analyzed off-line with a peak detection program (MiniAnalysis; Synaptosoft, Decatur, GA). The cumulative probability of the amplitude and interevent interval of mIPSCs and mEPSCs was compared using the Komoroger-Smirnov test, which estimates the probability that two distributions are similar. Analyses of the effect of drugs on the amplitude of eIPSCs and eEPSCs were performed using Clampfit (Axon Instruments Inc.). The effect of drug treatment on the postsynaptic currents and firing rate was determined by either a paired Student’s 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 mIPSCs in Labeled CeA Neurons**. In the presence of TTX and CNQX, bath application of 1 μM DAMGO significantly reduced the frequency of mIPSCs in 10 of 21 (47.6%) labeled CeA neurons examined (from 3.65 ± 0.61 to 1.92 ± 0.32 Hz; P < 0.05; Fig. 2, A–C). DAMGO did not significantly change the amplitude of mIPSCs in these cells (46.56 ± 2.28 versus 43.95 ± 2.24 pA; Fig. 2C). The decay phase of mIPSCs in these cells was best fitted to a double exponential function. DAMGO did not significantly change the fast (11.73 ± 1.29 versus 9.23 ± 1.39 ms) and the slow (30.12 ± 2.30 versus 31.40 ± 5.07 ms) components of the decay time constant of mIPSCs. The frequency of mIPSCs in these cells returned to near baseline values after approximately 6 to 10 min of washout (3.34 ± 0.41 Hz; Fig. 1, A and C). The cumulative probability analysis of mIPSCs revealed that the interevent interval distribution shifted to the right after DAMGO (Fig. 2B). However, DAMGO did not change the amplitude of mIPSCs in these 10 cells (Fig. 2B).

In another eight (38.0%) cells, DAMGO had no significant effect on the mIPSC frequency (from 2.81 ± 0.41 to 2.61 ± 0.40 Hz) and amplitude (56.14 ± 3.03 versus 53.49 ± 4.65 pA; Fig. 2D). In the remaining three labeled (14.3%) CeA neurons, bath application of DAMGO increased the mIPSC fre-
In 13 of 15 (86%) neurons tested above, 20 µM bicuculline completely abolished the mIPSCs (Fig. 2A). In one of the two remaining cells, bicuculline reduced the mIPSC frequency by 82% (from 3.37 to 0.63 Hz). In the remaining neuron, bicuculline alone reduced IPSCs by 46% (from 4.44 to 2.14 Hz). Subsequent application of 5 µM strychnine, a glycine receptor antagonist, completely abolished mIPSCs.

In a different population of labeled CeA neurons, the specificity of DAMGO was examined. In a total of seven neurons, the µ receptor antagonist, CTAP, completely blocked the effects of DAMGO on mIPSCs (Fig. 4). DAMGO significantly decreased the mIPSC frequency in all of the seven cells (from 2.75 ± 0.76 to 1.85 ± 0.60 Hz; P < 0.05; Fig. 4B). Bath application of 1 µM CTAP alone had no significant effect on the mIPSC frequency. Subsequent application of 1 µM DAMGO in the presence of CTAP failed to significantly change the frequency of mIPSCs (2.13 ± 0.70 versus 2.35 ± 0.76 Hz; Fig. 4B).

Effect of DAMGO on mEPSCs in Labeled CeA Neurons. The effect of 1 µM DAMGO on mEPSCs was examined in the presence of TTX and bicuculline in a total of 13 labeled CeA neurons. Bath application of DAMGO did not significantly change the mEPSC frequency (2.35 ± 0.55 versus 2.25 ± 0.47 Hz) and amplitude (38.98 ± 1.93 versus 39.04 ± 1.48 pA, Fig. 5D) in 9 of 13 (69%) labeled CeA neurons. In the remaining neuron, DAMGO caused a large increase in the frequency of mIPSCs (from 2.90 ± 0.47 to 4.24 ± 0.98 Hz) and the amplitude (66.06 ± 8.19 versus 78.43 ± 11.92 pA; Fig. 3). This potentiating effect was also washed out (2.80 ± 0.07 Hz) 6 to 10 min after DAMGO application was discontinued.
remaining 4 of 13 (31%) labeled cells, DAMGO significantly decreased the frequency (from 2.14 ± 0.16 to 1.10 ± 0.13 Hz; n = 4) but not amplitude (35.53 ± 1.42 versus 36.35 ± 1.37 pA; Fig. 5, A–C) of mEPSCs. The cumulative probability analysis of mEPSCs in the four DAMGO-responsive cells indicated that the distribution pattern of the interevent interval shifted to the right after treatment (Fig. 5B). DAMGO did not change the amplitude of mEPSCs in all the cells tested (Fig. 5, C and D).

In nine labeled CeA neurons tested above, 20 μM CNQX completely eliminated mEPSCs in six cells. In two of these nine cells, CNQX reduced the mEPSC frequency by 80% (1.26–0.25 Hz) in one labeled neuron and 88% (1.73–0.22 Hz) in another cell. In the remaining cell, CNQX reduced the frequency of mEPSCs by 45% (2.12–0.97 Hz). Subsequent application of 10 μM mecamylamine (a nicotinic receptor antagonist) in the presence of CNQX caused an additional 24% reduction (0.97–0.73 Hz) in the mEPSC frequency of this cell.

**Effect of DAMGO on eIPSCs in Labeled CeA Neurons.** The evoked IPSCs were elicited by placing a stimulating electrode approximately in the center of the BLA. This was done to determine the effect of μ receptor activation on afferent terminals originating from the BLA. Previous studies have shown that the BLA neurons project to the CeA (Smith and Millhouse, 1985), and this connection may be required for opioid analgesia initiated from the BLA (Tershner and Helmstetter, 2000). The effect of 1 μM DAMGO on eIPSC amplitude was examined in 13 labeled CeA neurons. In 9 of 13 (69%) cells, the eIPSC amplitude was significantly decreased by DAMGO (from 302.88 ± 33.64 to 146.44 ± 22.53 pA; P < 0.05, Fig. 6). This effect was washed out 6 to 10 min after discontinuing DAMGO application. In the remaining 4 (31%) cells, DAMGO had no significant effect (272.75 ± 63.0 versus 292.0 ± 78.0 pA; Fig. 6C) on the eIPSC amplitude.

To determine whether the GABA<sub>A</sub> receptor mediates eIPSCs, 11 of the above 13 neurons were tested with 20 μM bicuculline. The eIPSCs of all 11 cells were completely abolished by 20 μM bicuculline (Fig. 6A).

**Effect of DAMGO on eEPSCs in Labeled CeA Neurons.** The effect of 1 μM DAMGO on eEPSCs was examined in a total of 12 labeled CeA neurons. In 10 of 12 cells, DAMGO did not change significantly the eEPSC amplitude (288.70 ± 41.58 versus 267.90 ± 34.84 pA, Fig. 7, A and B). In the remaining two labeled neurons, DAMGO decreased eEPSC amplitude by 36% (from 156 to 101 pA) in one cell and 34% (from 220 to 146 pA) in another cell.

**Fig. 5.** Effect of DAMGO on mEPSCs in labeled CeA neurons. A, representative tracings showing mEPSCs during control, 1 μM DAMGO, washout, and 20 μM CNQX in a labeled RVM neuron. B, cumulative probability plots showing the distribution of interevent interval and the peak amplitude of the same neuron in A during control and DAMGO application. C, summary data showing DAMGO application decreased the frequency but not amplitude of mEPSCs in four labeled CeA cells. D, summary data showing the lack of effect of 1 μM DAMGO on the frequency and amplitude of mIPSCs in another nine labeled CeA neurons. Data presented as means ± S.E.M. *, P < 0.05 compared with control.

**Fig. 6.** Effect of 1 μM DAMGO on electrically evoked IPSCs in labeled CeA neurons. A, original tracings of evoked IPSCs in a labeled CeA neuron during control, 1 μM DAMGO, washout, and 20 μM bicuculline. B, summary data showing the inhibitory effect of DAMGO on the evoked IPSC amplitude in nine labeled CeA neurons. C, summary data showing lack of effect of 1 μM DAMGO on evoked IPSCs in another four labeled cells. Data presented as means ± S.E.M. *, P < 0.05 compared with control.
The effect of 20 μM CNQX on eEPSCs was tested in 11 of the above 12 neurons. In 10 of these cells, CNQX completely abolished the eEPSCs (Fig. 7A). In the remaining cell, CNQX reduced the eEPSC amplitude by 85% (400.0 versus 60.0 pA). DAMGO decreased the evoked firing rate from 17.9 ± 2.4 Hz to 6.4 Hz. In the remaining cell, DAMGO significantly attenuated the mIPSCs and eIPSCs (48 and 69%, respectively). The IPSCs were largely GABAergic in nature because the GABA_A receptor antagonist, bicuculline, almost abolished the IPSCs in most cells tested. On the other hand, DAMGO did not change mEPSCs or eEPSCs (69 and 83%, respectively) in the majority of cells tested. The EPSCs recorded in the CeA were glutamatergic since blockade of non-NMDA glutamate receptors completely abolished EPSCs in most cells. Furthermore, we found that the μ opioid receptor immunoreactivity was colocalized with the presynaptic marker synaptophysin in the CeA. Collectively, this study provides important new information that the presynaptic marker synaptophysin in the CeA. This is the first study examining the effect of μ opioid receptor stimulation on inhibitory and excitatory synaptic inputs to CeA neurons that project to the PAG. We found that in most labeled CeA neurons, the μ opioid receptor agonist, DAMGO, significantly attenuated the mIPSCs and eIPSCs (48 and 69%, respectively). The IPSCs were largely GABAergic in nature because the GABA_A receptor antagonist, bicuculline, almost abolished the IPSCs in most cells tested. On the other hand, DAMGO did not change mEPSCs or eEPSCs (69 and 83%, respectively) in the majority of cells tested. The EPSCs recorded in the CeA were glutamatergic since blockade of non-NMDA glutamate receptors completely abolished EPSCs in most cells. Furthermore, we found that the μ opioid receptor immunoreactivity was colocalized with the presynaptic marker synaptophysin in the CeA. Collectively, this study provides important new information that the presynaptic effect of the μ opioids is complex in the CeA, and activation of presynaptic μ opioid receptors decreases the GABAergic input to the majority of CeA neurons that project to the PAG.

Discussion

This is the first study examining the effect of μ opioid receptor stimulation on inhibitory and excitatory synaptic inputs to CeA neurons that project to the PAG. We found that in most labeled CeA neurons, the μ opioid receptor agonist, DAMGO, significantly attenuated the mIPSCs and eIPSCs (48 and 69%, respectively). The IPSCs were largely GABAergic in nature because the GABA_A receptor antagonist, bicuculline, almost abolished the IPSCs in most cells tested. On the other hand, DAMGO did not change mEPSCs or eEPSCs (69 and 83%, respectively) in the majority of cells tested. The EPSCs recorded in the CeA were glutamatergic since blockade of non-NMDA glutamate receptors completely abolished EPSCs in most cells. Furthermore, we found that the μ opioid receptor immunoreactivity was colocalized with the presynaptic marker synaptophysin in the CeA. Collectively, this study provides important new information that the presynaptic effect of the μ opioids is complex in the CeA, and activation of presynaptic μ opioid receptors decreases the GABAergic input to the majority of CeA neurons that project to the PAG.
The amygdala is a structurally and functionally heterogeneous region of the cerebral hemispheres. It is best known as a crucial center for fear and anxiety and is a component of the descending pathway that modulates nociception. The CeA is the major amygdaloid nucleus receiving nociceptive information, especially through the spinopontamygdaloid pathway (Bernard and Besson, 1990; Bester et al., 2000). This ascending nociceptive pathway is inhibited by \( \mu \) opioid receptors (Huang et al., 1993b). Although \( \delta \) and \( \kappa \) opioid receptors are also located in the CeA (Paden et al., 1987), their roles in opioid analgesia have not been studied previously. Furthermore, although there are more \( \mu \) opioid receptors in the BLA than CeA (Paden et al., 1987; Ding et al., 1996), lesions of the CeA but not BLA eliminate the analgesic effect of systemic morphine in both tail-flick and formalin tests in rats (Manning and Mayer, 1995a,b; Manning, 1998). Thus, the CeA is a region that plays an important role in opioid analgesia, an action likely mediated through its projection to the PAG (Rizvi et al., 1991; da Costa Gomez and Behbehani, 1995; Manning and Mayer, 1995b; Manning, 1998). However, the cellular mechanisms responsible for the opioid effect in the CeA are not known. Since the CeA contains interneurons and many types of outputs neurons, we used a retrograde tracer to identify and study CeA neurons projecting to the PAG in this study.

The exact mechanism through which activation of the amygdala by opioids results in modulation of the PAG neurons is unknown. It has been established that GABA is highly concentrated in the CeA (Nitecka and Ben-Ari, 1987). It is probable that CeA projection neurons are tonically inhibited by GABAergic inputs. In most of the cells examined, DAMGO significantly reduced the frequency of mIPSCs and the amplitude of eIPSCs but had little effect on EPSCs. The specific \( \mu \) opioid receptor antagonist CTAP completely blocked the inhibitory effect of DAMGO on GABAergic IPSCs. Furthermore, the immunocytochemical labeling experiments provide clear complementary evidence that \( \mu \) opioid receptors are located presynaptically to labeled CeA neurons. Thus, these data strongly suggest that activation of \( \mu \) opioid receptors attenuates synaptic GABA release onto most PAG-projecting CeA neurons. If the GABAergic inputs to the CeA were reduced by \( \mu \) opioid receptor stimulation, it would result in disinhibition of CeA-PAG neurons, thereby activating the descending inhibitory pathway. We attempted to determine the impact of GABAergic tone on the firing activity of labeled CeA neurons in this slice preparation. However, none of the labeled CeA neurons in the slice showed spontaneous discharges. Also, both DAMGO and bicuculline failed to elicit spontaneous firing of these CeA neurons. Furthermore, we observed that DAMGO or bicuculline did not significantly alter the firing frequency evoked by current injection in most labeled CeA neurons. These data suggest that in the thin brain slice preparation, the firing activity of CeA-PAG output neurons is not tonically controlled by the GABAergic input. Because most major tonic afferent (including GABAergic) activity had been removed during slice preparation, we were unable to further define the impact of GABAergic tone and the effect of DAMGO on the firing activity of CeA-PAG neurons using this in vitro thin slice preparation. It has been shown that disinhibition is the mechanism responsible for the action of opioids in the PAG and locus coeruleus (Vaughan and Christie, 1997; Pan et al., 2002, 2004). The present study extends these findings and supports the notion that \( \mu \) opioid receptors primarily decrease GABAergic synaptic inputs to neurons that project to the PAG.

We observed that the frequency of mIPSCs (~3 Hz) was higher than that of mEPSCs (~2 Hz), suggesting that there may be a larger GABAergic inhibitory tone than glutamatergic excitatory tone on CeA-PAG neurons. The precise sources of glutamatergic and GABAergic inputs to the CeA output neurons are not fully known. There are multiple sources of glutamatergic inputs to the CeA. Some glutamatergic inputs may arise from regions such as the frontal cortex (Carter, 1982). The parabrachial nucleus also sends glutamatergic projections to the CeA, which may be important for transmission of nociceptive information to the CeA (Takayama and Miura, 1992; Huang et al., 1993a,b). Although local interneurons release GABA, the intercalated amygdaloid nucleus (IAN) may play a more important role in the GABAergic tone in the CeA. For instance, the IAN contains a dense population of GABA-immunoreactive cell bodies that project to the CeA (Nitecka and Ben-Ari, 1987; Pare and Smith, 1993). Glutamatergic afferents from the BLA could activate GABAergic neurons in the IAN, which in turn, project to the CeA (Royer et al., 1999). We found that electrical stimulation of the BLA consistently evoked IPSCs in all labeled CeA neurons. Thus, the BLA is another important source of GABAergic inputs to CeA-PAG neurons. It should be noted that \( \mu \) opioid receptor activation has complex and diverse actions even in the labeled CeA neurons. We observed that DAMGO had no significant effect on GABAergic IPSCs in a small percentage of cells. The reasons for the different effects of DAMGO on labeled CeA neurons remain unclear. The CeA receives a diverse number of afferent inputs, ranging from the cerebral cortex, other amygdaloid nuclei, and negative feedback loops originating in the brainstem (Swanson and Petrovich, 1998). It is possible that different effects of DAMGO may depend on the presence of \( \mu \) receptors on GABAergic afferent terminals and their coupling mechanisms. The diverse effects of DAMGO on GABAergic IPSCs have been shown in projection neurons in the locus coeruleus and rostral ventrolateral medulla (Finnegan et al., 2004; Pan et al., 2004). Based on our observation that DAMGO significantly reduced the IPSCs evoked from the BLA in most cells, it appears that in the CeA, most afferent terminals from the BLA are endowed with functional \( \mu \) opioid receptors.

One unexpected finding in our study is that in a few labeled CeA cells, DAMGO increased the frequency of mIPSCs. This suggests that stimulating presynaptic \( \mu \) receptors may cause an increase in the synaptic GABA release onto a few PAG-projecting CeA neurons. Although it is not clear how stimulation of \( \mu \) opioid receptors increases synaptic GABA release, it is possible that DAMGO indirectly potentiates synaptic GABA release through neurotensin. In this regard, it has been shown that morphine increases neurotensin levels in the PAG (Stiller et al., 1997). Also, microdialysis studies have shown that neurotensin can increase extracellular GABA in various brain regions (Tanganelli et al., 1994; Ferraro et al., 1998; Koyama et al., 2002). The functional significance of this interaction is further supported by a behavioral study showing that neurotensin in the PAG is partially involved in analgesia produced by opioids injected into the amygdala (Tershner and Helmstetter, 2000). In humans and rodents, the amygdala has a dense distribution of both the neurotensin peptide and receptors (Tay et al., 1989; Benzing...
et al., 1992; Lantos et al., 1996). This peptide is localized at the soma, axons, and dendrites of neurons in the CeA and has been found in neurons that project to the PAG (Tay et al., 1989). Therefore, it is likely that DAMGO indirectly causes an increase in neurotransmitter release, which in turn augments synaptic GABA release in the CeA. Because DAMGO also increased the amplitude of mIPSCs, it is possible that the postsynaptic sensitivity to GABA may be increased by DAMGO in a few labeled CeA neurons. Nevertheless, the functional implications of this effect in the opioid analgesia are unclear and warrant further studies. The responses of CeA output neurons depend on the types and origin of afferent inputs (Royer et al., 1999). It has been shown that electrical and chemical stimulation of the CeA both activate and inhibit PAG neurons in anesthetized rats (da Costa Gomez and Behbehani, 1995). The CeA contains a wealth of neurotransmitters including neurotensin and enkephalin (Casassell et al., 1986; da Costa Gomez and Behbehani, 1995). Also, different CeA neurons may project to distinct types of neurons in the PAG (interneurons versus output neurons) (da Costa Gomez and Behbehani, 1995). Further studies are needed to determine what the phenotypes of the CeA-PAG neurons are and how these diverse actions on synaptic inputs in the CeA are integrated to produce opioid analgesia.

In summary, we found that activation of presynaptic μ opioid receptor in the CeA produced a preferential decrease in the GABAergic input to neurons that project to the vPAG. On the other hand, DAMGO had little effect on glutamatergic input in the majority of CeA cells. Such a finding is consistent with the hypothesis that μ opioid receptor agonists may disinhibit CeA neurons that project to the vPAG. These data suggest that stimulation of opioid receptors in the amygdala could result in excitation of CeA-PAG projection neurons, thereby activating the descending inhibitory circuitry to produce analgesia. The opioid actions in the CeA may also contribute in part to the inhibitory effect of opioids on the emotional affective aspects of pain.

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