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Actions of Cannabinoids on Membrane Properties and Synaptic Transmission in Rat Periaqueductal Gray Neurons In Vitro

CHRISTOPHER W. VAUGHAN, MARK CONNOR, ELENA E. BAGLEY, and MACDONALD J. CHRISTIE

Department of Pharmacology (C.W.V., M.C., E.E.B., M.J.C.) and the Medical Foundation (M.J.C.), University of Sydney, Sydney, New South Wales, Australia

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ABSTRACT

The midbrain periaqueductal gray (PAG) is a major site of cannabinoid-mediated analgesia in the central nervous system. In the present study, we examined the actions of cannabinoids on rat PAG neurons in vitro. In brain slices, superfusion of the cannabinoid receptor agonist WIN55,212-2 inhibited electrically evoked inhibitory and excitatory postsynaptic currents in all PAG neurons. The endogenous cannabinoid anandamide inhibited evoked inhibitory postsynaptic currents in the presence of the anandamide transport inhibitor AM404, but not in its absence. The stable anandamide analog R1-methanandamide also inhibited evoked inhibitory postsynaptic currents. WIN55,212-2 reduced the rate of spontaneous miniature inhibitory postsynaptic currents in normal and Ca^{2+}-free solutions, but had no effect on their amplitude distributions or kinetics. The WIN55,212-2-induced decrease in miniature inhibitory postsynaptic current rate was concentration dependent (EC_{50} = 520 nM). The effects of cannabinoids were reversed by the CB_1 receptor antagonist SR141716. WIN55,212-2 produced no change in membrane current or conductance in PAG neurons in brain slices and had no effect on Ca^{2+}-channel currents in acutely isolated PAG neurons. These findings suggest that cannabinoids act via CB_1 receptors to inhibit GABAergic and glutamatergic synaptic transmission in rat PAG, although the efficacy of endogenous cannabinoids is likely to be limited by uptake and breakdown. Like μ-opioids, cannabinoids act to reduce the probability of transmitter release from presynaptic terminals via a Ca^{2+}-independent mechanism. In contrast to μ-opioids, cannabinoids have no direct postsynaptic actions on PAG neurons. Thus, cannabinoids and μ-opioids are likely to produce analgesia within PAG in part by different mechanisms.

The active constituent of *Cannabis sativa*, Δ^9-tetrahydrocannabinol (Δ^9-THC) and a number of synthetic cannabinoid ligands produce pharmacological effects with potential therapeutic applications in the treatment of pain, nausea, muscle spasticity, and glaucoma (Howlett, 1995). Central and systemic administration of Δ^9-THC and synthetic cannabinoid agonists produces antinociception (Howlett, 1995), and synergistically enhances the analgesic actions of opioids (Smith et al., 1998). The identification of a cannabinoid CB_1 receptor and endogenous cannabinoid ligands, anandamide, and 2-arachidonoylglycerol in the brain (Devane et al., 1988, 1992; Matsuda et al., 1990; Mechoulam et al., 1995; Sugiura et al., 1995) suggests that endogenous cannabinoid transmitters might also have a role in the control of pain within the central nervous system (Smith et al., 1994; Adams et al., 1998; Walker et al., 1999).

The cannabinoid CB_1 receptor is present in a number of brain regions, including the midbrain periaqueductal gray (PAG) and the rostral ventromedial medulla (RVM) (Herkenham et al., 1991; Matsuda et al., 1993; Tsou et al., 1998), which play a critical role in the antinociceptive actions of opioids and cannabinoids (Fields et al., 1991; Martin et al., 1998; Meng et al., 1998). The PAG forms part of a descending antinociceptive pathway that, via the RVM, modulates nociceptive transmission at the level of the spinal cord (Fields et al., 1991). Microinjections of cannabinoid agonists into both the PAG and RVM produce analgesia (Lichtman et al., 1996; Martin et al., 1998; Meng et al., 1998).

It has been hypothesized that μ-opioids produce analgesia within the PAG and RVM by disinhibiting descending antinociceptive neurons within these brain regions (Fields et al., 1991). Disinhibition by μ-opioids occurs by two distinct cellular mechanisms within the PAG and RVM (Pan et al., 1990; Osborne et al., 1996; Vaughan and Christie, 1997). First, μ-opioids directly inhibit presumptive GABAergic interneurons and, secondly, presynaptically inhibit transmit-

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ABBREVIATIONS: Δ^9-THC, Δ^9-tetrahydrocannabinol; ACSF, artificial cerebrospinal fluid; eEPSC, electrically evoked excitatory postsynaptic current; eIPSC, electrically evoked inhibitory postsynaptic current; PAG, periaqueductal gray; mIPSC, spontaneous miniature IPSC; RVM, rostral ventromedial medulla; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; TTX, tetrodotoxin; I_{Ba}, calcium channel current.

ter release from the terminals of GABAergic neurons. It has recently been suggested that cannabinoid agonists also produce analgesia within the RVM by disinhibition (Meng et al., 1998); however, the cellular mechanisms of cannabinoid and opioid disinhibition in RVM differ (Vaughan et al., 1999). The cellular actions of cannabinoids within the PAG are unknown. The present study examined the somatic and synaptic actions of cannabinoid agonists and endogenous cannabinoids on PAG neurons using recordings from brain slices and acutely isolated cells.

Materials and Methods

Sprague-Dawley rats (16–40 days old) were anesthetized with halothane, decapitated, and horizontal midbrain PAG slices (250–300 μm) were cut in ice-cold artificial cerebrospinal fluid (ACSF), as described previously (Vaughan and Christie, 1997). The slices were maintained at 34°C in a submerged chamber containing ACSF equilibrated with 95% O_2 and 5% CO_2. For experiments on synaptic currents and postsynaptic K^+ currents, the slices were then transferred to a chamber and superfused continuously (2 ml/min) with ACSF (32°C) of composition: 126 mM NaCl; 2.5 mM KCl; 1.4 mM NaH_2PO_4; 1.2 mM $MgCl_2$; 2.4 mM $CaCl_2$; 11 mM glucose; 25 mM $NaHCO_3$. PAG neurons were visualized on an upright microscope (Olympus BH-2 with fixed stage modification; Olympus, New Hyde Park, NY) using infrared Nomarski optics. Whole-cell voltage-clamp recordings (Axopatch 1D; Axon Instruments, Foster City, CA) of synaptic currents (holding potential, −74 mV) were made using a CsCl-based internal solution of composition: 140 mM CsCl; 10 mM EGTA; 5 mM HEPES; 2 mM $CaCl_2$; and 2 mM MgATP (pH 7.3, osmolarity, 270–290 mOsmol/l^{-1}). Perforated patch-clamp recordings of postsynaptic K^+ currents (holding potential, −60 mV) were performed using a K^+-acetate-based internal solution of composition: 120 mM potassium acetate; 40 mM HEPES; 10 mM EGTA; 5 mM $MgCl_2$; containing 0.25 mg/ml Pluronic F-127 and 0.12 mg/ml amphotericin B. Series resistance (<15 mΩ for whole cell and <40 mΩ for perforated patch) was compensated by 80% and continuously monitored during experiments. Liquid junction potentials of −12 mV for K^+-acetate and −4 mV for CsCl-based internal solutions were corrected.

Electrically evoked inhibitory and excitatory postsynaptic currents (eIPSCs and eEPSCs) were elicited in the presence of 3 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 30 μM bicuculline, respectively, via bipolar tungsten-stimulating electrodes placed 200 to 600 μm from the recording electrode (rate, 0.05–0.067 Hz; stimuli, 5–70 V, 20–400 μs). Spontaneous miniature IPSCs (mIPSCs) were obtained in the presence of 0.3 μM tetrodotoxin (TTX) and 3 μM CNQX and recorded on video tape (via a Sony PCM501; Sony, Tokyo, Japan). IPSCs and EPSCs were filtered (1, 2 kHz low-pass filter) and sampled at 5, 10 kHz for on-line and later off-line analysis (Axograph 4.0, Axon), respectively. Miniature IPSCs above a preset threshold (4–6 S.D. above baseline noise) were automatically detected by a sliding template algorithm, then manually checked off-line. The mIPSCs were counted in 10- to 30-s epochs to construct time plots of event rate, and probability density functions of their amplitudes were constructed (bin width, 10–20 pA).

For experiments on postsynaptic Ca^{2+} currents, cells were dissociated as described previously (Connor and Christie, 1998). Slices were transferred to a dissociation buffer of composition: 82 mM Na_2SO_4; 30 mM K_2SO_4; 10 mM HEPES; 5 mM $MgCl_2$; 10 mM glucose; containing 20 U/ml papain, pH 7.3, and incubated for 2 min at 35°C. The slices were then placed in fresh dissociation buffer containing 1 mg/ml BSA and 1 mg/ml trypsin inhibitor, and the PAG region was subdissected from each slice with a fine tungsten wire. Cells were dissociated from the slices by gentle trituration, plated onto plastic culture dishes, and kept at room temperature in dissociation buffer.

Whole-cell patch-clamp recordings of currents through Ca^{2+} channels were made at room temperature (22–24°C) (Connor and Christie, 1998). Immediately before recording, dishes of cells were superfused with a buffer of composition: 140 mM NaCl; 2.5 mM KCl; 2.5 mM $CaCl_2$; 1.5 mM $MgCl_2$; 10 mM HEPES; 10 mM glucose; pH 7.3, to wash off the dissociation buffer. For calcium channel current (I_{Ba}) recordings, cells were perfused in solution containing: 140 mM tetraethylammonium chloride; 4 mM $BaCl_2$; 2.5 mM CsCl; 10 mM HEPES; 10 mM glucose; pH 7.3. Whole-cell patch recordings were made with an intracellular solution containing: 130 mM CsCl; 5 mM MgATP; 0.2 mM Na_2GTP; 10 mM EGTA; 2 mM $CaCl_2$; and 10 mM HEPES; pH 7.3. Series resistance (~4 mΩ) was compensated by 80% and continuously monitored during experiments. Leak current was subtracted on-line using a P/8 protocol; typically the leak conductance was of the order of 100 pS. I_{Ba} evoked by stepping the membrane potential from a holding potential of −90 mV were filtered at 2 kHz and sampled at 5 to 10 kHz for later analysis (PCLAMP, Axograph 3.5; Axon Instruments). Cells were exposed to drugs via a series of flow pipes positioned above the cells. The inhibition by drugs was quantified by measuring the current amplitude isochronically with the peak of the control I_{Ba}.

Stock solutions of all drugs were diluted to working concentrations using ACSF immediately before use and applied by superfusion. Stock solutions of cannabinoids were prepared in dimethyl sulfoxide or ethanol and diluted using ACSF to a final concentration of 0.01 to 0.1% dimethyl sulfoxide or ethanol and 0.05% BSA to decrease adsorption to the perfusion system. The superfusion system was dismantled and rinsed with ethanol after each recording that involved superfusion of a cannabinoid. Stock solutions of all other drugs were made in distilled water or added directly to the ACSF. Anandamide (arachidonyl ethanolamide), R1-methanandamide (5,8,11,14-eicosatetraenamide, *N*-(2-hydroxy-1-methylethyl)-,[*R*-(all-*Z*)]-), and AM404 ((5,8,11,14-eicosatetraenamide, *N*-(4-hydroxyphenyl)-,(all-*Z*)-) were obtained from Cayman (Ann Arbor, MI); methionine-enkephalin was obtained from Auspep (Melbourne, Australia); baclofen and bicuculline methiodide were obtained from Sigma (Sydney, Australia); CTAP (D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2) was obtained from Phoenix Pharmaceuticals (Mountain View, CA); CNQX was obtained from Tocris Cookson (Bristol, UK); naloxone hydrochloride and WIN55,212-2 mesylate were obtained from Research Biochemicals (Natick, MA); TTX was obtained from Alomone (Jerusalem, Israel); SR141716 (*N*-piperidino-5-(4-chlorophenyl)-*l*-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide) was donated by Sanofi Recherche. All pooled data are expressed as mean ± S.E., and statistical comparisons were made using paired Student's *t* tests.

Results

Cannabinoid Agonists Inhibit Evoked GABAergic Synaptic Currents. The effects of cannabinoids on eIPSCs in PAG neurons were examined using whole-cell patch recordings in brain slices. In the presence of the non-*N*-methyl-D-aspartate glutamate receptor antagonist CNQX (3 μM), local electrical stimulation produced eIPSCs in PAG neurons that were abolished by the γ-aminobutyric acid $(GABA)_A$ antagonist bicuculline (30 μM, Fig. 1, A and B). Superfusion of the cannabinoid agonist WIN55,212-2 (3 μM) reduced the amplitude of eIPSCs in PAG neurons by an average of 63 ± 4% (range, 42–93%; n = 18; Fig. 1, A and B). WIN55,212-2 inhibited eIPSCs in all neurons tested within the lateral (63 ± 6%; n = 10) and ventrolateral PAG (62 ± 6%; n = 8). The reduction in the amplitude of eIPSCs was not reversed by washout of WIN55,212-2 for periods of up to 40 min. However, the WIN55,212-2-induced reduction in eIPSC amplitude was reversed by the addition of the CB_1 receptor antagonist SR141716 (1–3 μM; 101 ± 3% of control; n = 10;

Fig. 1, A and B). In the same neurons, subsequent superfusion of the opioid agonist methionine-enkephalin (10 μM) produced a rapid reduction in the amplitude of eIPSCs (55 ± 5% inhibition; n = 6), which was reversed by the addition of naloxone (1 μM; n = 3; reversed to 99 ± 6% of control; Fig. 1, A and B). The application of WIN55,212-2 and methionine-enkephalin had no effect on the membrane current, or the conductance of the neurons at −74 mV (Cs-filled electrodes used).

IPSCs were evoked by two stimuli of identical strength in close succession (interstimulus interval, 40–70 ms) to determine whether the WIN55,212-2-induced inhibition of the first evoked response was associated with relative facilitation of the second evoked response. Under control conditions, the mean ratio of the amplitude of the paired eIPSCs was 1.26 ± 0.09 ($IPSC_2/IPSC_1$ range, 1.03–1.61; n = 6; Fig. 1C). Superfusion of WIN55,212-2 (3 μM) produced a significant increase in the mean ratio of $IPSC_2/IPSC_1$ to 1.57 ± 0.14 (125 ± 6% of control; P = .01; n = 6). Subsequent addition of SR141716 (3 μM) reduced the mean ratio of $IPSC_2/IPSC_1$ to 1.18 ± 0.04 (103 ± 6% of control; P = .8; n = 4; Fig. 1C).

Endogenous Cannabinoids Inhibit Evoked GABAergic Synaptic Currents. Superfusion of the endogenous cannabinoid ligand anandamide (30 μM) alone did not significantly reduce the amplitude of eIPSCs (3 ± 4% inhibition; n = 6; Fig. 2A). However, in the presence of the anandamide transport inhibitor AM404 (30 μM), superfusion of anandamide (30 μM) reduced the amplitude of eIPSCs by 23 ± 7% (n = 4; Fig. 2B). The inhibition of eIPSCs by anandamide in the presence of AM404 was reversed by SR141716 (1–3 μM; 96 ± 6% of control; n = 4; Fig. 2B). Superfusion of the metabolically stable anandamide analog R1-methanandamide (30 μM; n = 5) reduced the amplitude of eIPSCs by 41 ± 4%, and this was reversed by SR141716 (3 μM; 92 ± 8%; n = 3; Fig. 2C).

Cannabinoid Agonists Inhibit Evoked Glutamatergic Synaptic Currents. In the presence of the $GABA_A$ antagonist bicuculline (30 μM), local electrical stimulation produced eEPSCs in PAG neurons that were abolished by the non-*N*-methyl-D-aspartate glutamate receptor antagonist CNQX (3 μM; Fig. 3, A and B). Superfusion of the cannabinoid agonist WIN55,212-2 (3 μM) reduced the amplitude of eEPSCs by an average of 62 ± 7% (n = 9; range, 38–93%; Fig. 3, A and B) in neurons from the lateral (n = 4) and ventro-

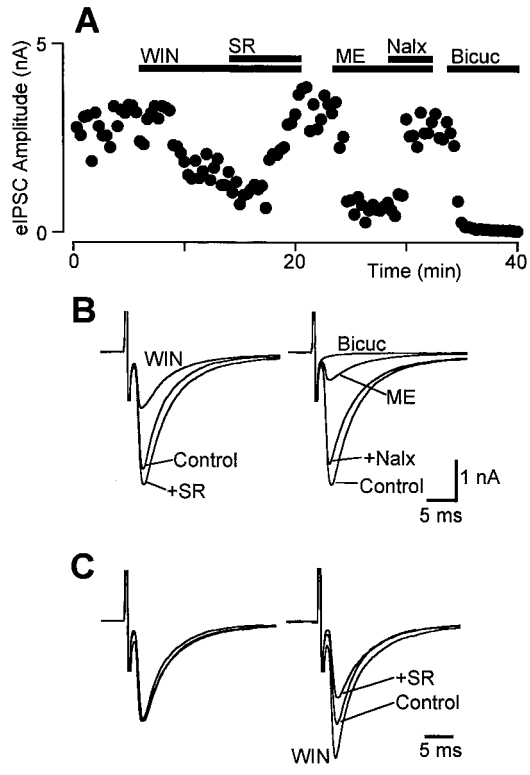

Fig. 1. WIN55,212-2 inhibits eIPSCs in PAG neurons. A, time course of eIPSC amplitude during application of 3 μM WIN55,212-2 (WIN) and addition of 3 μM SR141716 (SR), then during application of 10 μM methionine-enkephalin (ME) and addition of 1 μM naloxone (Nalx), then during 30 μM bicuculline (Bicuc). B, averaged eIPSCs before (Control) and during application of WIN55,212-2, then after addition of SR141716 (left); and before (Control) and during application of methionine-enkephalin, then after addition of naloxone (right). C, normalized average responses to identical paired stimuli (IPSC1–2 interval, 50 ms) for the traces in (B) with IPSC1 normalized (left) to demonstrate relative facilitation of IPSC2 during superfusion of WIN55,212-2 and its reversal by SR141716 (right). All traces were from the same neuron, which was voltage clamped at −74 mV. eIPSCs were elicited every 20 s in the presence of 3 μM CNQX.

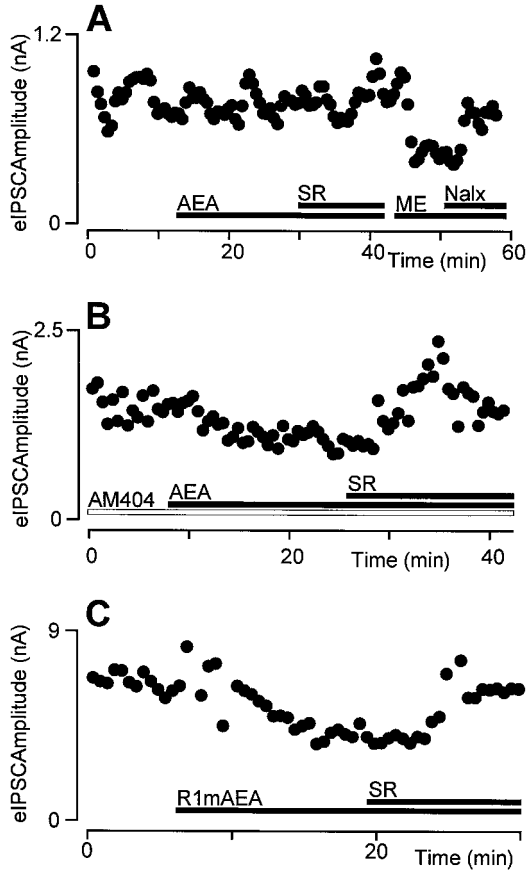

Fig. 2. Anandamide inhibits eIPSCs in PAG neurons. A, time course of eIPSC amplitude during application of 30 μM anandamide (AEA) and addition of 3 μM SR141716 (SR), then during application of 10 μM methionine-enkephalin (ME) and addition of 1 μM naloxone (Nalx). B, time course of eIPSC amplitude during application of 30 μM anandamide (AEA) and addition of 3 μM SR141716 (SR) in a neuron that had been maintained in 30 μM AM404. C, time course of eIPSC amplitude during application of 30 μM R1-methanandamide (R1 mAEA) and addition of 3 μM SR141716 (SR). Traces A to C were from the different neurons that were voltage-clamped at −74 mV. eIPSCs were elicited every 15 s in the presence of 3 μM CNQX. Each point is the mean of two consecutive eIPSCs.

lateral PAG ($n = 5$). In these neurons, addition of the opioid receptor antagonist naloxone (1 μM) had no effect on the WIN55,212-2-induced reduction in eEPSC amplitude (inhibition, 69 ± 11% in the presence of WIN55,212-2 plus naloxone; $n = 5$). The WIN55,212-2-induced reduction in eEPSC amplitude was reversed by the addition of the CB_1 receptor antagonist SR141716 (3 μM; 91 ± 6% of control; $n = 9$; Fig. 3, A and B).

Under control conditions, the mean ratio of the amplitude of the paired eEPSCs (interstimulus interval, 40–50 ms) was 1.06 ± 0.1 ($EPSC_2/EPSC_1$ range, 0.80–1.30; $n = 7$; Fig. 3C). Superfusion of WIN55,212-2 (3 μM) produced a significant increase in the mean ratio of $EPSC_2/EPSC_1$ (156 ± 25% of control; $P = .04$), which was reversed by the addition of SR141716 (3 μM; 108 ± 8% of control; $P = .2$; $n = 7$; Fig. 3C).

Cannabinoids Inhibit Miniature GABAergic Synaptic Currents. mIPSCs were readily observed during whole-cell voltage-clamp recordings in the presence of 3 μM CNQX and 0.3 μM TTX. This concentration of TTX prevented Na^+-dependent action potentials and abolished evoked postsynaptic currents (Vaughan and Christie, 1997). Superfusion of 3 μM WIN55,212-2 reduced the rate of mIPSCs (Fig. 4, A and B), but had no effect on their amplitude distributions or kinetics ($n = 9$; Fig. 4, C and D). On average, the mean mIPSC rate was reduced by 61 ± 4% during superfusion of WIN55,212-2, whereas the mean amplitude was increased by 5 ± 7% ($n = 9$). The inhibition of mIPSC rate produced by WIN55,212-2 was concentration dependent (EC_{50} = 520 ± 240 nM; Figs. 4A and 5) and was reversed by the addition of SR141716 (1–3 μM; 104 ± 18% of control; $n = 6$; Fig. 4, A and B). In Ca^{2+}-free solutions (0 mM Ca^{2+}, 10 mM Mg^{2+}), the mean rate of mIPSCs was reduced by 52 ± 6% during superfusion of WIN55,212-2 (3 μM), whereas the mean amplitude was increased by 1 ± 7% ($n = 4$).

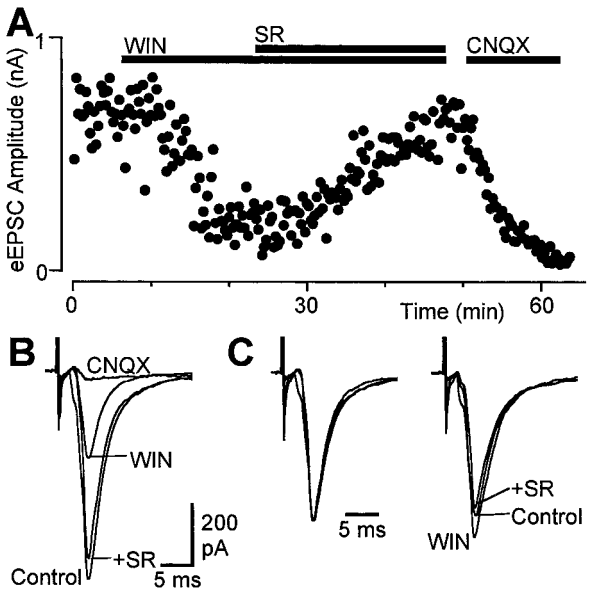

Fig. 3. WIN55,212-2 inhibits eEPSCs in PAG neurons. A, time course of eEPSC amplitude during application of 3 μM WIN55,212-2 (WIN) and addition of 3 μM SR141716 (SR), then during 3 μM CNQX. B, averaged eEPSCs before (Control) and during application of WIN55,212-2, then after addition of SR141716 and CNQX. C, normalized average responses to identical paired stimuli (EPSC1–2 interval, 50 ms) for the traces in (B) with EPSC1 normalized (left) to demonstrate relative facilitation of EPSC2 during superfusion of WIN55,212-2 and its reversal by SR141716 (right). All traces were from the same neuron, which was voltage-clamped at −74 mV. eEPSCs were elicited every 15 s in the presence of 30 μM bicuculline.

Superfusion of the anandamide analog R1-methanandamide (30 μM) reduced the rate of mIPSCs by an average of 21 ± 4%, but had no effect on their mean amplitude (4 ± 10% reduction; $n = 5$). The reduction in mIPSC rate produced by R1-methanandamide was reversed by the addition of SR141716 (3 μM; 107 ± 13% of control; $n = 5$).

Cannabinoids Do Not Affect Postsynaptic K^+ and Ca^{2+} Conductances. The effect of cannabinoids on postsynaptic K^+ currents in PAG neurons was examined using perforated patch recordings in brain slices. When neurons were voltage clamped to a potential of −60 mV, superfusion of the cannabinoid agonist WIN55,212-2 (3 μM) produced no significant membrane current in neurons (0 ± 1 pA; $n = 15$; Fig. 6A) from the lateral ($n = 9$) and ventrolateral PAG ($n = 6$). Subsequent addition of the cannabinoid CB_1 receptor antagonist SR 141716 (3 μM) produced no significant membrane current ($n = 13$; Fig. 6A). In these neurons, superfusion of the $GABA_B$ agonist baclofen (10 μM) produced a reversible outward current (Fig. 6A; 54 ± 10 pA; $n = 14$). In addition, superfusion of the opioid agonist methionine-enkephalin (10 μM) produced a reversible outward current in 12 of 13 of these neurons (Fig. 6A; 24 ± 4 pA), which was abolished by naloxone (1 μM; $n = 8$) and the μ-opioid receptor antagonist CTAP (1 μM; $n = 3$).

The resting membrane conductance of PAG neurons was 3.1 ± 0.6 nS and 3.8 ± 0.8 nS when measured between −60 to −90 mV and −110 to −130 mV, respectively ($n = 7$; Fig. 6B). Superfusion of 3 μM WIN55,212-2 had no significant effect on the conductances when measured over the same potentials (3.1 ± 0.7 and 3.8 ± 1.0 nS; $P > .4$, paired Student's t test; $n = 7$; Fig. 6B). Addition of 3 μM SR141716 also had no significant effect on the conductances when measured over the same potentials (3.3 ± 0.8 and 4.3 ± 1.3 nS; $P > .2$, paired Student's t test; $n = 7$; Fig. 6B). Baclofen (10 μM) increased the conductances to 4.6 ± 1.1 and 6.2 ± 1.9 nS ($P < .04$, paired Student's t test; $n = 6$) when measured over the same potentials (Fig. 6B). The baclofen-induced current reversed at −113 ± 7 mV ($n = 6$; Fig. 6B).

The effect of cannabinoids on Ba^{2+}-mediated Ca^{2+} channel currents in PAG neurons was examined using whole-cell recordings in acutely isolated PAG neurons. WIN55,212-2 (300 nM) had no effect on I_{Ba} (2 ± 1% inhibition; $n = 19$; Fig. 7). In these neurons, 10 μM baclofen produced a rapid and reversible inhibition of I_{Ba} (45 ± 4% inhibition; $n = 5$; Fig. 7).

Discussion

This study demonstrates that cannabinoid agonists act via CB_1 cannabinoid receptors to inhibit GABAergic and glutamatergic synaptic transmission in rat PAG. The endogenous cannabinoid, anandamide, also acts via CB_1 receptors to inhibit GABAergic synaptic transmission, although its activity is limited by uptake and degradation. Like μ-opioids, cannabinoid inhibition of GABAergic synaptic transmission is mediated by a presynaptic Ca^{2+}-independent mechanism. However, unlike μ-opioids, cannabinoids do not modulate postsynaptic K^+ and Ca^{2+} conductances in PAG neurons. These findings indicate that cannabinoids and μ-opioids are likely to produce analgesia within the PAG by distinct, but partially overlapping, disinhibitory mechanisms.

In this study, it was demonstrated that cannabinoid agonists act via CB_1 receptors to inhibit GABAergic synaptic transmission in PAG, as previously demonstrated in the RVM (Vaughan et al., 1999). Inhibition of GABAergic synaptic transmission is thought to produce analgesia within these brain regions (see below). The inhibition of synaptic transmission by the cannabinoid agonist WIN55,212-2 was mediated by cannabinoid CB_1 receptors because it was reversed by the CB_1-specific antagonist SR141716 (Rinaldi-Carmona et al., 1994), but was unaffected by the opioid antagonist naloxone. Like μ-opioids (Vaughan and Christie, 1997), cannabinoids inhibited GABAergic and glutamatergic synaptic transmission in all neurons throughout the lateral and ventrolateral PAG. These results are consistent with the presence of CB_1-like immunoreactivity (Tsou et al., 1998), cannabinoid radioligand [^{3}H]CP55,940 binding sites (Herkenham et al., 1991), and CB_1 mRNA throughout the PAG (Matsuda et al., 1993). Inhibition of GABAergic and glutamatergic synaptic transmission has previously been demonstrated in the hippocampus, cerebellum, and basal ganglia (Shen et al., 1996; Chan et al., 1998; Levenes et al., 1998; Szabo et al., 1998).

The potency of WIN55,212-2 (EC_{50} = 520 nM) was similar to that previously observed in other studies using brain slices (Chan et al., 1998; Levenes et al., 1998; Szabo et al., 1998; Vaughan et al., 1999), hippocampal cultures (Deadwyler et al., 1993), and oocytes (Henry and Chavkin, 1995). However, the potency of WIN55,212-2 was much greater in other studies using hippocampal cultures (Shen et al., 1996; Twitchell et al., 1997) and transfected cells (Mackie et al., 1995). The difference in agonist potency between slices, cultures, and transfected cells may be due to the lipophilic nature of cannabinoids, which results in adsorption to the perfusion system, reduced penetration into the slice, and reduced access to the synaptic cleft. The differences may also be due to overexpression of cannabinoid receptors in transfected cells and variations in receptor reserve between different brain regions (Herkenham et al., 1991).

The inhibition of GABAergic and glutamatergic synaptic transmission by cannabinoids in PAG was likely to be mediated by a presynaptic mechanism, as previously demonstrated in the basal ganglia (Chan et al., 1998; Szabo et al., 1998), cerebellar purkinje cells (Levenes et al., 1998), rostroventral medial medulla (Vaughan et al., 1999), and in hippocampal cultures (Shen et al., 1996). First, WIN55,212-2 and R1-methanandamide produced a reduction in the rate of mIPSCs without any effect on their amplitude distributions or kinetics, which is indicative of a presynaptic site of action. Second, WIN55,212-2 had no effect on postsynaptic K^+ or Ca^{2+} conductances (see below). Last, the inhibition of eIPSCs and eEPSCs by WIN55,212-2 was associated with an increase in paired pulse facilitation, which arises from an increase in the probability of presynaptic transmitter release (del Castillo and Katz, 1954). Thus, like μ-opioids (Vaughan and Christie, 1997), cannabinoids are likely to act presynaptically to reduce the probability of transmitter release from GABAergic and glutamatergic terminals in PAG. The CB_1 receptor-induced inhibition of miniature GABAergic postsynaptic currents was likely to be mediated via a Ca^{2+}-independent mechanism because the WIN55,212-2-induced reduction in mIPSC rate was similar in normal and Ca^{2+}-free/high Mg^{2+} solutions, as previously demonstrated for μ-opioid-mediated inhibition in PAG (Vaughan and Christie, 1997). However, a role for presynaptic Ca^{2+} conductances in CB_1-mediated inhibition of evoked GABA release cannot be excluded. It was not determined whether the cannabinoid CB_1 presynaptic inhibition of evoked and miniature GABAergic synaptic transmission in PAG was mediated by a lipoxygenase-coupled 4-aminopyridine-sensitive K^+ conductance, similar to μ-opioids (Vaughan et al., 1997).

Although the endogenous cannabinoid anandamide produces analgesia, it is less potent and efficacious than Δ^9-THC and other cannabinoid agonists (Smith et al., 1994; Felder et al., 1995). The reduced analgesic potency of anandamide is likely to be the result of degradation that occurs within cells after uptake by a selective transport system (Deutsch and Chin, 1993; Abadji et al., 1994; Di Marzo et al., 1994). Thus, the analgesic activity of anandamide is enhanced by the

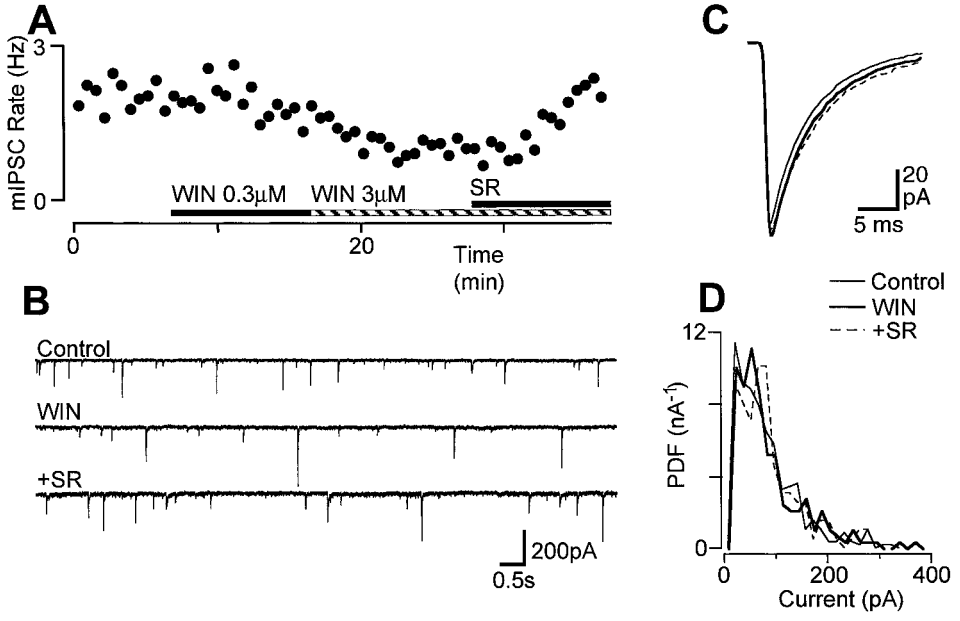

Fig. 4. WIN55,212-2 decreases the rate of mIPSCs. A, time course of mIPSC rate during superfusion of 0.3 and 3 μM WIN55,212-2 (WIN) and addition of 3 μM SR141716 (SR). B, raw current traces of mIPSCs before (Control) and during superfusion of 3 μM WIN55,212-2 and addition of 3 μM SR141716. C, averaged traces of mIPSCs before and during superfusion of 3 μM WIN55,212-2, then after addition of SR141716 (number of events = 393, 192, and 132 for 200-, 200-, and 75-s epochs of Control, 3 μM WIN, and SR, respectively). D, probability density distributions of mIPSC amplitude (bin width, 15 pA) for the epochs indicated in (C). (A) to (D) are taken from one neuron that was voltage-clamped at −74 mV in the presence of 3 μM CNQX and 0.3 μM TTX.

anandamide transport inhibitor, AM404 (Beltramo et al., 1997). In the present study, anandamide inhibited evoked GABAergic synaptic transmission in the presence, but not in the absence, of AM404. Furthermore, the metabolically stable analog R1-methanandamide inhibited GABAergic synaptic transmission, a finding that also indicates that the effectiveness of anandamide may be limited by breakdown. However, maximal inhibition produced by anandamide and R1-methanandamide was less than that produced by WIN55,212-2, suggesting that the endogenous cannabinoid has relatively low intrinsic activity. The relatively low activity of anandamide was likely to have been exacerbated by poor penetration into the slice (see above) and its low affinity for the CB_1 receptor (Abadji et al., 1994; Felder et al., 1995).

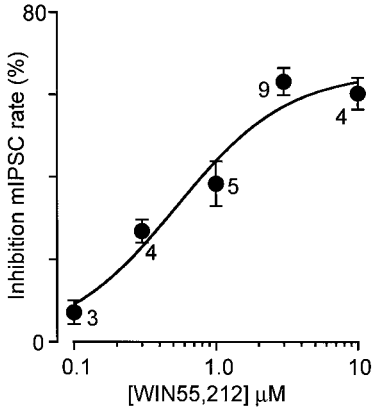

Fig. 5. Concentration-response relationship for percent inhibition of mIPSC rate produced by WIN55,212-2. Each point shows the mean ± S.E. of responses of several different neurons, with the number of neurons indicated adjacent to each point. A logistic function was fitted (Kaleidograph; Synergy Software, Reading, PA) to determine the EC_{50} (520 ± 240 nM; slope factor = 1.1 ± 0.4).

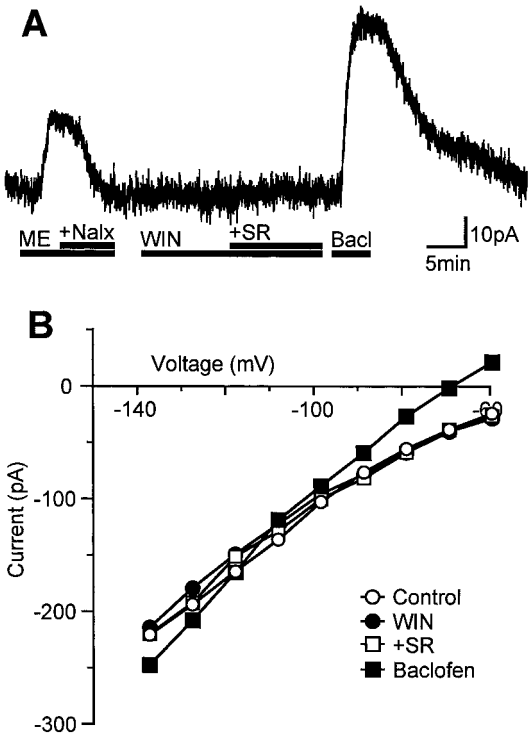

Fig. 6. WIN55,212-2 does not affect postsynaptic membrane currents in PAG neurons. A, membrane current trace of a PAG neuron during superfusion of 10 μM methionine-enkephalin (ME), 1 μM naloxone (Nalx), 3 μM WIN55,212-2 (WIN), 3 μM SR141716 (SR), and 10 μM baclofen (Bacl). B, current-voltage relationship for control (○), WIN55,212-2 (●), WIN55,212-2 + SR141716 (□), and baclofen (■) is plotted from the amplitudes of evoked currents. Membrane currents were evoked by voltage command steps in 10-mV increments from a holding potential of −60 to −140 mV (250-ms duration). (A) and (B) are from the same neuron, which was voltage-clamped at −60 mV.

Unlike μ-opioid agonists (Chieng and Christie, 1994; Osborne et al., 1996; Connor and Christie, 1998), the cannabinoid agonist WIN55,212-2 did not increase an inwardly rectifying K^+ conductance or inhibit Ca^{2+} conductances in PAG neurons. The absence of postsynaptic actions of cannabinoid agonists on PAG neurons is consistent with the prevalence of CB_1 receptors within PAG fibers rather than cell bodies demonstrated using immunohistochemistry (Tsou et al., 1998). The lack of postsynaptic cannabinoid effect was unlikely to be due to cell damage because these neurons responded to μ-opioid and $GABA_B$ receptor agonists. Furthermore, the proportion of neurons directly inhibited by μ-opioid receptor agonists in the present study using perforated patch recordings (92% of neurons) was greater than in previous studies using intracellular recordings or in whole-cell patch recordings from PAG-RVM projection neurons (40% of neurons). The lack of direct postsynaptic cannabinoid inhibition in PAG is similar to that previously reported in RVM (Vaughan et al., 1999), but differs from the hippocampus and substantia nigra, where cannabinoids have both pre- and postsynaptic actions (Deadwyler et al., 1993; Twitchell et al., 1997; Chan et al., 1998).

The PAG forms a component of a descending inhibitory network that, via the RVM, modulates nociceptive neurotransmission at the level of the dorsal horn of the spinal cord (Fields et al., 1991). It has been proposed that μ-opioids produce antinociception within the PAG and RVM by reduc-

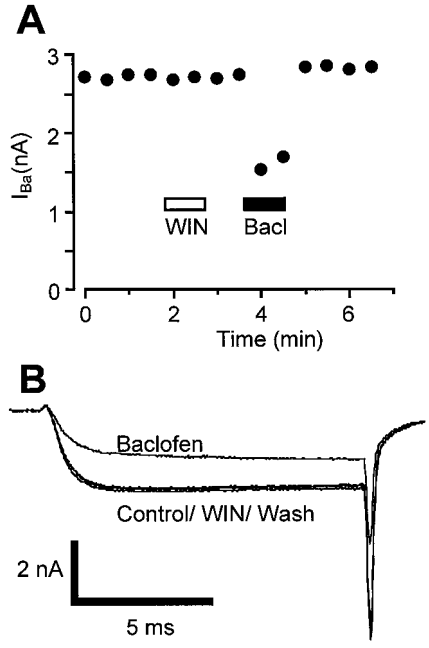

Fig. 7. WIN55,212-2 does not affect postsynaptic Ca^{2+} currents in acutely isolated PAG neurons. A, time plot of the peak amplitude of I_{Ba} during superfusion of 300 nM WIN55,212-2 (WIN) and 10 μM baclofen (Bacl). B, representative traces of evoked I_{Ba} before (Control), during (WIN, Bacl), and after washout of drugs (wash). I_{Ba} was evoked by repetitively stepping the membrane potential from −90 to 0 mV (10-ms duration) every 30 s.

ing inhibitory GABAergic influences on PAG and RVM output projection neurons (disinhibition). The present and previous results suggest that cannabinoid CB_1 receptor-mediated antinociception might also be produced by disinhibition in the PAG and RVM (Meng et al., 1998; Vaughan et al., 1999). However, the mechanisms of cannabinoid and μ-opioid disinhibition differ. Disinhibition by μ-opioids occurs by two distinct cellular mechanisms within the PAG. μ-Opioids directly inhibit the cell bodies of a subpopulation of PAG neurons (presumptive GABAergic interneurons) by activating an inwardly rectifying K^+ conductance and by inhibiting Ca^{2+} conductances (Chieng and Christie, 1994; Osborne et al., 1996; Connor and Christie, 1998). μ-Opioids also act on the presynaptic terminals of GABAergic neurons to inhibit transmitter release (Vaughan and Christie, 1997). The present findings indicate that cannabinoid disinhibition is restricted to presynaptic inhibition of GABA release because cannabinoids had no direct somatic effects on PAG neurons. These findings parallel those in the RVM where cannabinoid disinhibition is exclusively mediated by presynaptic GABAergic inhibition (Vaughan et al., 1999), and μ-opioid disinhibition is mediated by both pre- and postsynaptic GABAergic inhibition (Pan et al., 1990). Differences in the mechanisms and regional distribution of action of cannabinoids and μ-opioids could contribute to their synergistic analgesic interactions (Smith et al., 1998). The finding that cannabinoids inhibited both GABAergic and glutamatergic synaptic transmission throughout the lateral and ventrolateral PAG suggests that cannabinoids might also modulate the other behavioral and autonomic functions of the PAG (Bandler and Shipley, 1994).

Although the exogenous application of anandamide and cannabinoid agonists produces analgesia (Smith et al., 1994; Lichtman et al., 1996; Adams et al., 1998), recent findings suggest that endogenously released anandamide might also produce analgesia within the PAG. Pain of superficial origin produced an increase in the release of anandamide within the PAG (Walker et al., 1999). The cellular actions of exogenously applied anandamide (present study) and the analgesic actions of endogenously released anandamide (Walker et al., 1999) within the PAG are mediated by cannabinoid CB_1 receptors and are likely to be disinhibitory. These findings differ from other studies that suggest the analgesic actions of anandamide are not mediated by CB_1 receptors (Adams et al., 1998).

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Send reprint requests to: Dr. C.W. Vaughan, Department of Pharmacology, University of Sydney, Sydney, New South Wales 2006, Australia. E-mail: chrisv@pharmacol.usyd.edu.au