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# Presynaptic $\mu$ -opioid receptors regulate a late step of the secretory process in rat ventral tegmental area GABAergic neurons

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## Abstract

$\gamma$ -Aminobutyric acid (GABA)-containing interneurons of the ventral tegmental area (VTA) regulate the activity of dopaminergic neurons. These GABAergic interneurons are known to be innervated by synaptic terminals containing enkephalin, an endogenous ligand of  $\mu$ -opioid receptors. Bath application of  $\mu$ -opioid receptor agonists inhibits the activity of VTA GABAergic neurons but the mechanism whereby  $\mu$ -opioid receptors regulate synaptic GABA release from these neurons has not been directly identified. Using cultured VTA neurons we have confirmed that  $\mu$ -opioid receptor agonists inhibit synaptic GABA release. DAMGO, a selective  $\mu$ -opioid receptor agonist, had four distinct effects on GABAergic IPSCs: (1) it inhibited the frequency and amplitude of spontaneous IPSCs (sIPSCs), (2) it reduced the amplitude of IPSCs evoked by single action potentials, (3) it inhibited the frequency, but not the amplitude of miniature IPSCs (mIPSCs), and (4) DAMGO inhibited mIPSCs evoked by ionomycin, a  $\text{Ca}^{2+}$  ionophore. The inhibition of action potential-evoked IPSCs and of spontaneous and ionomycin-evoked mIPSCs by DAMGO was prevented by the  $\text{K}^+$  channel blocker, 4-aminopyridine (4-AP). In conclusion, our work shows that one of the mechanisms through which  $\mu$ -opioid receptors inhibit GABA release by VTA neurons is through inhibition of the secretory process at the nerve terminal level. In addition, considering that ionomycin stimulates exocytosis through a mechanism that should be insensitive to membrane polarization, our experiments with 4-AP suggest that  $\text{K}^+$  channels are implicated in the inhibition of the efficacy of the secretory process by  $\mu$ -opioid receptors. © 2002 Elsevier Science Ltd. All rights reserved.

**Keywords:** Synapse; Exocytosis; GABA; Opioids; Patch-clamp; Culture; Ventral tegmental area

## 1. Introduction

The ventral tegmental area (VTA), a part of the mesolimbic dopamine system, is known for its implication in the reinforcing properties of many drugs of abuse, including opioids (Wise, 1996; Koob and Le Moal, 1997). Experiments have shown that rats will learn to lever press for microinjections of opioids directly into the VTA (Bozarth and Wise, 1981; Devine and Wise, 1994), and VTA microinjections of opioids will produce conditioned place preference (Phillips and LePiane, 1980; Phillips et al., 1983). It has also been demonstrated that microinjections of opioids into the VTA produce an increase in spontaneous locomotor activity in rats

(Broekkamp et al., 1979; Kelley et al., 1980). Evidence suggests that these opioid-induced rewarding and motor-stimulant effects are mediated by an increased activity of VTA dopaminergic (DAergic) neurons. For example, morphine increases the firing rate of DAergic neurons when administered directly into the VTA (Gysling and Wang, 1983). Furthermore, microinjections of enkephalin analogs in the VTA increase dopamine release in the mesolimbic dopamine terminal fields (Kalivas and Duffy, 1990; Devine et al., 1993).

The activity of VTA DAergic neurons is known to be regulated by the activity of GABAergic neurons, which provide inhibitory synaptic input to the DAergic cells (Johnson and North, 1992a, 1992b; Steffensen et al., 1998). Some of this input arises from GABAergic neurons intrinsic to the VTA (Johnson and North, 1992b). Electrophysiological studies have shown that  $\mu$ -opioid agonists inhibit the firing of GABAergic neurons in the VTA slice preparation (Johnson and North, 1992a). Fur-

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thermore, the extracellular concentration of  $\gamma$ -aminobutyric acid (GABA) is reduced after microdialysis of morphine into the VTA of conscious rats (Klitenick et al., 1992). Based upon these findings, it has been suggested that the effect of opioids on VTA GABAergic neurons results in a disinhibition of DAergic neurons, and thus produces an increase in mesolimbic DAergic transmission (Gysling and Wang, 1983; Johnson and North, 1992a, 1992b).

The mechanism by which opioids inhibit VTA GABAergic neurons is not well defined. Intracellular recordings of VTA cells have shown that local application of opioids hyperpolarizes GABAergic neurons by increasing a somatic  $K^+$  conductance (Johnson and North, 1992a). However, the disinhibitory action of opioids could also occur via opioid receptors located on GABAergic terminals in the VTA. Indeed, evidence suggests the presence of  $\mu$ -opioid receptors on VTA GABAergic neurons (Dilts and Kalivas, 1989), and the direct apposition of GABA- and enkephalin-immunolabeled terminals in the VTA is consistent with a presynaptic location of this receptor (Sesack and Pickel, 1995). Furthermore, presynaptic inhibition of neurotransmitter release by opioids has been shown in many brain regions. For example, presynaptic modulation of GABA release by  $\mu$ -opioid receptors has been observed in the hippocampus (Cohen et al., 1992; Rekling, 1993; Capogna et al., 1993; Lupica, 1995), the globus pallidus (Stanford and Cooper, 1999) and the periaqueductal gray (Prisco et al., 1994; Vaughan and Christie, 1997; Vaughan et al., 1997). In the latter structure, the inhibition of GABA release from GABAergic interneurons by  $\mu$ -opioid agonists seems to be mediated by the activation of a 4-aminopyridine (4-AP)-sensitive voltage-dependent  $K^+$  channel located at the presynaptic terminal (Vaughan et al., 1997). In the hippocampus, a mechanism implicating a direct modulation of the secretory machinery has been suggested for the  $\mu$ -opioid receptor-mediated presynaptic inhibition of GABA release from GABAergic interneurons of the CA3 area (Capogna et al., 1993, 1996).

The aim of the present study was therefore to test the hypothesis that the inhibition of GABAergic neurons of the VTA following activation of  $\mu$ -opioid receptors involves a presynaptic modulation of GABA release and to investigate the mechanism responsible for this presynaptic inhibition.

## 2. Methods

### 2.1. Cell culture

Primary cultures of rat VTA neurons were prepared according to recently described protocols (Michel and Trudeau, 2000; Congar et al., 2002; Bourque and

Trudeau, 2000) derived from Cardozo (Cardozo, 1993) and Sulzer et al. (Sulzer et al., 1998). For the two types of cultures that were prepared (standard and micro-dot cultures), dissociated VTA neurons were plated on pre-established midbrain astrocytic monolayers on pre-coated glass coverslips. To prepare astrocyte cultures, neonatal (P0 to P3) Sprague-Dawley rats were cryoanesthetized. The brain was quickly removed from the skull and placed in ice-cold dissociation solution. A 1 mm<sup>2</sup> block of midbrain tissue was rapidly isolated from a 1 mm slice cut at the level of the midbrain flexure. The tissue was then digested with papain for 60 min at 37 °C before being gently triturated using Pasteur pipettes of decreasing diameter until a single-cell suspension was obtained. The cells were grown in culture flasks for 5 to 10 days. After the first 48 hours, they were vigorously washed with cold medium to dislodge most neurons, leaving only tightly adherent astrocytes. After reaching confluence, astrocytes were gently trypsinized, washed, collected, counted and plated at a concentration of 60 000 to 80 000 (micro-dot cultures) or 120 000 (standard cultures) living cells per milliliter on collagen/poly-L-lysine-coated coverslips (standard cultures) or on 0.15% agarose-covered coverslips sprayed with collagen/poly-D-lysine (micro-dot cultures). The later approach permitted the formation of small groups of isolated cells. Twenty-four hours after they were plated, astrocytes covered most of the collagen/polylysine surface and further division was inhibited with 5-fluoro-2-deoxyuridine (FUDR). To prepare neurons, neonatal (P0 to P3) Sprague-Dawley rats were cryoanesthetized and their VTA was rapidly isolated using a custom tissue micro-punch from a 1 mm slice cut at the level of the midbrain flexure. As for the astrocyte preparation, the tissue was digested with papain for 30 min at 37 °C, before being gently triturated. The dissociated cells were then collected by centrifugation, counted and diluted at a density titrated to optimize neuronal viability in standard culture conditions (240 000 living cells per milliliter) and to maximize the number of single neurons in micro-dot cultures (100 000 living cells per milliliter) before being plated on coverslips with a pre-established midbrain astrocyte monolayer. Further astrocytic division was inhibited by a second addition of FUDR 24 hours after neurons were plated. Kynurenate (0.5 mM) was added to the culture medium 7 days after neurons were plated in order to block excitotoxicity. Cell cultures were incubated at 37 °C in a 5% CO<sub>2</sub> atmosphere and maintained in Basal Medium Eagle with Earl's Salts (Gibco, Burlington, Ontario, Canada) supplemented with 5% Fetal Calf Serum (Gibco) and Mito+ serum additive (VWR Canlab, Montréal, Québec, Canada). Experiments were performed on neurons between 10 and 30 days after plating.

## 2.2. Electrophysiology

Whole-cell voltage clamp recordings were performed using a Warner PC-505 patch-clamp amplifier (Warner Instruments Corp., Hamden, CT, USA). Signals were filtered at 1 kHz, digitized at 5 kHz and recorded and analyzed using Pclamp7 software (Axon Instruments, Foster City, CA, USA). Except for the experiments in Fig. 4B, which were performed at 30 °C, all recordings were performed at room temperature (~21 °C). Patch pipettes were prepared with borosilicate glass and had a resistance of ~5 M $\Omega$ . Cells were perfused with an extracellular solution containing (in mM): NaCl 140, KCl 5, MgCl<sub>2</sub> 2, CaCl<sub>2</sub> 2, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid) (HEPES) 10, glucose 10, sucrose 6, at a pH of 7.35. Tetrodotoxin (TTX; 0.5  $\mu$ M) and cyano-7-nitroquinoline-2,3-dione (CNQX; 10  $\mu$ M) were added to the extracellular bathing solution while recording miniature inhibitory postsynaptic currents (mIPSCs), in order to block respectively fast sodium spikes and glutamatergic non-*N*-methyl-D-aspartate (NMDA) excitatory postsynaptic currents. CNQX was also added to the bathing solution while recording spontaneous inhibitory postsynaptic currents (sIPSCs). Spontaneous IPSCs and mIPSCs were recorded using a cesium gluconate intrapipette solution containing (in mM): Cs gluconate 117.5, NaCl 10, MgCl<sub>2</sub> 4, ethylene glycol bis( $\beta$ -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) 5, adenosine triphosphate (ATP) (Mg salt) 2, guanidine triphosphate (GTP) (Tris salt) 0.2, HEPES 15 (pH 7.35), or using a cesium methylsulfate intrapipette solution containing (in mM): Cs methane sulfonate 120, CsCl 20, NaCl 5, EGTA 1, ATP (Mg salt) 4, GTP (Tris salt) 0.5, HEPES 10 (pH 7.35). To evoke autaptic inhibitory postsynaptic currents (autaptic IPSCs), isolated neurons were stimulated every 10–15 s with a brief (1 ms) voltage command pulse to 20 mV from a holding potential of –50 to –40 mV, and evoked postsynaptic currents were recorded. Evoked autaptic IPSCs were recorded with a K<sup>+</sup> methylsulfate intrapipette solution containing (in mM): KMeSO<sub>4</sub> 140, NaCl 10, EGTA 0.1, ATP (Mg salt) 4, GTP (Tris salt) 0.5, HEPES 10 (pH 7.35). Spontaneous IPSCs and mIPSCs were analyzed with Mini Analysis Software (version 5.4) by Synaptosoft Inc. (Leonia, NJ, USA). Data are expressed as mean  $\pm$  standard error of the mean (SEM). Unless otherwise indicated, data were analyzed for statistical significance using the paired Student's *t*-test, with a probability value of  $P < 0.05$  used as the criterion. The Kolmogorov–Smirnov test was used to compare cumulative probability distributions. Considering the high sensitivity of this test, a probability value of  $P < 0.01$  was used as a criterion in order to be more stringent.

## 2.3. Immunocytochemistry

The identity of the neurons as GABAergic was confirmed in some experiments by post-recording immunofluorescent labeling for glutamic acid decarboxylase (GAD). Unlike dopaminergic neurons, such GABAergic neurons were never found to be labeled with an antibody directed against tyrosine hydroxylase (not shown). Cells were fixed with 4% paraformaldehyde in phosphate buffer, pH 7.4, for 30 min at room temperature and then rinsed several times. After a 20 minute long permeabilization with 0.1% Triton X100 and blocking with bovine serum albumin and goat serum, cells were incubated overnight at 4 °C with primary antibodies against GAD. The cells were then rinsed several times and incubated for 1 h with an Alexa-488 conjugated secondary antibody. Coverslips were mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA) and observed by epifluorescence microscopy. Images of immunofluorescent labeling were acquired using a Hamamatsu Orca-II digital cooled CCD camera and an Invision workstation using ISee software (Invision Corporation, Raleigh, NC, USA).

## 2.4. Drugs

All chemicals and primary antibodies were obtained from Sigma Chemicals Co. (St Louis, MO, USA) except for TTX, which was obtained from Alomone Laboratories (Jerusalem, Israel). The secondary antibodies were obtained from Molecular Probes Inc. (Eugene, OR, USA).

## 3. Results

### 3.1. DAMGO inhibits the frequency and amplitude of spontaneous IPSCs

The effect of the selective  $\mu$ -opioid receptor agonist DAMGO ([D-Ala<sup>2</sup>,N-Me-Phe<sup>4</sup>,Gly<sup>5</sup>-ol]-enkephalin; 1  $\mu$ M) on the frequency and amplitude of sIPSCs was first investigated (Fig. 1A, B). Recordings were performed from randomly selected neurons in the presence of CNQX (10  $\mu$ M) using standard VTA cultures. DAMGO significantly reduced the frequency and amplitude of sIPSCs. The frequency was reduced by  $57.7 \pm 9.9\%$  ( $n = 11$ ,  $P < 0.005$ ) and the amplitude by  $38.9 \pm 12.1\%$  ( $n = 11$ ,  $P < 0.05$ ) (Fig. 1). Both effects were reversible after washout of DAMGO (Fig. 1B). In these experiments, percent inhibition was calculated by comparing the frequency or amplitude of events in the presence of DAMGO to the mean value of the frequency or amplitude during the 4 minutes baseline period and after washout of DAMGO. All further effects were calculated in the same way to account for any gradual change in

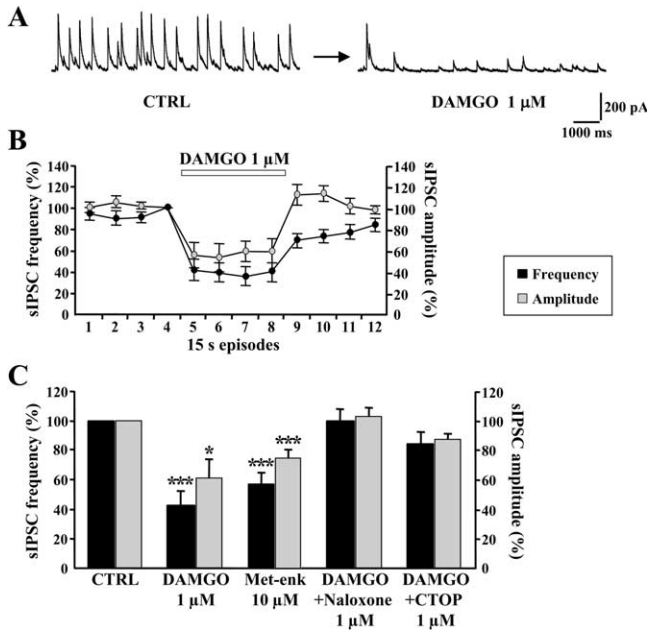


Fig. 1. Effect of DAMGO on spontaneous inhibitory postsynaptic currents (sIPSCs). (A) Whole-cell recording of sIPSCs under control conditions and in the presence of DAMGO (1  $\mu$ M). The frequency and the amplitude of the sIPSCs were markedly decreased by DAMGO (right current trace). (B) Time course showing the effect of DAMGO on sIPSC frequency (black circles) and amplitude (gray circles) ( $n = 11$ ). The data were normalized to the value of the last control measurement for each experiment. (C) Summary histogram of the mean sIPSC frequency (black bars) and amplitude (gray bars) expressed as percentage of control. The sIPSC frequency was decreased by DAMGO ( $n = 11$ ) and by met-enkephalin (10  $\mu$ M) ( $n = 10$ ) (\*\*\*,  $P < 0.005$ ). The sIPSC amplitude was also decreased by DAMGO ( $n = 11$ ) (\*\*\*,  $P < 0.005$ ), and by met-enkephalin ( $n = 10$ ) (\*,  $P < 0.05$ ). However, DAMGO failed to significantly decrease the frequency and amplitude of sIPSCs in the presence of the opioid receptor antagonists naloxone (1  $\mu$ M) ( $n = 13$ ) or CTOP (1  $\mu$ M) ( $n = 6$ ).

the baseline event frequency. The  $\mu$ -opioid agonist met-enkephalin (10  $\mu$ M; Fig. 1C) mimicked the effect of DAMGO by reducing sIPSC frequency by  $43.1 \pm 7.7\%$  ( $n = 10$ ,  $P < 0.005$ ) and the amplitude by  $25.5 \pm 6.2\%$  ( $n = 10$ ,  $P < 0.005$ ).

In the presence of the non-selective opioid receptor antagonist naloxone (1  $\mu$ M; Fig. 1C), DAMGO caused no significant change of sIPSC frequency or amplitude. In the presence of naloxone, the sIPSC frequency following application of DAMGO was at  $100.0 \pm 7.5\%$  of the control value ( $n = 13$ ,  $P > 0.05$ ), while the sIPSC amplitude was at  $102.8 \pm 5.9\%$  of the control value ( $n = 13$ ,  $P > 0.05$ ). The effect of DAMGO was also blocked by the selective  $\mu$ -opioid receptor antagonist CTOP (D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH<sub>2</sub>) (1  $\mu$ M; Fig. 1C). The inhibition of sIPSC frequency by DAMGO was of  $16.1 \pm 7.8\%$  in the presence of CTOP ( $n = 5$ ,  $P > 0.05$ ) as opposed to  $31.2 \pm 7.5\%$  when DAMGO was applied alone in the same cells ( $n = 5$ ,  $P < 0.05$ ; data not shown). Moreover, the decrease

of sIPSC amplitude by DAMGO was of  $13.3 \pm 4.5\%$  in the presence of CTOP ( $n = 5$ ,  $P > 0.05$ ) as opposed to a decrease of  $41.0 \pm 5.3\%$  in its absence ( $n = 5$ ,  $P < 0.005$ ; data not shown). Overall, these results confirm that in cultured VTA GABAergic neurons, DAMGO inhibits GABA release by acting on  $\mu$ -opioid receptors.

### 3.2. DAMGO inhibits GABAergic autaptic currents

The effect of DAMGO (1  $\mu$ M) on synaptic transmission from VTA GABAergic neurons was examined by recording GABAergic synaptic currents evoked by single action potentials. Experiments were performed on single VTA GABAergic neurons in micro-dot cultures (Fig. 2A). The identity of the neurons as GABAergic was confirmed in some experiments by post-recording immunofluorescent labeling for glutamic acid decarboxylase (GAD), which strongly labeled the abundant synaptic terminals of isolated GABAergic neurons (Fig. 2B). Depolarizing voltage steps evoked a fast inward sodium current followed by a slow outward GABA<sub>A</sub> receptor-mediated IPSC (autaptic IPSC) (Fig. 2C). In most experiments, the GABAergic nature of the evoked autaptic current was confirmed by its sensitivity to SR 95531 (5  $\mu$ M), a GABA<sub>A</sub> receptor antagonist (Fig. 2D). DAMGO inhibited autaptic IPSCs in 18 of the 45 GABAergic cells tested, thus demonstrating some heterogeneity in cell properties. Sensitivity to DAMGO was essentially all or none. Cells were considered sensitive to DAMGO if the evoked IPSC was inhibited by at least 15% relative to the control period. In these 18 cells, DAMGO reduced the amplitude of autaptic IPSCs by  $49.8 \pm 4.4\%$  (Fig. 2C, E). This effect was reversible upon washout of DAMGO (Fig. 2C, E).

### 3.3. Lack of postsynaptic action

To determine whether the inhibition of IPSC amplitude caused by  $\mu$ -opioid receptor activation was associated with a postsynaptic modification, GABA (500  $\mu$ M) was directly applied by brief (3.5 ms) pressure ejection onto the processes of isolated GABAergic neurons. Autaptic IPSCs and membrane currents mediated by exogenously applied GABA were evoked with a 500 ms delay and measured in parallel (Fig. 2D). The GABA<sub>A</sub> receptor antagonist SR95531 (5  $\mu$ M) was always applied at the end of the experiments to ensure that the IPSC and the GABA-evoked membrane currents were both mediated by the GABA<sub>A</sub> receptor. Only results from neurons with IPSCs that were inhibited by DAMGO were further analyzed (six out of 10 neurons tested). While IPSCs were robustly inhibited by DAMGO ( $48.9 \pm 9.7\%$  inhibition;  $n = 6$ ,  $P < 0.05$ ), membrane currents evoked in parallel with exogenous GABA were unaffected ( $2.3 \pm 5.4\%$ ;  $n = 6$ ,  $P > 0.05$ ). This finding argues against the hypothesis that DAMGO acts through

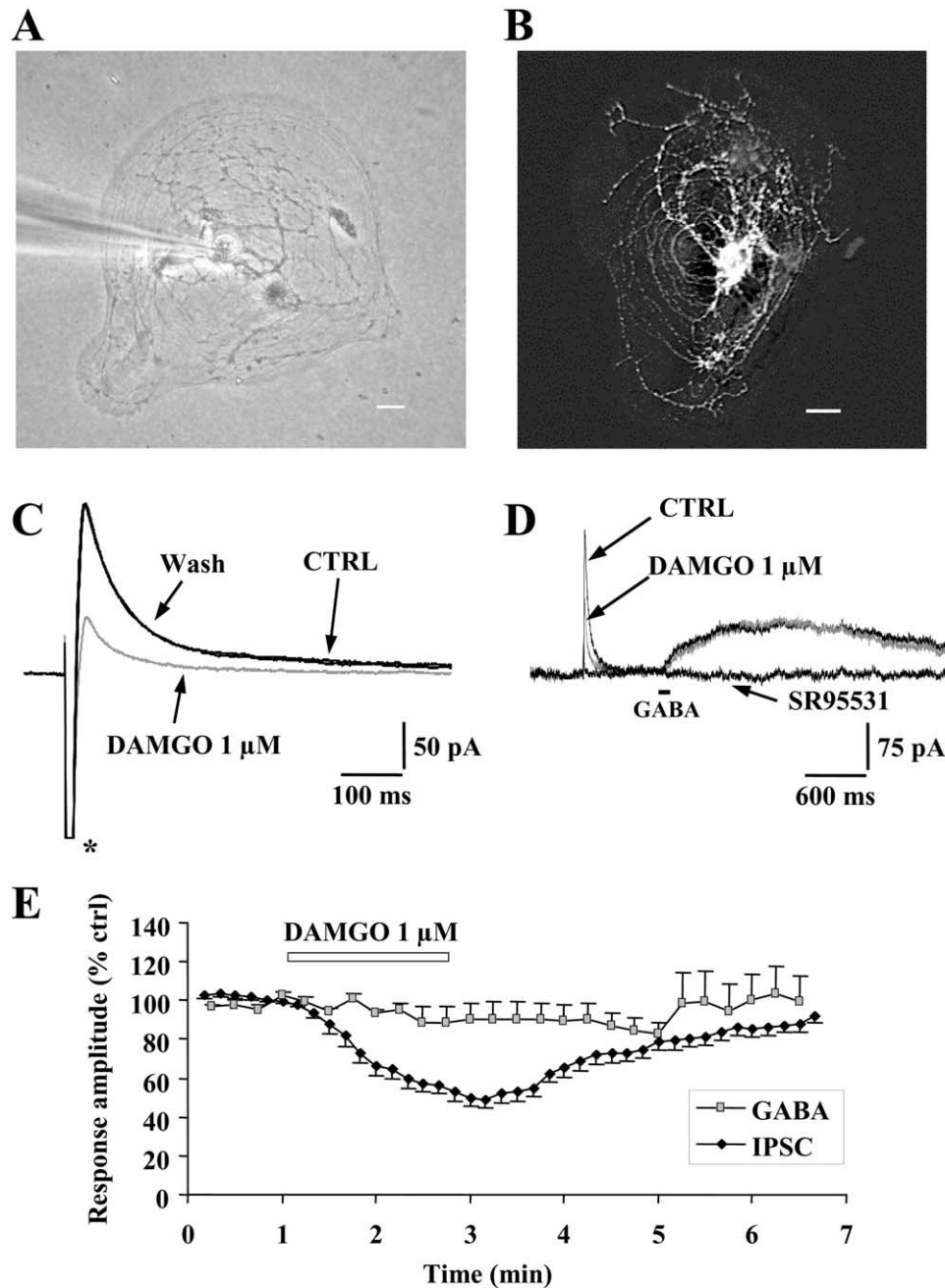


Fig. 2. Effect of DAMGO on autaptic current amplitude and GABA-mediated responses in VTA GABAergic neurons in micro-dot culture. (A) Phase contrast image of an isolated VTA neuron (note patch pipette on the left). White bar represents 10  $\mu\text{m}$ . (B) Image of an isolated VTA neuron sensitive to DAMGO after immunostaining for glutamic acid decarboxylase, to confirm the cell's GABAergic phenotype. This cell was also immuno-negative for tyrosine hydroxylase (not shown). White bar represents 10  $\mu\text{m}$  (different neuron than in A). (C) Whole-cell recording of an autaptic IPSC from an isolated GABAergic neuron (different neuron than in B). Stimulation with a brief voltage step ( $-40$  mV to 20 mV for 1 ms) triggered a fast inward sodium current (\*) followed by a slow outward GABA<sub>A</sub> receptor-mediated IPSC. DAMGO (1  $\mu\text{M}$ ) markedly decreased the IPSC amplitude (gray trace) and this effect was reversible (Wash). (D) Membrane current recording in a different isolated GABA neuron. The neuron was stimulated to evoke an autaptic IPSC. After a delay of 500 ms, GABA (500  $\mu\text{M}$ ) was applied to the dendrites of the same neuron through pressure application (15 psi, 3.5 s), from a locally positioned pipette (black bar). This generated an outward current. The IPSC was reduced by DAMGO (1  $\mu\text{M}$ ) but the GABA membrane current was unchanged (gray trace). Both the IPSC and the GABA current were blocked by SR95531 (5  $\mu\text{M}$ ), a GABA<sub>A</sub> receptor antagonist. Each trace is an average of 3–5 sweeps. The sodium current was subtracted out of the traces for clarity. (E) Time course of autaptic IPSC and exogenous GABA-mediated current amplitude expressed as percentage of control (IPSC,  $n = 18$ ; GABA current,  $n = 6$ ). DAMGO significantly reduced IPSC amplitude but had no effect on GABA-mediated currents.

a postsynaptic mechanism to inhibit GABA-mediated IPSCs.

### 3.4. DAMGO inhibits the frequency of mIPSCs

As an alternate strategy to determine whether  $\mu$ -opioid receptor activation inhibited evoked GABA IPSCs through a pre- or postsynaptic mechanism, the effect of DAMGO (1  $\mu$ M) on spontaneous GABAergic mIPSCs was examined. Recordings were performed on standard VTA cultures in the presence of CNQX (10  $\mu$ M) to block glutamate-mediated synaptic currents and TTX (0.5  $\mu$ M) to eliminate action potential-evoked GABAergic events. Experiments were performed at a holding potential between  $-10$  mV and  $10$  mV, at which mIPSCs appeared as outward currents, as illustrated in Fig. 3A. DAMGO significantly decreased the frequency of mIPSCs but not their amplitude. The frequency decreased by  $48.2 \pm 6.5\%$  ( $n = 14$ ,  $P < 0.05$ ; Fig. 3A, B) while the amplitude was unaffected ( $n = 14$ ,  $P > 0.05$ ). The average frequency of mIPSCs was  $5.2 \pm 1.8$  Hz in control, as opposed to  $2.6 \pm 1.0$  Hz in the presence of DAMGO. This decrease in frequency could be observed as a reversible shift of the cumulative probability distribution of mIPSC inter-event intervals (Fig. 3C). DAMGO caused a significant change of the inter-event intervals in 10 of the 14 cells tested (Kolmogorov–Smirnov test,  $P < 0.01$ ). Analysis of mIPSC amplitude cumulative probability distributions showed that, during these experiments, no significant

change in mIPSC amplitude was detected in 11 of the 14 cells tested (Kolmogorov–Smirnov test,  $P > 0.01$ ), as illustrated in Fig. 3D. Hence, these results suggest that DAMGO acts on  $\mu$ -opioid receptors located at the presynaptic terminals of GABAergic neurons of the VTA.

Although mIPSCs are often independent of the entry of  $\text{Ca}^{2+}$  in the synaptic terminal, mIPSCs that are dependent on the entry of  $\text{Ca}^{2+}$  in the terminal via voltage-dependent  $\text{Ca}^{2+}$  channels have been observed in certain preparations (Ogura and Kita, 2000; Koyama et al., 2000). To determine which of these two classes of mIPSCs are present under our experimental conditions, we tested the effect of the broad spectrum  $\text{Ca}^{2+}$  channel antagonist cadmium ( $\text{Cd}^{2+}$ ) on mIPSC frequency (Fig. 4A). Application of  $\text{Cd}^{2+}$  (100  $\mu$ M) decreased the mIPSC frequency by  $54.7 \pm 7.1\%$  ( $n = 10$ ,  $P < 0.05$ ). In this set of experiments, the overall mIPSC frequency was  $3.2 \pm 1.0$  Hz in control condition and  $1.0 \pm 0.3$  Hz in the presence of  $\text{Cd}^{2+}$ . These results suggest that a component of the basal frequency of mIPSCs was  $\text{Ca}^{2+}$ -dependent in control conditions. Since the rate of release is low in the presence of  $\text{Cd}^{2+}$ , it is to be expected that the detection of a decrease in basal mIPSC frequency might become difficult under such conditions. To circumvent this problem, we performed additional experiments at a bath temperature of  $\sim 30$  °C based on the long-standing observation that the frequency of miniature synaptic events increases with temperature (Fatt and Katz, 1952). As expected, this caused a marked increase in basal

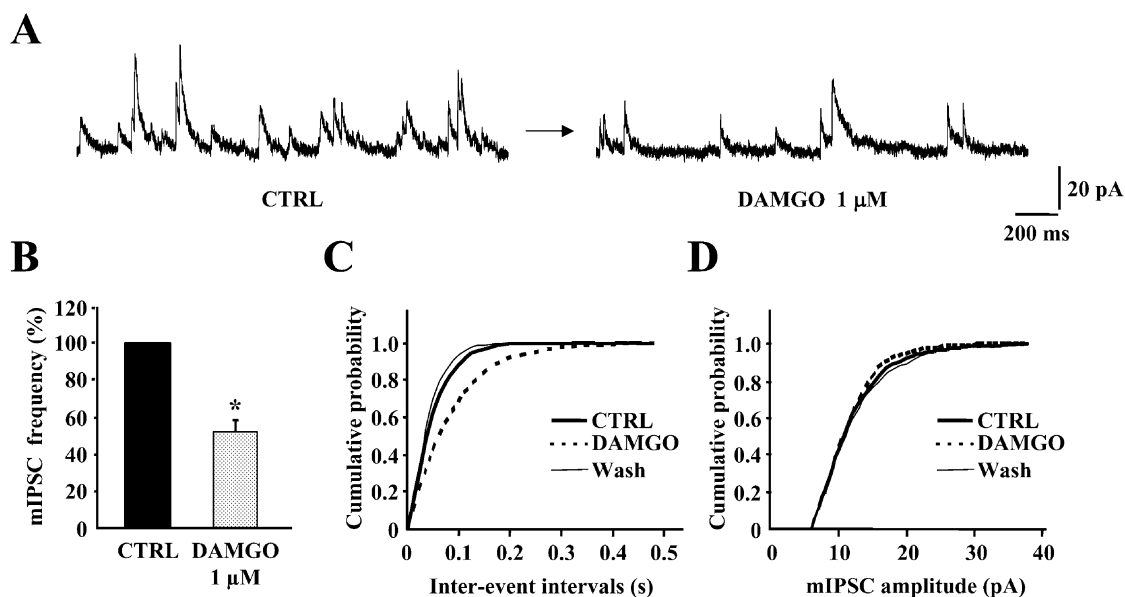


Fig. 3. Effect of DAMGO on spontaneous miniature inhibitory postsynaptic currents (mIPSCs). (A) Whole-cell recording of mIPSCs from a representative experiment under control conditions and in the presence of DAMGO (1  $\mu$ M). The frequency of spontaneous mIPSCs was markedly decreased by DAMGO (right). (B) Summary histogram of the mean mIPSC frequency expressed as percentage of control. The mIPSC frequency was decreased by DAMGO ( $n = 14$ ) (\*,  $P < 0.05$ ). (C) Cumulative probability distribution of inter-event intervals from a representative experiment. DAMGO caused a significant change in inter-event intervals and this effect was reversible (Wash). (D) Cumulative probability distribution of mIPSC amplitudes from the same experiment as in (C). No significant change in mIPSC amplitude was caused by DAMGO.

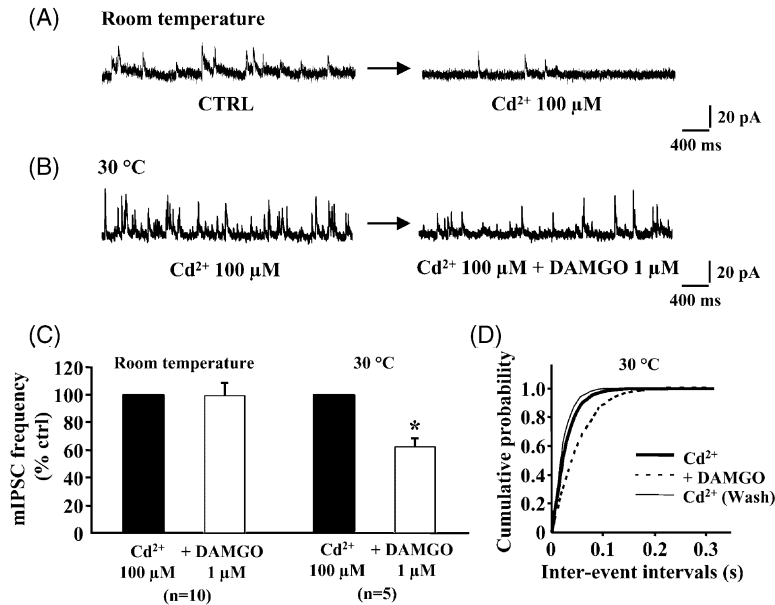


Fig. 4. Effect of Ca<sup>2+</sup> channel blockade on mIPSC modulation by DAMGO. (A) Whole-cell recording of mIPSCs at room temperature from a representative experiment. The frequency of mIPSCs was significantly decreased in the presence of the Ca<sup>2+</sup> channel blocker Cd<sup>2+</sup> (100 μM; right). (B) Whole-cell recording of mIPSCs in the presence of Cd<sup>2+</sup> (100 μM) at 30 °C. At such higher temperature, the frequency of spontaneous mIPSCs was markedly decreased by DAMGO in the presence of Cd<sup>2+</sup> (right). (C) Summary histogram of the mean mIPSC frequency expressed as percentage of control. DAMGO failed to decrease mIPSC frequency in the presence of Cd<sup>2+</sup> at room temperature ( $n = 10$ ). However, at 30 °C, DAMGO caused a significant decrease of mIPSC frequency in the presence of Cd<sup>2+</sup> ( $n = 5$ ) (\*,  $P < 0.05$ ). (D) Cumulative probability distribution of inter-event intervals from a representative experiment. DAMGO caused a significant change in inter-event intervals in the presence of Cd<sup>2+</sup> at 30 °C. This effect was reversible after washout of DAMGO (Wash).

mIPSC frequency (to  $27.5 \pm 7.2$  Hz) (Fig. 4B). This was accompanied by a decrease in the time constant of decay of these events which was  $17.3 \pm 0.2$  ms at room temperature (9189 events analyzed in five cells), as opposed to  $12.0 \pm 0.1$  ms at 30 °C (19 340 events analyzed in five cells). We then examined the ability of DAMGO to inhibit mIPSC frequency in the presence of Cd<sup>2+</sup>. At room temperature, no significant decrease in mIPSC frequency was observed following the application of DAMGO in the presence of Cd<sup>2+</sup> (Fig. 4C). The mIPSC frequency in the presence of DAMGO was  $98.8 \pm 9.1\%$  of the control value ( $n = 10$ ,  $P > 0.05$ ) and the analysis of mIPSC inter-event intervals showed that DAMGO caused no significant change of the inter-event intervals in the 10 cells tested (Kolmogorov–Smirnov test,  $P > 0.01$ ; not shown). However, at 30 °C, DAMGO was able to reliably decrease mIPSC frequency by  $38.2 \pm 6.3\%$  in the presence of Cd<sup>2+</sup> ( $n = 5$ ,  $P < 0.05$ ; Fig. 4B, C). This decrease in frequency could be observed as a reversible shift of the cumulative probability distribution of mIPSC inter-event intervals (Fig. 4D). DAMGO caused a significant change of the inter-event intervals in all five cells tested (Kolmogorov–Smirnov test,  $P < 0.01$ ). Overall, these results suggest that one of the mechanisms by which DAMGO acts on presynaptic terminals is independent of the activation of voltage-dependent Ca<sup>2+</sup> channels.

### 3.5. DAMGO inhibits ionomycin-evoked mIPSCs

The decrease in mIPSC frequency evoked by μ-opioid receptor stimulation in the absence of Cd<sup>2+</sup> could be due to either of two mechanisms. A first mechanism is that μ-opioid receptor activation may produce a hyperpolarization of nerve terminals leading to a reduction of Ca<sup>2+</sup> influx through voltage-dependent Ca<sup>2+</sup> channels. A second possibility is that μ-opioid receptor activation leads to a direct inhibition of the secretory process. Although our results with Cd<sup>2+</sup> provide evidence for the second model, we performed additional experiments to distinguish between these two hypotheses. We first examined the effect of DAMGO (1 μM) on the frequency of asynchronous mIPSCs evoked by the Ca<sup>2+</sup> ionophore ionomycin (2.5 μM). Ionomycin is known to elicit neurotransmitter release by directly producing an elevation of intra-terminal Ca<sup>2+</sup>. Its effect is fully independent of the activation of voltage-dependent Ca<sup>2+</sup> channels in the presynaptic terminal and completely insensitive to the Ca<sup>2+</sup> channel blocker Cd<sup>2+</sup> (Capogna et al., 1996). A marked increase of mIPSC frequency was observed following a brief (2–3 min) application of ionomycin (2.5 μM) (Fig. 5A, B). This increase was reversible within a 5 min washout period (Fig. 5B). After 10 min of washout and a recovery of the mIPSC frequency, a second stimulation with ionomycin was then

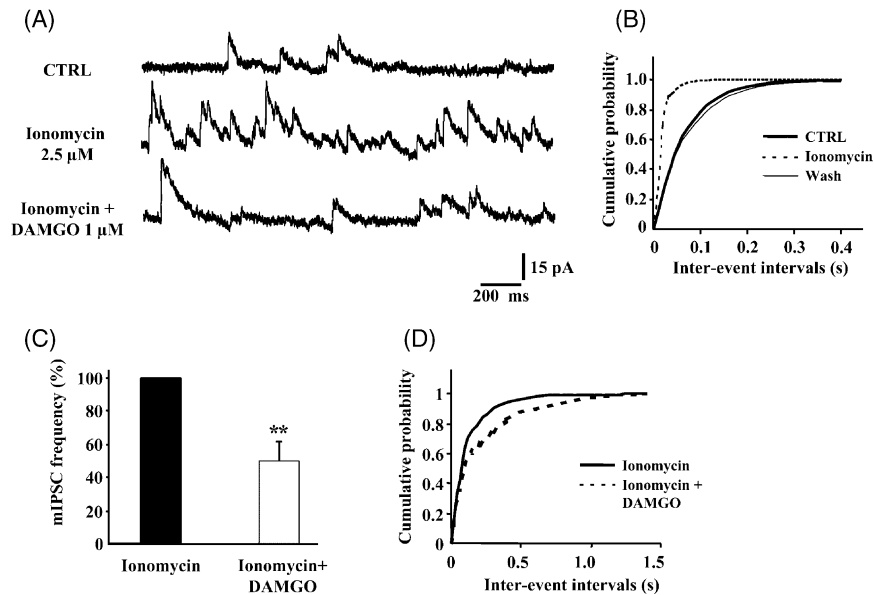


Fig. 5. Effect of DAMGO on mIPSCs triggered by ionomycin. (A) Whole-cell recording of mIPSCs. Perfusion of ionomycin (2.5  $\mu$ M) increased the frequency of mIPSCs. The frequency of ionomycin-evoked mIPSCs was markedly decreased by DAMGO (1  $\mu$ M). (B) Cumulative probability distribution of inter-event intervals from a representative experiment. Ionomycin caused a leftward shift of the distribution curve and this effect was reversible (Wash). (C) Summary histogram. DAMGO caused a significant decrease in ionomycin-evoked release ( $n = 5$ ) (\*\*,  $P < 0.01$ ). (D) Cumulative probability distribution of inter-event intervals from a representative experiment. DAMGO caused a rightward shift in the distribution curve of inter-events intervals of ionomycin-evoked events.

performed in the presence of DAMGO (1  $\mu$ M). Finally, after an additional 10 min washout of DAMGO, a third application of ionomycin was performed. To quantify the ionomycin-induced increase of mIPSCs frequency, we counted the number of mIPSCs during a 60 seconds period at the peak of the ionomycin effect and compared it with the number of mIPSCs 60 seconds before ionomycin application, thus calculating a delta score. This was performed for each stimulation. The delta value of the stimulation with ionomycin in the presence of DAMGO was compared to the mean delta value of the first and third stimulations. We found that DAMGO reduced ionomycin-induced release by  $50.6 \pm 12.4\%$  ( $n = 5$ ,  $P < 0.01$ ; Fig. 5C, D). These results confirm that DAMGO can inhibit quantal GABA release by a mechanism that does not involve presynaptic voltage-dependent  $\text{Ca}^{2+}$  channels.

### 3.6. 4-Aminopyridine inhibits the effects of DAMGO

It has been previously suggested that  $\mu$ -opioid receptor activation can decrease GABA release in the periaqueductal gray nucleus by activating 4-AP-sensitive  $\text{K}^+$  channels leading to the hyperpolarization of nerve terminals (Vaughan et al., 1997). To test the possibility that the action of DAMGO could be mediated by such a voltage-dependent  $\text{K}^+$  channel, we first examined the effect of the voltage-dependent  $\text{K}^+$  channel blocker 4-aminopyridine (4-AP; 100  $\mu$ M and 1 mM) on the DAMGO-induced inhibition of autaptic IPSCs. In these experi-

ments, DAMGO was applied twice to isolated GABAergic neurons: it was first applied in the presence of the antagonist, then again alone after washout of the antagonist. By itself, 4-AP often produced an increase in autaptic IPSC amplitude (Fig. 6A, left). This effect was highly variable. At 100  $\mu$ M, the increase was  $32.9 \pm 15.6\%$ , while at 1 mM, it was  $86.2 \pm 51.3\%$ . In the presence of 4-AP, the ability of DAMGO to inhibit IPSC amplitude was significantly reduced (Fig. 6B). In experiments where 4-AP was used at 100  $\mu$ M, DAMGO reduced IPSC amplitude by  $27.5 \pm 8.9\%$  in comparison to  $56.4 \pm 4.8\%$  in the absence of 4-AP ( $n = 8$ ,  $P < 0.02$ ; Fig. 6C). Approximately 50% of the effect of DAMGO was thus blocked. In the presence of 1 mM 4-AP, the block was almost complete ( $8.2 \pm 3.4\%$  inhibition versus  $43.3 \pm 7.5\%$ ,  $n = 6$ ,  $P < 0.01$ ; Fig. 6B, C). It appears that the block of DAMGO's ability to inhibit IPSCs was not caused by some kind of "saturation" of evoked release due to the enhancement of IPSC amplitude by 4-AP alone. This is illustrated by the complete lack of correlation between the ability of 4-AP to increase IPSC amplitude by itself and its ability to reduce DAMGO's presynaptic inhibition, both at 100  $\mu$ M 4-AP ( $r = -0.03$ , Pearson,  $P = 0.99$ ) and at 1 mM ( $r = -0.32$ , Pearson,  $P = 0.53$ ) (Fig. 6A, right). These results are compatible with the involvement of a 4-AP-sensitive  $\text{K}^+$  channel in the effect of DAMGO in VTA GABAergic neurons.

Because  $\mu$ -opioid receptors can also activate G protein-gated inward rectifying  $\text{K}^+$  (GIRK) channels, at least



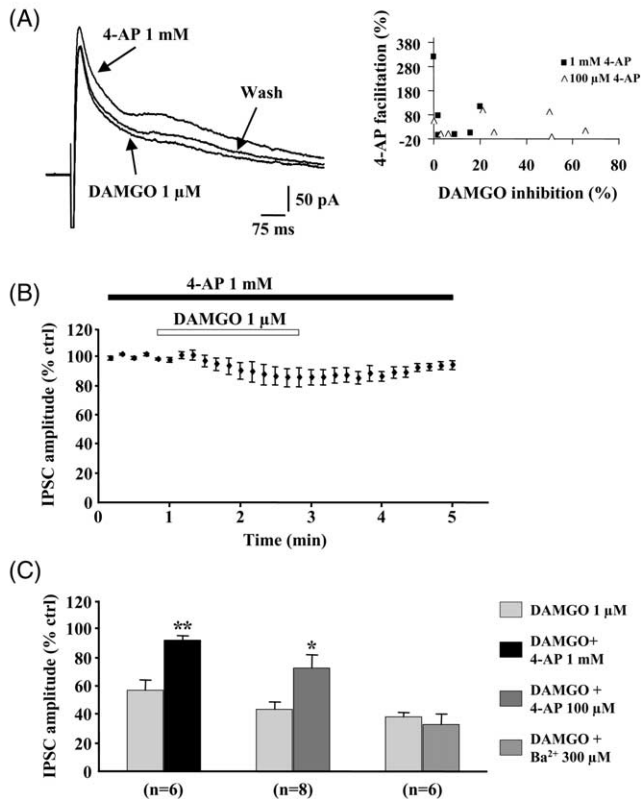


Fig. 6. Mechanism of autaptic current amplitude decrease by DAMGO. (A) Whole-cell recordings of autaptic IPSCs in the presence of 4-AP (1 mM) from the same cell as in Fig. 2C. DAMGO failed to decrease IPSC amplitude in the presence of 4-AP. The graph on the right shows that there is no correlation between the ability of 4-AP to increase IPSC amplitude and its ability to block DAMGO-mediated inhibition of IPSCs. (B) Time course of autaptic IPSC amplitude in the presence of 4-AP (1 mM) expressed as percentage of control ( $n = 6$ ). (C) Summary histogram of mean IPSC amplitude expressed as percentage of control. The effect of DAMGO was significantly blocked by 1 mM 4-AP ( $n = 6$ ) (\*\*,  $P < 0.01$ ) and by 100  $\mu\text{M}$  4-AP ( $n = 8$ ) (\*,  $P < 0.05$ ). However,  $\text{Ba}^{2+}$  (300  $\mu\text{M}$ ) failed to block the effect of DAMGO ( $n = 6$ ).

in somatodendritic compartments (Williams et al., 1988; Johnson and North, 1992a; Wimpey and Chavkin, 1991; Han et al., 1999), we next used barium ( $\text{Ba}^{2+}$ ), a general blocker of GIRK channels, to determine whether a component of DAMGO's effect was dependent on a GIRK-type channel. At 300  $\mu\text{M}$ , usually a maximally effective concentration,  $\text{Ba}^{2+}$  failed to significantly modify IPSC amplitude by itself. The amplitude of IPSCs in the presence of  $\text{Ba}^{2+}$  was at  $96.7 \pm 3.3\%$  of the control value ( $n = 6$ ,  $P > 0.05$ ; not shown). Barium also failed to significantly diminish the ability of DAMGO to reduce IPSC amplitude. In the presence of  $\text{Ba}^{2+}$ , DAMGO reduced IPSC amplitude by  $66.6 \pm 6.5\%$  in comparison to  $62.2 \pm 4.1\%$  in the absence of the antagonist ( $n = 6$ ,  $P > 0.05$ ; Fig. 6C). These results argue against the implication of a GIRK-type  $\text{K}^+$  channel in the inhibition of GABA release by  $\mu$ -opioid receptors in isolated GABAergic neurons.

We next tested the effect of 4-AP (1 mM) on the DAMGO-induced inhibition of spontaneous mIPSC frequency. 4-AP itself caused no significant change in mIPSC frequency ( $102.2 \pm 6.5\%$ ,  $n = 7$ ,  $P > 0.05$ ; not shown). We found that DAMGO failed to cause any significant change of mIPSC frequency in the presence of 4-AP (Fig. 7A). The mIPSC frequency in the presence of DAMGO was at  $94.7 \pm 8.4\%$  of the control value ( $n = 7$ ,  $P > 0.05$ ). Thus, DAMGO caused an average frequency reduction of 5% in the presence of 4-AP ( $n = 7$ ), as opposed to a 48% inhibition in its absence ( $n = 14$ ; Fig. 7A).

Considering on the one hand that 4-AP is expected to reduce presynaptic inhibition by preventing permeation through  $\text{K}^+$  channels and membrane hyperpolarization,

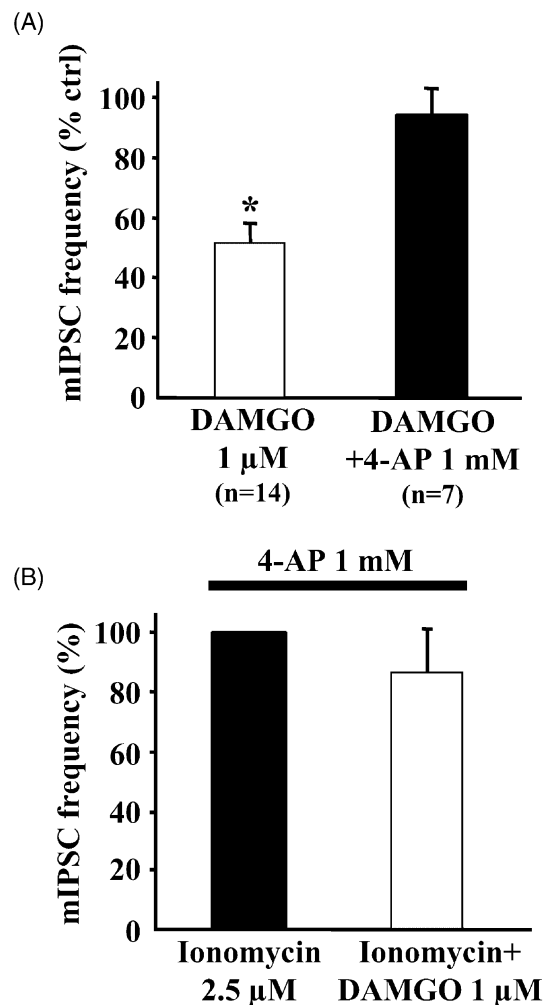


Fig. 7. Mechanism of mIPSC frequency decrease by DAMGO. (A) Histogram of mean mIPSC frequency expressed as percentage of control. DAMGO significantly decreased mIPSC frequency ( $n = 14$ ) (\*,  $P < 0.05$ ) (same data as in Fig. 3B). This effect was blocked in the presence of 4-AP (1 mM) ( $n = 7$ ). (B) Histogram of ionomycin-evoked release expressed as percentage of control. The DAMGO-induced decrease of ionomycin-evoked release was abolished in the presence of 4-AP (1 mM) ( $n = 4$ ).

and on the other hand that ionomycin evokes mIPSCs independently of membrane polarization, a prediction would be that the modulation of ionomycin-evoked mIPSCs by DAMGO should not be blocked by 4-AP. Unexpectedly, we found that in the presence of 4-AP (1 mM), DAMGO failed to cause a significant change of ionomycin-induced release. The ionomycin-evoked release in the presence of DAMGO was at  $86.7 \pm 14.4\%$  of the control value ( $n = 4$ ,  $P > 0.05$ ; Fig. 7B).

#### 4. Discussion

In the present work we have used cultured VTA neurons to investigate how  $\mu$ -opioid receptors inhibit GABA release. We found that GABA-mediated IPSCs evoked by single action potentials were reversibly inhibited by DAMGO, a selective  $\mu$ -opioid receptor agonist. This was accompanied by a decrease in the frequency but not the amplitude of both spontaneous and ionomycin-evoked mIPSCs, suggesting that  $\mu$ -opioid receptors localized on GABAergic nerve terminals directly inhibit quantal GABA release. In addition, we found that this presynaptic inhibition is blocked by 4-AP, a  $K^+$  channel blocker. Taken together, these results suggest that  $\mu$ -opioid receptor activation inhibits the efficacy of the secretory process in GABAergic nerve terminals by a mechanism that is influenced by  $K^+$  channels.

##### 4.1. Presynaptic site of action

It has been established that exogenously applied  $\mu$ -opioid receptor agonists can inhibit the activity of VTA GABAergic neurons through a postsynaptic mechanism of action, i.e. somatodendritic hyperpolarization (Johnson and North, 1992a). However,  $\mu$ -opioid receptors could also be localized on presynaptic terminals and directly regulate GABA release (Sesack and Pickel, 1995). In the present study, we report that the activation of  $\mu$ -opioid receptors located on the terminals of VTA GABAergic neurons reduces GABA release through a presynaptic mechanism.

The  $\mu$ -opioid agonists DAMGO and met-enkephalin decreased the frequency of sIPSCs. Because these events are generated by spontaneously firing GABAergic neurons, this observation is consistent with the previously described reduction in cellular excitability of VTA GABAergic neurons induced by  $\mu$ -opioid receptor agonists (Johnson and North, 1992a). However, our finding that the amplitude of sIPSCs and of evoked autaptic IPSCs is decreased by DAMGO and by met-enkephalin suggests that  $\mu$ -opioid receptor agonists also decrease GABA release through other excitability-independent mechanisms. Our data demonstrate that this  $\mu$ -opioid receptor-mediated inhibition of GABA release is most

likely to involve, at least in part, a presynaptic mechanism of action. Indeed, DAMGO reduced the frequency of spontaneous mIPSCs without altering their amplitude, providing good evidence that functional  $\mu$ -opioid receptors are present on the synaptic terminals of VTA GABAergic neurons. In addition, DAMGO failed to decrease the amplitude of membrane currents evoked by pressure application of exogenous GABA. The effects described in the current study were most likely due to the activation of  $\mu$ -opioid receptors since the DAMGO-mediated inhibition of sIPSC frequency and amplitude was blocked by the opioid receptor antagonist naloxone and by the highly selective  $\mu$ -opioid receptor antagonist CTOP. It should be noted that, in the present study, IPSCs were measured either in randomly selected neurons (sIPSCs) or in GABAergic neurons themselves (autaptic IPSCs). Although our experiments have allowed us to analyze the general mechanism by which terminal  $\mu$ -opioid receptors inhibit GABA release in these neurons, we have not explored the possibility that such a mechanism could be different whether GABAergic synapses are established on GABAergic neurons or on dopaminergic neurons, the latter being their suspected natural targets *in vivo*. We have also not explored the nature of the heterogeneity in the sensitivity of GABAergic neurons in our model to  $\mu$ -opioid receptor activation. We have observed that 18 out of 45 isolated GABAergic neurons were sensitive to DAMGO. The lack of response from the insensitive neurons could be due to a genuine heterogeneity in the properties of VTA GABAergic neurons, although we are not aware of any additional data directly addressing this issue. Alternately, it may be hypothesized that in a micro-culture system, neurons may lack some kind of trophic support required to maintain high levels of  $\mu$ -opioid receptor expression. The all-or-none nature of the sensitivity to DAMGO argues in favor of the first possibility.

##### 4.2. Direct inhibition of the secretory process

Several presynaptic mechanisms could account for the modulation of GABA release by terminal  $\mu$ -opioid receptors in the VTA. It has been proposed that in general, presynaptic G-protein-linked receptors can inhibit neurotransmitter release via three major mechanisms: (1) direct inhibition of  $Ca^{2+}$  channels located in the terminals (Yawo and Chuhma, 1993; Bourinet et al., 1996; Wu and Saggau, 1994); (2) activation of presynaptic  $K^+$  channels leading to action potential shunting and indirect reduction in  $Ca^{2+}$  influx (Thompson et al., 1993; Simmons and Chavkin, 1996); and (3) direct modulation of the release machinery (Trudeau et al., 1996; Bouron, 2001; Capogna et al., 1996). In the present work, we demonstrate that a direct inhibition of the release machinery occurs following the activation of  $\mu$ -opioid receptors located on the terminals of VTA GABAergic

neurons. Such a conclusion is based on the following two observations. First, in the presence of the voltage-dependent  $\text{Ca}^{2+}$  channel blocker  $\text{Cd}^{2+}$ , DAMGO still caused a decrease in mIPSC frequency (at 30 °C). Second, in experiments where ionomycin was used to trigger  $\text{Ca}^{2+}$ -dependent exocytosis under conditions where  $\text{Ca}^{2+}$  channel activation was bypassed, we found that the  $\mu$ -opioid receptor agonist DAMGO still caused a decrease in mIPSC frequency. Considering these observations, it is somewhat surprising that the frequency of spontaneously generated mIPSCs was not decreased by DAMGO at room temperature in the presence of  $\text{Cd}^{2+}$ . A reasonable explanation for this finding is that under conditions of low release rate, one's ability to detect a decrease in basal mIPSC frequency is limited. We may have been incapable of detecting a small decrease in basal mIPSC frequency under such conditions. Our finding that DAMGO inhibits mIPSC frequency at 30 °C provides good evidence that this explanation is correct.

In summary, our findings on spontaneous and ionomycin-evoked mIPSCs suggest that  $\mu$ -opioid receptor activation directly inhibits the secretory process in VTA GABAergic neurons. Although most of the literature suggests that spontaneously generated miniature synaptic currents are usually modulated in the same way as action potential-evoked synaptic currents, the specific function of miniature events is not completely defined (Bouron, 2001) and it is difficult to reject the possibility that, under some circumstances, both types of events could be modulated differently. Nonetheless, the mechanism identified in the present work using mIPSC recordings is thus likely to contribute, at least in part, to the inhibition of action potential-evoked GABA release. In addition, it is important to note that the experiments performed with ionomycin involved the measurement of synaptic events which, like action potential-evoked IPSCs, were directly triggered by a rise in intracellular  $\text{Ca}^{2+}$  (although independently from voltage-dependent  $\text{Ca}^{2+}$  channels). The demonstration that such events are inhibited to about the same extent as action potential-evoked IPSCs increases the likelihood that the detected modulation is physiologically relevant.

#### 4.3. Implication of presynaptic $\text{K}^+$ channels

A modulation of 4-AP-sensitive  $\text{K}^+$  channels by opioid receptors has been reported in several areas of the brain such as the hippocampus (Wimpey and Chavkin, 1991; Simmons and Chavkin, 1996), the periaqueductal gray (Vaughan et al., 1997), the VTA (Manzoni and Williams, 1999), and the supraoptic nucleus (Muller et al., 1999). Based on these reports, we investigated the possibility that such  $\text{K}^+$  channels could mediate the effect of DAMGO on VTA GABAergic neurons. We demonstrated that the  $\text{K}^+$  channel blocker 4-

AP abolished the inhibition of autaptic IPSCs by DAMGO. This block is not likely to have been caused by some kind of occlusion due to the enhancement of GABA release caused by 4-AP itself. This can be concluded because the ability of 4-AP to cause an increase in IPSC amplitude was highly variable and there was no correlation between the ability of 4-AP to increase IPSC amplitude and its ability to block DAMGO-mediated inhibition of IPSCs. Indeed, in a number of neurons, the DAMGO-mediated inhibition was blocked in cells which showed little if any increase in IPSC amplitude in the presence of 4-AP alone. A 4-AP-sensitive modulation of IPSCs by  $\mu$ -opioid receptor agonists has been previously described in other brain areas such as the periaqueductal gray (Vaughan et al., 1997) and in glutamatergic projections to the VTA (Manzoni and Williams, 1999).

#### 4.4. Implication of presynaptic $\text{Ca}^{2+}$ channels

The inability of DAMGO to reduce autaptic IPSCs in the presence of 4-AP provides evidence which suggests that a direct inhibition of  $\text{Ca}^{2+}$  channels in nerve terminals is not necessarily involved in presynaptic inhibition mediated through the  $\mu$ -opioid receptor in our preparation. Indeed, if  $\mu$ -opioid receptor activation directly inhibited  $\text{Ca}^{2+}$  influx through voltage-dependent  $\text{Ca}^{2+}$  channels in synaptic terminals, one would have expected a significant fraction of the inhibitory effect of DAMGO to remain in the presence of 4-AP. This observation argues against the idea that  $\text{Ca}^{2+}$  channel modulation is critical for the presynaptic effects of  $\mu$ -opioid receptors. However, it is important to note that we have not directly evaluated the activity of terminal  $\text{Ca}^{2+}$  channels in our experiments and thus cannot formally exclude that DAMGO, in addition to inhibiting the secretory process, also caused some reduction in terminal  $\text{Ca}^{2+}$  influx during action potentials.

#### 4.5. Role of $\text{K}^+$ channel modulation in secretory process inhibition

We found that 4-AP also blocked the DAMGO-induced decrease of mIPSC frequency. These results suggest that the  $\mu$ -opioid receptor-mediated decrease of GABA release involves the activation of a 4-AP-sensitive  $\text{K}^+$  channel located on presynaptic terminals of VTA GABAergic neurons. Taken alone, this finding suggests a mechanism whereby  $\mu$ -opioid receptor-mediated inhibition of GABA release is due to the hyperpolarization of the GABAergic terminals following the activation of a presynaptic  $\text{K}^+$  conductance. This would in turn decrease  $\text{Ca}^{2+}$  influx in the terminal and thus reduce synaptic vesicle exocytosis. Such a mechanism would cause a decrease in mIPSC frequency only if a component of the basal mIPSC frequency was  $\text{Ca}^{2+}$ -mediated. Although

this was the case under our experimental conditions, this simple mechanism cannot account for the rest of our observations. Indeed, as mentioned above, we found that the  $\mu$ -opioid receptor agonist DAMGO inhibited ionomycin-evoked mIPSCs. Because events evoked in such a way are not expected to be influenced by the opening of  $K^+$  channels and membrane hyperpolarization, our results suggest that the inhibition of GABA release induced by  $\mu$ -opioid receptor activation is mediated through a direct regulation of the secretory process. The ability of 4-AP to block the modulation of ionomycin-evoked GABA release is thus difficult to explain unless the  $K^+$  channel subunit(s) to which 4-AP binds in nerve terminals directly interacts with the secretory apparatus and is able to influence the efficacy of exocytosis through mechanisms other than membrane hyperpolarization. It is interesting to note that recent work from Linial and colleagues has demonstrated that some 4-AP-sensitive  $K^+$  channel subunits are able to directly interact with the synaptic protein syntaxin (Fili et al., 2001), a key component of the molecular complex involved in mediating synaptic vesicle exocytosis. The physiological significance of this observation is not fully understood, but it could provide a molecular basis for our current results as well as those obtained in the periaqueductal gray and on glutamatergic afferents to VTA dopaminergic neurons (Vaughan et al., 1997; Manzoni and Williams, 1999).

Because 4-AP (1 mM) blocked essentially all of DAMGO's effect on action potential-evoked IPSCs and on mIPSC frequency, it can be considered unlikely that other 4-AP-insensitive classes of  $K^+$  channels are also involved in mediating presynaptic inhibition induced by  $\mu$ -opioid receptor activation. Although  $\mu$ -opioid receptors are well known to activate somatodendritic G protein-gated inward rectifying  $K^+$  channels (GIRKs) (Williams et al., 1988; Johnson and North, 1992a; Wimpsey and Chavkin, 1991; Han et al., 1999), these are not known in most preparations to be localized on nerve terminals. In agreement with these results, we found that the GIRK-type antagonist  $Ba^{2+}$  failed to decrease the ability of  $\mu$ -opioid receptor activation to inhibit autaptic IPSCs. In the present work we have not attempted to identify the molecular nature of the  $K^+$  channels involved. Nonetheless, our finding that these channels are insensitive to  $Ba^{2+}$  and sensitive to relatively low concentrations of 4-AP (100  $\mu$ M) suggests the implication of A-type ( $I_A$ )-like  $K^+$  channels. Much additional work will be required to identify further the specific types of  $K^+$  channels involved. Finally, an alternative explanation that cannot yet be excluded is that 4-AP binds to other molecular targets in nerve terminals besides voltage-dependent  $K^+$  channels. To our knowledge, no other such targets have been identified.

Several presynaptic G protein-linked receptors have been reported to act directly on the release machinery,

including the A1 adenosine receptor (Scholz and Miller, 1992; Trudeau et al., 1996; Capogna et al., 1996), the GABA<sub>B</sub> receptor (Capogna et al., 1996), the somatostatin receptor (Boehm and Betz, 1997), the 5-HT<sub>1A</sub> serotonin receptor (Koyama et al., 1999) and the dopamine D2 receptor (Congar et al., 2002). In addition, a mechanism implicating a modulation of the secretory process has also been suggested for  $\mu$ -opioid receptor-mediated presynaptic inhibition of GABA release from GABAergic interneurons of the CA3 area of the hippocampus (Capogna et al., 1993, 1996). Although our results do not directly exclude the implication of a modulation of terminal  $Ca^{2+}$  channels as a contributing mechanism, taken together, our present results provide evidence that a late-step mechanism is involved in the ability of terminal  $\mu$ -opioid receptors to inhibit the synaptic release of neurotransmitter from VTA GABAergic interneurons. In addition, our results suggest the possibility that presynaptic inhibition mediated by  $\mu$ -opioid receptors is under the regulatory control of a novel type of mechanism implicating voltage-dependent  $K^+$  channels, but not necessarily  $K^+$  ion flux and membrane hyperpolarization. Such a mechanism of presynaptic inhibition could be broadly expressed in the central nervous system. In support of such a possibility, we have recently reported that the dopamine D2 receptor causes presynaptic inhibition through a very similar mechanism in cultured dopamine neurons (Congar et al., 2002). Further experiments will be required to determine the precise molecular mechanism involved.

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