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DAMGO DEPRESSES INHIBITORY SYNAPTIC TRANSMISSION VIA DIFFERENT DOWNSTREAM PATHWAYS OF μ OPIOID RECEPTORS IN VENTRAL TEGMENTAL AREA AND PERIAQUEDUCTAL GRAY

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Abstract—Opioid-induced rewarding and motorstimulant effects are mediated by an increased activity of the ventral tegmental area (VTA) dopamine (DA) neurons. The excitatory mechanism of opioids on VTA-DA neurons has been proposed to be due to the depression of GABAergic synaptic transmission in VTA-DA neurons. However, how opioids depress GABAergic synaptic transmission in VTA-DA neurons remain to be studied. In the present study, we explored the mechanism of the inhibitory effect of [D-Ala^2, N-Me-Phe^4, Gly^5-ol]-enkephalin (DAMGO) on GABAergic synaptic transmission in VTA-DA neurons using multiple approaches and techniques. Our results showed that (1) DAMGO inhibits GABAergic inputs in VTA-DA neurons at presynaptic sites; (2) effect of DAMGO on GABAergic inputs in VTA-DA neurons is inhibited by potassium channel blocker 4-aminopyridine (4-AP) and G_i protein inhibitor *N*-ethyl maleimide (NEM); (3) phospholipase A_2 (PLA_2) does not mediate the effect of DAMGO on GABAergic inputs in VTA-DA neurons, but mediates it in the periaqueductal gray (PAG); (4) multiple downstream signaling molecules of μ receptors do not mediate the effect of DAMGO on GABAergic inputs in VTA-DA neurons. These results suggest that DAMGO depresses inhibitory synaptic transmission via μ receptor-G_i protein-K_v channel pathway in VTA-DA neurons, but via μ receptor-PLA_2 pathway in PAG neurons.

Key words: DAMGO, μ receptors, PLA_2, VTA, PAG, GABA release.

INTRODUCTION

Many drugs of abuse, including opioids, are known to act at the ventral tegmental area (VTA) to produce their reinforcing properties. Microinjection of opioids into VTA produced conditioned place preference and an increase in spontaneous locomotor activity in rats (Devine and Wise, 1994; Badiani et al., 1995). Further experiments showed that these opioid-induced rewarding and motorstimulant effects were mediated by an increased activity of VTA dopamine (VTA-DA) neurons (Matthews and German, 1984; Johnson and North, 1992; Devine et al., 1993). For example, [D-Ala^2, N-Me-Phe^4, Gly^5-ol]-enkephalin (DAMGO), a hydrolysis-resistant derivative of endogenous enkephalin, could induce an increase in the firing rate of DA neurons when administered directly into VTA (Johnson and North, 1992).

The excitatory mechanism of opioids on VTA-DA neurons has been proposed to be due to the depression of GABAergic synaptic transmission in VTA-DA neurons (Johnson and North, 1992; Hjelmstad et al., 2013). Opioids first depressed GABAergic synaptic transmission in VTA-DA neurons by activating μ receptors and then disrupted the balance between inhibition and excitation on VTA-DA neurons in favor of excitatory side (Jalabert et al., 2011), thus promoting VTA-DA neuron excitation. However, how opioids depress GABAergic synaptic transmission in VTA-DA neurons remains to be studied.

Previous study showed that the site of the inhibitory effect of DAMGO on GABAergic synaptic transmission in cultured neurons of VTA was at the presynaptic site to inhibit GABA release from terminals of GABAergic neurons (Bergevin et al., 2002; Nugent et al., 2007). They also showed that the K_v channel blocker 4-aminopyridine (4-AP) could prevent DAMGO-induced inhibition of GABA release, suggesting that 4-AP-sensitive K_v channels might be implicated in the inhibition of GABA release by DAMGO. These results were consistent with the report from Johnson and North (1992) which showed that DAMGO could hyperpolarize

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Abbreviations: 4-AP, 4-aminopyridine; AA, arachidonic acid; $AACOCF_3$, arachidonyl trifluoromethyl ketone; AC, adenylyl cyclase; BIS I, bisindolylmaleimide I; DA, dopamine; DAMGO, [D-Ala^2,N-Me-Phe^4,Gly^5-ol]-enkephalin; DL-AP 5, DL-2-amino-5-phosphonopentanoic acid; DNQX, 6,7-dinitroquinoxaline-2,3-dione; DTT, DL-dithiothreitol; EDTA, ethylenediaminetetra acetic acid; EGTA, ethyleneglycoltetra acetic acid; GABA, γ-aminobutyric acid; H-89, *N*-[2-(p-bromocinnamylamino) ethyl]-5-isoquinolinesulfonamide dihydrochloride; HEPES, N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid; IPSC, inhibitory postsynaptic currents; NEM, *N*-ethylmaleimide; PAG, periaqueductal gray; PKA, protein kinase A; PKC, protein kinase C; PKG, protein kinase G; PLA_2, phospholipase A_2; PLC, phospholipase C; PPP, paired pulse ratio; PTX, pertussis toxin; TTX, tetrodotoxin; VTA, ventral tegmental area.

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VTA GABAergic neurons. These evidences strongly suggested that DAMGO, a selective μ-opioid receptor agonist, could inhibit presynaptic GABA release in VTA-DA neurons and 4-AP-sensitive K_v channel might be involved in. However, up to now, intracellular signaling pathways downstream of the activation of μ-opioid receptors have not been studied.

Since DAMGO had a similar inhibitory effect on presynaptic GABA release in neurons of periaqueductal gray (PAG) (Vaughan et al., 1997) and phospholipase A_2 (PLA_2) had been shown to be involved in the effect of DAMGO in PAG (Vaughan et al., 1997), here we studied whether PLA_2 was also involved in the effect of DAMGO in VTA-DA neurons. As a comparison, we also confirmed the role of PLA_2 in the inhibitory effect of DAMGO on GABAergic synaptic transmission in PAG neurons. In addition, we explored the possible involvement of other downstream signaling molecules of μ receptors in the DAMGO–induced inhibition of GABAergic synaptic transmission in VTA-DA neurons.

EXPERIMENTAL PROCEDURES

Preparation of slices and whole-cell recording

Male Sprague–Dawley rats (15–20 days old) were anesthetized with chloral hydrate (400 mg/kg, i.p.). All experimental procedures conformed to Fudan University and international guidelines on the ethical use of animals. All efforts were made to minimize animal suffering and to reduce the number of animals used. Brain slices were prepared according to procedures described previously (Li et al., 2011). Following decapitation, the brain was quickly removed and submerged in ice-cold cerebrospinal fluid (ACSF) containing (in mM): 126 NaCl, 21.4 $NaHCO_3$, 2.5 KCl, 1.2 NaH_2PO_4, 2.4 $CaCl_2$, 1.2 $MgSO_4$, 11.1 glucose, 0.4 ascorbic acid, saturated with 95% O_2/5% CO_2 (pH 7.4). A block of tissue containing VTA or PAG was cut and placed on a layer of moistened filter paper glued to the cutting stage of a vibratome (VT1200 S; Leica). Horizontal VTA slices or coronal PAG slices (250 μm thick) were prepared, incubated for at least one hour at 32 °C and transferred to a recording chamber where the slice was submerged in warmed ACSF.

Neurons of VTA or PAG were visualized using infrared Nomarski optics. Patch pipettes were filled with (in mM): 121 KCl, 6.8 NaCl, 2 $MgCl_2$, 2 ATP-K_2, 0.3 GTP-Na_3, 0.6 EGTA, and 10 HEPES (pH 7.2). Voltage and current signals were recorded with an Axopatch 200B amplifier (Axon) connected to a Digidata1400A interface (Axon). Data were digitized and stored on disks using pClamp (version 10.2; Axon). I_h and action potential duration were used to distinguish dopaminergic from GABAergic neurons in VTA. Resting membrane potential and action potentials were observed under the current-clamp mode and I_h was recorded under the voltage-clamp mode. If the steady-state h-current was greater than 60 pA during a step from −50 to −100 mV (Nugent et al., 2007) and the action potential duration was larger than 1.2 ms (Chieng et al., 2011), the neuron was considered to be a DA neuron. These electrophysiological criteria for the identification of DA neurons are established because they are matched with neurochemical identity by tyrosine hydroxylase (TH) immunohistochemistry (Margolis et al., 2003; Chieng et al., 2011). To record inhibitory postsynaptic currents (IPSCs), cells were held at −70 mV under a voltage-clamp mode. A concentric stimulating electrode (FHC) was placed 200–300 μm to the recording site in VTA or PAG at 0.1 Hz (single-pulse duration 100 μs). In all experiments recording IPSCs, 6,7-dinitroquinoxaline-2,3-dione (DNQX) (10 μM) and strychnine (1 μM) were added to block AMPA- and glycine-mediated synaptic currents, respectively. To observe paired-pulse ratio (PPR), two synaptic responses were evoked by a pair of stimuli given at short intervals (50 ms) at 0.1 Hz. Miniature IPSCs (mIPSCs) were recorded in sweeps of 2 s at a holding potential of −70 mV under the voltage-clamp mode in additional tetrodotoxin (TTX, 0.5 μM) and DL-2-amino-5-phosphonopentanoic acid (DL-AP 5) (50 μM) to block voltage-dependent sodium channels and NMDA receptors, respectively. The cell series resistance was monitored throughout the experiment. The experiments were discarded if the value of series resistance changed by more than 15% during the experiment.

Synaptosome preparation

Male Sprague–Dawley rats (200–240 g) were anesthetized with chloral hydrate (400 mg/kg, i.p.). Synaptosomes were prepared as described previously (Dong et al., 2005; Yang et al., 2014). The VTA and PAG tissues (from six rats) were dissected and homogenized in 0.32 M sucrose solution at 4 °C using the Art-Miccra D-8 tissue grinder with a motor-driven pestle rotating at 900 rpm. The homogenate was centrifuged at 3000*g* for 3 min at 4 °C. The supernatant was centrifuged at 14,500*g* for 12 min at 4 °C. The pellet was resuspended and loaded onto Percoll gradients consisting of three steps of 23%, 10%, and 3% Percoll in 0.32 M sucrose additionally containing 1 mM EDTA and 250 μM DL-dithiothreitol (DTT). The gradients were centrifuged at 32,500*g* for 6.5 min at 4 °C. Synaptosomes were harvested from the interface between the 23% and 10% Percoll layers and washed in ACSF (described before). Washed synaptosomes were centrifuged at 27,000*g* for 15 min at 4 °C.

PLA_2 activity assay

VTA and PAG synaptosomes were incubated with DAMGO for 30 min. Controls were incubated for an equivalent length of time without DAMGO. The assay protocol was adapted from the instruction of a PLA_2 assay kit (Cayman Chemical) and from previous studies (Reynolds et al., 1994; Zhang and Pan, 2010). Control and DAMGO-treated synaptosomes were homogenized in a cold buffer (50 mM HEPES, pH 7.4, with 1 mM EDTA) and centrifuged at 10,000*g* for 15 min at 4 °C. The supernatant was collected for assay and stored on ice. The following assay groups were used: (1) blank (no-enzyme control), (2) bee venom PLA_2 (positive control), and (3) sample. The substrate solution (200 μl)