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The Inhibitory Effect of Opioid on the Hyperpolarization-Activated Cation Currents in Rat Substantia Gelatinosa Neurons

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The action of opioid on the hyperpolarization-activated cation current (I_h) in substantia gelatinosa neurons were investigated by using whole-cell voltage-clamp recording in rat spinal brain slices. Hyperpolarizing voltage steps revealed slowly activating currents in a subgroup of neurons. The half-maximal activation and the reversal potential of the current were compatible to neuronal I_h. DAMGO (1 μM), a selective-opioid agonist, reduced the amplitude of I_h reversibly. This reduction was dose-dependent and was blocked by CTOP (2 μM), a selective μ-opioid antagonist. DAMGO shifted the voltage dependence of activation to more hyperpolarized potential. Cesium (1 mM) or ZD 7288 (100 μM) blocked I_h and the currents inhibited by cesium, ZD 7288 and DAMGO shared a similar time and voltage dependence. These results suggest that activation of μ-opioid receptor by DAMGO can inhibit I_h in a subgroup of rat substantia gelatinosa neurons.

Key Words: Substantia gelatinosa, Spinal cord, Hyperpolarization-activated cation current (I_h), Opioid, DAMGO, Pain

INTRODUCTION

The substantia gelatinosa (SG) of the dorsal horn is the important site of termination of primary afferents that respond to noxious stimuli (Kumazawa & Perl, 1978; Light & Perl, 1979; Sugiura et al, 1989), and the site for the analgesic actions of administered opioid agonists (Duggan et al, 1977; Johnson & Duggan, 1981). In addition, the SG contains opioid peptides that may play a role in endogenous control of pain (Merchenthaler et al, 1986). Many works have implicated opioids inhibit both the increased excitability and the pain associated with inflammation (Ferreira & Nakamura, 1979; Ferreira et al, 1982; Russell et al, 1987; Stein et al, 1989).

Opioids have been known to inhibit adenylyl cyclase and reduce cellular levels of cAMP in various neuronal tissues and cell lines (Duman et al 1988; Johnson & Fleming 1989, Ingram & Williams, 1994). Opioid receptors are members of Gi/Go-coupled receptors that inhibit both adenylyl cyclase and voltage-dependent Ca^{2+} channels (VDCCs) in central and peripheral neurons (Childers, 1993; Moises et al, 1994). In addition to these actions, opioids activate an inwardly rectifying potassium current (I_{kir}), resulting in the hyperpolarization of neurons throughout the CNS (Williams et al, 1982; Wimpey & Chavkin, 1991; Schneider et al, 1998). These opioid effects on ion channels are generally considered to inhibit neuronal activity.

Hyperpolarization-activated cation current (I_h) acts as a modulator of firing characteristics in neuronal activity, and the primary current underlying cardiac

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pacemaker activity (DiFrancesco, 1993; Pape, 1996). The hippocampal interneurons found in stratum oriens is also known as I_h (Maccaferri & McBain, 1996). This slowly developing inward cation current is activated by hyperpolarization, combines Na^+/K^+ current, and does not inactivate even with prolonged hyperpolarization (Mayer & Westbrook, 1983; Maccaferri & McBain, 1996). Because of these properties, I_h probably contributes to the resting membrane potential and the generation of rhythmic pacemaker-like depolarizations in central neurones and cardiac cells (DiFrancesco, 1981; Maccaferri & McBain, 1996; Pape, 1996; Gasparini & DiFrancesco, 1997). Therefore, modulation of I_h by neurotransmitters would be expected to alter the oscillatory activity of individual neurons and, in turn, the network of cells with which they communicate (Freund & Buzsaki, 1996; Pape, 1996). Among the several neuromodulators that modulate I_h, the opioids inhibit this current by inhibiting adenylyl cyclase in peripheral neurons (Ingram & Williams, 1994). And the modulation of I_h by opioids can contribute to the inhibition of interneuron activity in the hippocampus (Svoboda & Lupica, 1998).

However, studies on the opioid modulation of I_h in SG neurons have not been thoroughly performed yet. In the present study, we attempted to assess the presence of hyperpolarization-activated currents in rat SG neurons of the dorsal horn and their possible modulation by μ-opioid receptor agonist, DAMGO.

METHODS

Spinal cord slices were prepared from the postnatal (P9-P13) rats (Sprague-Dawley) which were sacrificed by rapid decapitation. The spinal cords were removed and placed in ice-cold oxygenated artificial cerebrospinal fluid (aCSF) containing (in mM): 130 NaCl, 26.2 NaHCO3, 1.25 NaH_2PO_4, 3.0 KCl, 1.5 $MgSO_4$, 2.5 $CaCl_2$, 10.0 glucose, 20.0 sucrose, pH adjusted to 7.4, 320 mOsm. A segment from L4 to L6 was isolated, attached to agarose block (2.5% in aCSF), and cut transverse to the anterior-posterior axis with 300 μM nominal thickness, using a vibraslicer (Vibratome 3000, TPI). Then, a slice was transferred to a submerged chamber, where both the aCSF and the aerating gas were warmed to 32°C for 1 hour and then maintained at room temperature (20~23°C) in aCSF bubbled continuously with 95% O_2~5% CO_2.

Whole cell currents were recorded from SG neurons using glass micropipettes pulled on a microelectrode puller (Narishige, pp-83) with Kimax- 51 borosilicate capillary tubes (Kimble, USA). The pipette resistance was 3~5 MΩ with tip diameter of 1~2 micrometer. The patch pipette was filled with an internal solution containing (in mM): 126 K-gluconate, 10 NaCl, 1 MgCl2, 11 EGTA, 10 HEPES, 2 Mg-ATP, 0.1 Na-GTP, pH adjusted to 7.3 with KOH. For recordings, we used an N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES)-based external solution that contained (in mM): 140 NaCl, 5.4 KCl, 0.5 $MgCl_2$, 1.8 $CaCl_2$, 10 HEPES, 5 glucose, pH adjusted to 7.4 with Tris-NaOH. The solution in the recording chamber was perfused under gravity and simultaneously removed by suction connected to the recording chamber.

The whole-cell configuration was achieved by "visual" approach. The SG neurons were visually identified using a Olympus fixed-stage microscope (BX50WI) with Nomarski optics and a 40X water-immersion objective. SG was identified as a translucent band in the outer part of the dorsal horn.

The electrical signals were acquired and processed using EPC-9 patch-clamp amplifier and Pulse 8.30 software (both from HEKA, FRG). Currents were filtered at 1 kHz and sampled at 3 kHz. Series resistance was typically less than 20 MΩ and was compensated by about 70%. The liquid junction potential between bath and low chloride internal solution (8 mV) was corrected. All experiments were done in room temperature.

All drugs were applied to the bath after dilution into HEPES-based external solution from concentrated stock solutions. [D-Ala2, N-Me-Phe4, Gly5-ol]-enkephalin (DAMGO), D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr Amide (CTOP), CsCl, BaCl2 were purchased from Sigma St. Louis, MO, USA, except ZD 7288 (Tocris Cookson). Solutions were applied with a multibarrel delivery system in which five polyethylene tubes were packed into the end of a glass tube tapered to a 50~100μ M diameter. The close apposition of the perfusion tip near the cells allowed fast exchange of bath within one second.

Results are expressed as mean$\pm$SEM. Comparison was made using Student's *t-test*. A difference was considered to be significant when $P < 0.05$.

RESULTS

All of the recordings were obtained from neurons that have somata located within lamina II of the spinal dorsal horn. The substantia gelatinosa in a transverse slice of the spinal cord is clearly visible translucent area. Slowly activating I_h was observed in a subgroup of neurons (112 neurons from 427 recordings; Fig. 1A). The resting membrane potential of I_h-positive neurons measured in current-clamp mode was -60 ± 1.1 mV (n=108). Neurons displayed action potential amplitudes of 60 ± 1.4 mV (n=50). The input resistance measured by hyperpolarizing the cells in voltage-clamp mode was 1.0 ± 0.2 GΩ

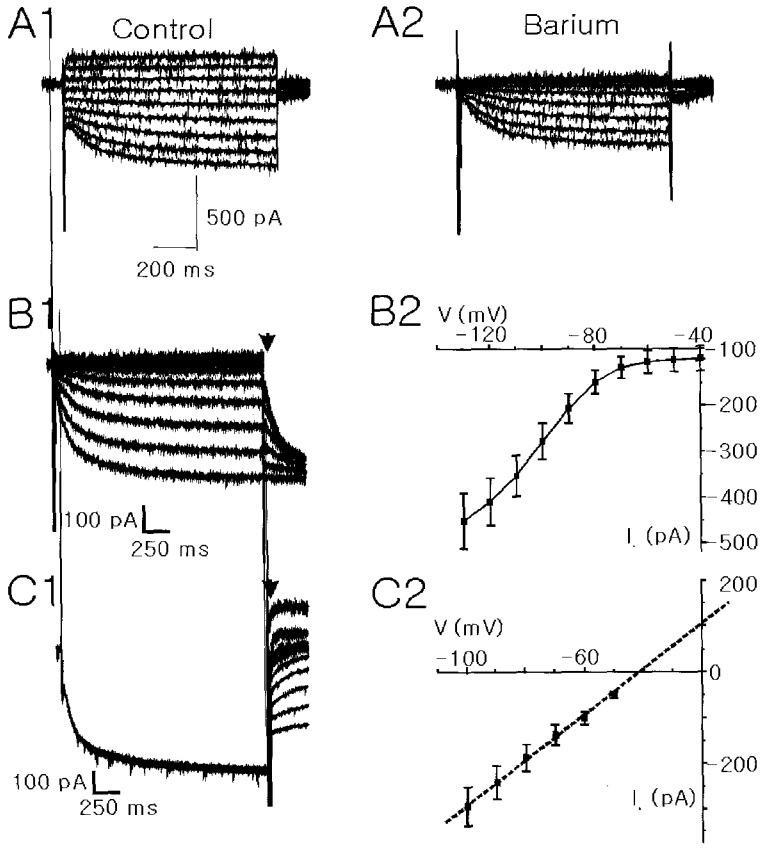

Fig. 1. Electrophysiological properties of the Ih in SG neurons measured in whole-cell clamp. (A1) Whole cell currents in control condition were activated by step hyperpolarization from -130 to -40 mV at holding potential of -60 mV. (A2) extracellular barium (1 mM) blocks the time-independent current. (B1, B2) Determination of the voltage dependence of Ih activation. (B1) Cells were voltage-clamped from a holding potential of -60 mV to the various voltages (ranging from -130 mV to -20 mV, in 10 mV increments); this was followed by a step to -130 mV. (B2) Tail current analysis of Ih activation. The amplitude of tail-current traces measured immediately after the voltage step to -130 mV was plotted as function of the preceding membrane potential (indicated by the arrow in Fig. B1). (C1, C2) Determination of the *I/V* relationship for fully activated Ih. (C1) Steps to test voltages ranging from -100 mV to 0 mV, in 10 mV increments, were either delivered after Ih was fully activated for 1.5 sec by a step from -20 mV to -130 mV. (C2) *I/V* relationship for the fully activated current obtained by tail currents. The tail current amplitudes were measured immediately after the voltage clamp to the indicated test voltages was settled (arrow in Fig. C1). The range positive to -50 mV could not be explored due to interference of other components (n=7).

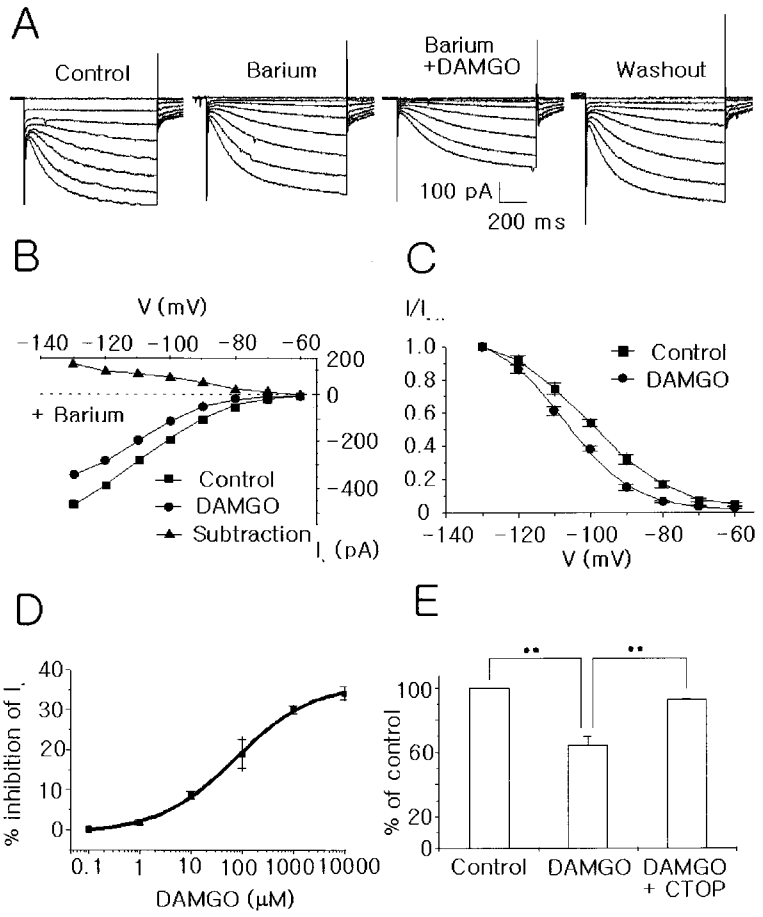

Fig. 2. Effect of DAMGO a selective -opioid agonist, on Ih of SG neurons. (A) Current responses obtained from a single SG neuron before (Control) and during DAMGO application. Voltage steps (1 sec in duration, 10 mV increments) from −130 mV to −60 mV were used to produce these responses. (B) Effect of -opioid agonist on current-voltage (*I/V*) relationships obtained by measuring difference between the amplitudes of steady-state current and instantaneous current. All data were normalized to the largest current response that was obtained by using a 2 sec voltage step to −130 mV. This and subsequent figures indicate the drug-induced change in current obtained by subtracting the control points from those measured during drug application. (C) Activation curves of the hyperpolarization-activated conductance. Plot of the activation curves were generated from amplitude of between steady-state current and instantaneous current normalized to the maximum current amplitude for drug application (I/I max). Activation curves were fitted with Boltzmann equation ($I/I_{max} = 1/(1+\exp((V-V_{0.5}) / k)$, where I_{max} is the maximum current, I is the calculated current at a membrane potential of V, $V_{0.5}$ is the midpoint of activation, and k is the slope factor), n=8. (D) A dose-response curve was generated from data of four neurons receiving five concentrations of DAMGO (1 nM, 10 nM, 100 nM, 1 M and 10 M) by the difference of amplitude of between steady-state current and instantaneous current at −130 mV. Solid line is a fit of the data to a logistic equation with an EC_{50} near 73.3 nM (n=6). (E) The effect of DAMGO on Ih of SG neurons was blocked by CTOP (2 μM). Significance was tested with Student's *t-test* ; ** $p<0.01$, n=5. All experiments were conducted at V_{hold}= −60 mV in the presence of barium 1 mM.

(n=50). The activation curve of I_h was estimated from the amplitude of the tail currents recorded following a series of prepulses from 40 to −130 mV in 10 mV steps (Fig. 1B). In 10 SG neurons, the threshold for I_h activation was between 60 and −70 mV. At −130 mV, I_h appeared to be fully activated and the half-maximal activation occurred at about −100 mV. The reversal potential of I_h was measured by using the method described by Maccaferri et al (1993) and Ludwig et al (1998). It was measured from the tail-current amplitude evoked by the repolarization from −100 mV to −20 mV (Fig. 1C). The range positive to −50 mV could not be explored due to interference of other ionic components. In 7 cells, mean value for the reversal potential of Ih was −40.0 ±2.0 mV.

DAMGO consistently decreased the whole-cell conductance and slowed the rate of I_h activation in 40/50 neurons (Fig. 2). The rate of I_h activation demonstrated strong voltage dependence, with time constant of 196±54 msec at 130 mV voltage step in control condition (n=8). DAMGO significantly slowed the activation of I_h, such that the time constant was 240±57 msec at 130 mV ($p<0.05$). In the presence of DAMGO, the amplitude of I_h was reduced by 26.2 ±3.6% (n=40). The effect of DAMGO on the voltage-dependency of I_h activation also was determined in a subset of opioid-sensitive neurons. The currents were then normalized to the maximum current measured within each drug application (I/Imax; Fig. 2C). DAMGO significantly shifted the activation curves to the left by 6.8 0.6 mV (measured at the V0.5, n=8, $p<0.05$). DAMGO inhibited I_h in a concentration-dependent manner. A dose-response curve of DAMGO (n=6) revealed an EC50 of 73.3±11.8 nM, suggesting that the opioid inhibition resulted from activation of a -opioid receptor (Fig. 2D). The effect of DAMGO on I_h was antagonized by a selective μ -opioid antagonist, CTOP (2 μM). Current responses were obtained from SG neuron before (control), during DAMGO, and during DAMGO and CTOP application. When DAMGO was superfused prior to the application of CTOP, I_h in SG neurons was greatly depressed in magnitude, as seen in Fig. 2E (peak amplitude: 64.2±5.6% of control, n=5). And then, after CTOP application, CTOP reversed the inhibitory effects of DAMGO, nearly similar to the state of control (peak amplitude: 93.1±0.2% of control, n=5, $p<0.01$).

I_h is classically blocked by cesium in the millimolar concentration range. Cesium blocked only the time-dependent current evoked by hyperpolarization, leaving the residual leak currents (Fig. 3A). In the presence of cesium, the inhibitory effect of opioid was not observed (data not shown). The currents inhibited by cesium and ZD 7288 are similar in its kinetic activation and voltage dependence to that inhibited by DAMGO. Exponential time constants fit to these currents at the largest hyperpolarizing voltage steps also indicate that the time course of these inhibited currents was similar (Fig. 3B). This suggests that all of these agents inhibited a current possessing the same temporal characteristics as I_h.

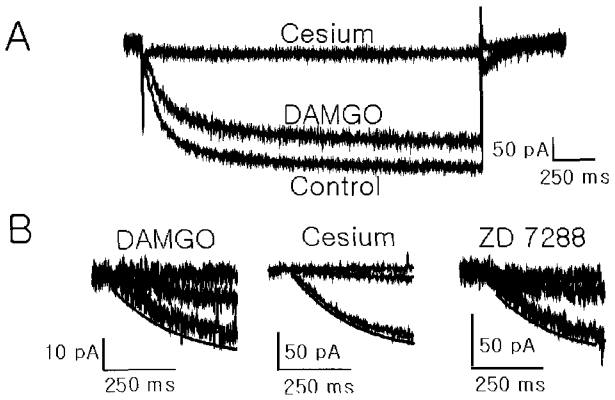

Fig. 3. Opioid effects during the blockade of Ih by extracellular cesium on Ih of SG neurons Currents inhibited by DAMGO, cesium, and ZD 7288 share similar time and voltage dependence. (A) Current records obtained during 2 sec hyperpolarizing voltage steps at −130 mV. Note that both cesium and ZD 7288 had a larger effect on the steady-state versus the instantaneous currents and that both inhibited a similar current with an activation threshold near −70 mV. (B) The currents shown in A~B were obtained by subtracting traces obtained during the application of the indicated modulator from control traces, using 2 sec hyperpolarizing voltage steps to −60, −100, and −130 mV. Each trace thus represents the current that was inhibited by ZD 7288 (50 μM), Cesium (2 mM), or DAMGO (1 μM) at these step potentials. The currents obtained at the −130 mV voltage steps then were fit by using exponential time constants and are indicated by solid lines. Currents inhibited by DAMGO exhibit typical Ih voltage and time dependence (τ = 213 ms). Currents inhibited by cesium (τ = 205 ms). Currents inhibited by ZD 7288 (τ = 234 ms). Note that the currents inhibited by DAMGO, cesium, and ZD 7288 share similar time- and voltage-dependent properties.

DISCUSSION

SG neurons receive the synaptic inputs from pain-transmitting primary sensory neurons and integrate the noxious information. The excitability of these neurons determines the threshold of pain in spinal cord and many neurotransmitters are involved in the synaptic transmission and integration. The major findings of this study are as follows. 1) The voltage- and time-dependent characteristics of I_h in rat SG neurons are revealed. 2) Activation of μ-opioid receptor by DAMGO, a selective μ-opioid agonist, reduced the amplitude of I_h in SG neurons. 3) Extracellular application of CTOP, a selective μ-opioid antagonist, blocked μ-opioid agonist effects. 4) During cesium and ZD7288 application, the inhibitory effect of opioid was not observed and the currents inhibited by cesium, ZD 7288 and DAMGO shared a similar time and voltage dependence.

Electrophysiological properties of I_h in SG neurons

The hyperpolarization-activated cation current, I_h, is known to activate slowly and displays virtually no time-dependent inactivation during a voltage step (Mayer & Westbrook, 1983; Maccaferri & McBain, 1996). A slowly activating, voltage-dependent inward current was evoked in some neurons during hyperpolarizing voltage steps from a holding potential of -60 mV. Our data indicate that 1) a hyperpolarization-activated current (I_h) presents at SG neurons of dorsal horn, 2) I_h has a reversal potential of about -40 mV, 3) the current activation threshold lays negative potential than -60 mV, implying that I_h is activated at resting voltages, 4) the possible tonic activation of I_h at resting potentials may affect the cell ability to discharge and promote excitability.

The reversal potentials measured in this study seems to be slightly more positive than those reported in previous work in central and peripheral neurons (Ludwig et al, 1998; Maccaferri et al, 1993) but are in accordance with measurement in thalamic relay neurons (McCormick & Pape, 1990).

Current-clamp experiments in several laboratories have indicated that the blockade of I_h by cesium or ZD 7288 can result in the hyperpolarization of neurons held near the resting membrane potential (Harris & Constanti, 1995; Maccaferri & McBain, 1996; Gasparini & DiFrancesco, 1997). However, in the present study, resting membrane potential were around -60 mV in SG neurons, others have reported these values to range between approximately -52 mV and -66 mV in stratum oriens interneurons (Lacaille et al, 1987; McBain et al, 1994; Bergles et al, 1996). Furthermore, Maccaferri & McBain (1996) demonstrated in these interneurons that I_h made a substantial contribution to the membrane conductance when the cells were held at -60 mV, which is likely close to rest. These data suggest the possibility that the inward current contributed by I_h may help to set the resting membrane potentials of these cells.

Functional significance of I_h modulation by opioid

The evidence of the modulation I_h by DAMGO was derived from several observations, including (1) DAMGO reduced the magnitude of I_h and the rate at which it activates, (2) DAMGO had a larger effect on the steady-state *I/V* relationship, in which I_h is more fully active, versus the instantaneous *I/V* relationship. (3) extracellular cesium and ZD 7288 blocked I_h and eliminated the opioid-induced decrease in membrane conductance.

Opioids only inhibited I_h in the presence of forskolin in guinea pig nodose ganglion neurons (Ingram & Williams, 1994). In present study, I_h might be reduced by inhibition of opioid directly and/or indirectly via adenylyl cyclase in SG neurons. In addition, the finding that the inhibition of currents was generated in the majority of neurons by DAMGO suggests that most SG neurons express μ-opioid receptor and that some of these cells express receptor subtype.

In some cells the opioid modulation of I_h was apparent in the absence of the blockade of I_{kir} by barium, suggesting either that I_{kir} was not coupled to opioid receptors in these neurons or that the coupling of the opioid receptor to I_h was greater. However, in the majority of SG neurons the modulation of I_h was not obvious until the competing K^+ channel response was blocked by barium. Together, these data add pharmacological substrates to the previously described physiological actions of the opioids in the spinal cord, including the activation of I_{kir} (Schneider et al, 1998) and the inhibition of glutamate release (MacDonald & Nelson, 1978; Kangrga & Randic, 1991) and substance P release (Mudge et al, 1979; Hirota et al, 1985). The inhibition of I_h by opioid receptors may provide an additional site to reduce the excitability of the SG neurons and alter the activity of

the spinal cord network. We suggest that the action of DAMGO Ih on is a cellular mechanism which can explain the known analgesic action of DAMGO in the spinal cord.

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