Pre- and Post synaptic Inhibitory Actions of Methionine-Enkephalin on Identified Bulbospinal Neurons of the Rat RVL

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Hayar, Abdallah and Patrice G. Guyenet. Pre- and postsynaptic inhibitory actions of methionine-enkephalin on identified bulbospinal neurons of the rat RVL. J. Neurophysiol. 80: 2003–2014, 1998. The effects of methionine-enkephalin (ME) on visualized bulbospinal neurons of the rostral ventrolateral medulla (RVL) were characterized in thin slices at 32°C using the whole cell patch-clamp technique. Thirty-five percent of the recorded neurons were found to be tyrosine hydroxylase immunoreactive (C1 neurons). In voltage-clamp recordings, ME (3 μM) induced an outward current in 66% of RVL bulbospinal neurons. A similar percentage of C1 and non-C1 neurons were opioid sensitive. The current induced by ME was inwardly rectifying, reversed close to the potassium equilibrium potential, and was blocked by barium. Most spontaneous postsynaptic currents recorded in these neurons were tetrodotoxin (TTX)-resistant miniature postsynaptic currents (mPSCs). Approximately, 75% of mPSCs had rapid kinetics (decay time = 4.7 ms) and were glutamatergic [miniature excitatory postsynaptic currents (mEPSCs)] because they were blocked by 6-cyano-7-nitroquinolinoxide-2,3-dione (10 μM). The remaining mPSCs had much slower kinetics (decay time = 19.6 ms) and were GABAergic [miniature inhibitory postsynaptic currents (mIPSCs)] as they were blocked by gabazine (3 μM) but not by strychnine (3–10 μM). ME decreased the frequency of mEPSCs and mIPSCs by 69% and 43%, respectively. The inhibitory effects of ME were mimicked by the selective μ-opioid receptor agonist endomorphin-1 (EM, 3 μM) and were blocked by naloxone (1 μM). In the absence of TTX, excitatory PSCs evoked by focal electrical stimulation were isolated by application of gabazine and strychnine. EM reduced the amplitude of the evoked PSCs by 41% without changing their decay time. We conclude that opioids inhibit the majority of RVL C1 and non-C1 bulbospinal neurons by activating a potassium conductance postsynaptically and by decreasing the presynaptic release of glutamate. These cellular mechanisms could explain the depressive cardiovascular effects and the sympathoinhibition produced by opioid transmitters in the RVL, in particular during hypotensive hemorrhage.

INTRODUCTION

Endogenous opioids are present in many brain stem nuclei implicated in the regulation of sympathetic tone and arterial pressure (Sapru et al. 1987). The decrease in sympathetic tone observed during hypotensive hemorrhage may be due to the release of enkephalin peptides in the medulla oblongata, a phenomenon that may account for the beneficial effects of naloxone in preventing circulatory collapse after hemorrhage (Elam et al. 1984; Ludbrook and Rutter 1988; Sandor et al. 1987).

A likely site for inhibition of sympathetic tone by endogenous opioids is the rostral ventrolateral medulla (RVL), a region that harbors the main excitatory projection to sympathetic vasomotor preganglionic neurons (for reviews, see Guyenet et al. 1996; Sun 1995, 1996). Exogenous application of opioids either by intravenous administration or by direct injection into the RVL induces potent hypotensive effects (Punnen and Sapru 1986; Rhee et al. 1992; Sun et al. 1996; White et al. 1995). Moreover, some RVL bulbospinal neurons with presumed sympathoexcitatory function are inhibited by iontophoretically applied morphine (Baraban et al. 1995). In addition, the RVL contains a dense plexus of enkephalinergic fibers, and the catecholaminergic (C1) neurons constitute one of the primary targets of this innervation (Milner et al. 1989, 1990). The enkephalin-containing terminals present in RVL may originate from local neurons or from the nucleus tractus solitarius (Morilak et al. 1989). Finally, the region of the rostroventral medulla contains low to moderate level of both μ and delta opioid receptors (Harstand et al. 1988; Kaluzhny et al. 1996).

Opioid peptides activate a potassium conductance in many brain areas, including the locus coeruleus (Williams et al. 1988) and rat periaqueductal gray neurons (Chiang and Christie 1994). Moreover, they inhibit transmitter release in the dorsal horn neurons (Jefﬁnja 1988), the rat CA1 (Cohen et al. 1992; Rekling 1993) and CA3 (Coppola et al. 1993) areas of the hippocampus, the periaqueductal gray (Vaughan and Christie 1997), and the dorsal raphe nucleus (Jol and Aghajanian 1997).

The cellular mechanisms responsible for the hypotensive actions of opioids in the RVL are still unknown. In this study, we used a retrograde marker in combination with the patch-clamp technique (Kangrga and Loewy 1994; Li et al. 1995; Osborne et al. 1996) to record in vitro from identiﬁed RVL bulbospinal neurons suspected to play a sympathoexcitatory role. This population includes C1 catecholaminergic neurons. Our aim was to investigate the pre- and postsynaptic effects of methionine-enkephalin and endomorphin-1 on these cells. Endomorphin-1 is a newly discovered and highly specific μ-opiate receptor agonist (Zadina et al. 1997) with potent vasodepressin effects (Champion et al. 1997; Czapla et al. 1998).

METHODS

Slice preparation

Sprague-Dawley rat pups (2 to 3 days old) were anesthetized by hypothermia, and a suspension of fluorescein isothiocyanate (FITC) microspheres (0.3–0.5 μl; Lumafluor) was injected bilater-
ally into the upper thoracic spinal cord. This procedure was designed to label RVL cells retrogradely for later identification in the slice. One to 8 days later, the pups (4 to 11 days old) were anesthetized deeply by hypothermia and decapitated. The brain stem was blocked and immersed in sucrose-artificial cerebrospinal fluid (sucrose-ACSF) equilibrated with 95% O₂-5% CO₂ (pH = 7.38). The sucrose-ACSF had the following composition (in mM): 26 NaHCO₃, 1 NaH₂PO₄, 3 KCl, 5 MgSO₄, 0.5 CaCl₂, 10 glucose, and 248 sucrose.

Coronal slices (180-μm thick) were cut with a Microslicer (Ted Pella, Redding, CA). The slices then were incubated until used at room temperature (22°C) in lactic acid-ACSF equilibrated with 95% O₂-5% CO₂ (composition was (in mM): 124 NaCl, 26 NaHCO₃, 5 KCl, 1 NaH₂PO₄, 2 MgSO₄, 2 CaCl₂, 10 glucose, and 4.5 lactic acid). For recording, a single slice containing the RVL was placed in a recording chamber on an upright epifluorescent microscope (Olympus BH-2). The selected slices (1 or 2 per brain) were identified under a ×10 objective by their characteristic pattern of retrograde labeling (Li et al. 1995). In this chamber, the slice was superfused continuously at the rate of 1.5 ml/min with normal ACSF equilibrated with 95% O₂-5% CO₂, and all recordings were made at 31–32°C.

Electrophysiological recording

Neurons containing microbeads were identified under epifluorescence illumination and viewed with a water-immersion ×40 objective using a closed circuit television camera. Patch pipettes were pulled from borosilicate glass capillaries with an inner filament (1.5 mm OD, Clark, UK) on a pipette puller (Sutter P87) and were filled with a solution of the following composition (in mM): 114 K-gluconate, 17.5 KCl, 4 NaCl, 4 MgCl₂, 10 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid, 0.2 ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 3 Mg₂ATP, 0.3 NaGTP, and 0.02% Lucifer yellow (Molecular Probes). Osmolarity was adjusted to 270 mOsm and pH to 7.3. The pipette tips were coated with silicone elastomer (Sylgard), and their resistance was 4–7 MΩ. Whole cell current-clamp (fast clamp mode) and voltage-clamp recordings were made with an Axopatch-200B amplifier. Liquid junction potential was 9–10 mV, and all reported voltage measurements have been corrected for these potentials. No series resistance compensation was performed.

Electrical stimulation was performed using two tungsten wires, Teflon-coated except at their tips (50 μm in diameter, A-M Systems, Everett, WA). They were positioned 100 μm apart and were placed on the surface of the slice 300–400 μm dorsal to the recorded neurons, near the nucleus ambiguus. The stimulation parameters were adjusted to obtain a maximal evoked postsynaptic current with the minimum intensity of stimulation (potential: 30–50 V, duration: 100–200 μs, frequency: 0.2–0.5 Hz).

Tyrosine-hydroxylase (TH) immunostaining

After recording, images of the recorded neurons (labeled with Lucifer yellow) were stored on videotape or digitized using a video card (Snappy video snapshot, Play, Rancho Cordova, CA) and stored in the computer hard disk using JPEG format. This procedure was useful to confirm the identity of the recorded neurons after histological processing, in particular when recordings were performed from several neurons in the same slice. The slices were fixed in freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3). Immunostaining for TH was done using an avidin-biotin-based reaction (mouse anti-TH monoclonal antiserum from Chemicon; dilution 1:750; biotinylated goat anti-mouse antisem from Vector; 1:150 dilution; and avidin-conjugated Texas red from Molecular Probes, 1:200 dilution). The neurons that displayed detectable TH immunoreactivity were considered catecholaminergic and were assumed to be C1 adrenergic cells because in double-labeling studies of the RVL region, nearly all bulbospinal TH-immunoreactive cells are also phenylethanolamine N-methyltransferase (PNMT) immunoreactive (Tucker et al. 1987). We preferred to use TH rather than PNMT immunostaining to identify C1 cells because our TH antibody consistently provides a more reliable and intense staining than is possible with PNMT antibodies currently available.

Reagents

Drugs and solutions of different ionic content were applied to the slice by switching the perfusion with a three-way electronic valve system. The following drugs were used: tetrodotoxin (TTX), strychnine from Sigma (St. Louis, MO). Methionine-enkephalin (ME), endomorphin-1 (EM), gabazine (SR95531), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), naltroxone hydrochloride, and bacofoen were obtained from Research Biochemicals International (Natick, MA). The actual concentration of barium chloride used in our experiments was unknown because some of barium ions might have precipitated with negative divalent ions like sulfate. However, the uncertainty about the free-barium concentration should not affect the interpretation of our data because barium blocked the postsynaptic response to opioids without altering the presynaptic response.

Data analysis

During experiments, analog signals were low-pass filtered at 2 kHz (Axopatch 200B), digitized at 48 kHz (Vetter, A. R. Vetter, Rebersburg, PA), and stored on a videotape for later analysis. Off-line, selected recordings of spontaneous postsynaptic currents (PSCs) were collected through a Digidata-1200A Interface and were digitized at 4–5 kHz using the Fetchex module of pClamp6 software (Axon Instruments, Foster City, CA). For the detection of PSCs, we used a program Mepp26 written according to algorithms for detection of spontaneous events by S. Cochran (1993). Each event had to satisfy at least two criteria that were set at the beginning of each analysis protocol. The rising phase had to exceed a minimum slope, and the amplitude of the events had to exceed a detection threshold that was usually set at the maximum of the noise on a sweep having no spontaneous synaptic events. Threshold criteria were adjusted to guarantee that no false events would be identified as confirmed by visual inspection for each analysis. These conservative criteria necessitated that a small percentage of probably true events were rejected. The program automatically determines, among other properties, the rise time and decay time of each detected PSC. The rise time was defined as the time from 10 to 90% of the PSC amplitude. The decay time was determined by fitting a single exponential from peak to baseline.

For frequency analysis of events in long stretches of data (15–20 min), we used the Fetchan module of pClamp6. Event detection was based on the first derivative of the signal after appropriate filtering (100–500 Hz), and the threshold for detection was set just above noise level (usually between 1.5 and 3 pA/s, for ≃1 ms duration). The detected events subsequently were grouped and binned (10–70 s) using the Pstat module of pClamp6. The presynaptic effects of opioids were calculated as changes in the frequency of TTX-resistant PSCs by comparing the baseline frequency of PSCs to the number of events within 2 min after 4–5 min of the drug application. To determine the drug effect on PSCs evoked by focal electrical stimulation, we averaged 5–15 consecutive evoked synaptic currents 1 min before and 3 min after drug application.

Further statistics, plots, and histograms were performed using Axoscope 1.1 (Axon Instruments) and Origin 4.0 programs (Microcal Software, Northampton, MA). Data were expressed throughout the text as means ± SD and analyzed statistically using...
Thirty-five percent of the recovered neurons had detectable properties between TH-ir and non-TH-ir neurons (Table 1). Most RVL bulbospinal neurons (34 of 48, 71%) exhibited tonic firing activity at resting membrane potential. In our conditions (recordings at 32°C), the mean firing rate of the active neurons was 3.9 ± 1.8 Hz (range 2–8 Hz, n = 34), which was slightly higher than the value obtained at room temperature (2.5 Hz) (Li et al. 1995). The remaining cells either were completely silent (n = 10) or displayed irregularly firing action potentials (<1 Hz). A typical recording from a spontaneously active RVL neuron is shown in Fig. 2. The action potentials of all 48 neurons had an overshoot of 34.7 ± 7.3 mV, an amplitude of 79.3 ± 7.3 mV (range 67 to 94 mV), and a duration of 2.6 ± 0.6 ms (range 1.6–4.7 ms) calculated from the threshold for spike generation (~44.6 ± 3.5 mV).

The membrane properties of these neurons (summarized in Table 1) were evaluated in current clamp 4–10 min after establishing the whole cell configuration when the cells had reached stability in discharge frequency (active cells) or membrane potential (silent cells). ME (3–10 μM) or EM (3 μM) were tested on 53 bulbospinal neurons. Thirty-five of 53 bulbospinal neurons (66%) responded to ME or EM (either in current clamp or voltage clamp) and were therefore considered opioid-sensitive. Among the electrophysiological properties analyzed (Table 1), only the overshoot and amplitude of the action potential in opioid-insensitive neurons were found to be significantly higher than in opioid-sensitive neurons. This difference, even though small (~6 mV), is unlikely due to a higher viability state of the opioid-sensitive neurons because the membrane input resistance and the duration of the spike were not significantly different between the opioid-sensitive and insensitive groups. Seventeen of 48 (35%) immunocytochemically recovered neurons and tested for opioid sensitivity were catecholaminergic, and 12 of these neurons (71%) were opioid-sensitive. Similarly, 21 of the 31 (68%) noncatecholaminergic neurons were opioid sensitive. We found no significant difference in electrophysiological properties between TH-ir and non-TH-ir neurons (Table 1). Moreover, the anatomic location within the RVL did not differ between opioid-insensitive and -sensitive neurons.

To determine the effect of opioids on the tonic firing of RVL neurons, we applied ME (3 μM) and/or EM (3 μM) paired or unpaired t-test. In all cases, significance was accepted if \( P < 0.01 \).

**RESULTS**

Whole cell recordings were made exclusively from RVL bulbospinal neurons that were identified by the presence of retrogradely transported FITC-tagged microbeads after injection in the spinal cord. This report focuses on 48 neurons tested for sensitivity to opioid peptides that were recovered after processing the tissue for immunocytochemical detection of tyrosine hydroxylase. Neurons were recorded in whole cell configuration for 1–4 h (mean 2 h). Five additional neurons, recorded but not recovered histologically also will be discussed. Histologically identified neurons were confirmed to be in the RVL by their location in the region containing the largest number of the retrogradely labeled TH-immunoreactive (TH-ir) neurons (Li et al. 1995). Thirty-five percent of the recovered neurons had detectable levels of TH-ir. Examples of one TH-ir and one non-TH-ir bulbospinal RVL neurons are shown in Fig. 1 (A and B). There was no detectable difference between the structure of TH-ir neurons and other recorded neurons.

**TABLE 1. Comparison between the electrophysiological properties of TH and non-TH, opioid-sensitive and -insensitive RVL bulbospinal neurons**

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Number of Silent Cells</th>
<th>Number of Active Cells</th>
<th>Threshold, mV</th>
<th>Overshoot, mV</th>
<th>Amplitude, mV</th>
<th>Duration, ms</th>
<th>Input Resistance, MΩ</th>
</tr>
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<tbody>
<tr>
<td>Non TH</td>
<td>31</td>
<td>9 (29)</td>
<td>22 (71)</td>
<td>−45.1 ± 3.5</td>
<td>34.7 ± 6.3</td>
<td>79.8 ± 6.5</td>
<td>2.5 ± 0.6</td>
<td>600 ± 147</td>
</tr>
<tr>
<td>TH</td>
<td>17</td>
<td>5 (29)</td>
<td>12 (71)</td>
<td>−43.8 ± 3.6</td>
<td>34.9 ± 9.0</td>
<td>78.5 ± 8.7</td>
<td>2.7 ± 0.6</td>
<td>640 ± 199</td>
</tr>
<tr>
<td>Opioid-sensitive</td>
<td>33</td>
<td>9 (27)</td>
<td>26 (73)</td>
<td>−45.4 ± 3.8</td>
<td>36.7 ± 6.6*</td>
<td>81.2 ± 6.9**</td>
<td>2.5 ± 0.6</td>
<td>632 ± 175</td>
</tr>
<tr>
<td>Opioid-insensitive</td>
<td>15</td>
<td>5 (33)</td>
<td>10 (67)</td>
<td>−44.8 ± 3.0</td>
<td>30.5 ± 7.2*</td>
<td>75.3 ± 7.3**</td>
<td>2.7 ± 0.8</td>
<td>575 ± 143</td>
</tr>
<tr>
<td>All neurons</td>
<td>48</td>
<td>14 (29)</td>
<td>34 (71)</td>
<td>−44.6 ± 3.5</td>
<td>34.7 ± 7.3</td>
<td>79.3 ± 7.3</td>
<td>2.6 ± 0.6</td>
<td>615 ± 160</td>
</tr>
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Values are means ± SE. Parentheses enclose percentages. Values with asterisks (* and **) indicate a significant difference using unpaired t-test (level \( P > 0.01 \)). TH, tyrosine hydroxylase; RVL, rostral ventrolateral medulla.
The remaining cell did not respond, and there was no effect voltage. This was accomplished by using slow ramp voltage a relatively slow and irregular firing of the neuron. The cell activity to ME than in less sensitive neurons (Fig. 2). Recovery from ME and EM, the membrane potential displayed current was plotted as a function of membrane potential. clamped at a holding potential (HP) of 10 mM (Fig. 3). The drug then was washed to prevent desensitization of opioid receptors. Neurons were considered insensitive if ≤5 pA outward current could be observed even when the concentration of ME was increased ≤10 μM.

When baclofen (10 μM) was tested on two neurons that were considered opioid insensitive, it induced an outward current of 30–40 pA. Because all RVL neurons were responsive to baclofen, as shown in a previous study (Li and Guyenet 1996), the lack of effect of ME on some RVL neurons is unlikely due to the dialysis of the cells by the pipette intracellular solution. When a 15- to 20-min delay was allowed between two consecutive applications of ME, the response to the second application was reproducible with <15% reduction in its amplitude compared with the first response (n = 3). In opioid-sensitive neurons, an outward current in response to ME could be obtained for ≈3 h of recordings from a single cell, indicating that the mechanism of action of opioids probably does not involve intracellular messenger subject to dialysis.

The mean amplitude of the peak response to ME (3 μM) was 37.5 ± 22.5 pA (n = 30) in opioid-sensitive neurons at a HP of −70 mV (Fig. 2B). The majority of RVL bulbospinal neurons responded with 19–25 pA, although another group responded with >50 pA of current. During washout of ME, the decay phase of the response had two components that could be distinguished in particular in highly responsive neurons (e.g., Figs. 2B, inset, 3B, and 7B). It is possible that the second relatively faster component occurs because the endogenous peptidases become less saturated with ME after washout and may participate in the opioid breakdown and removal. However, the reason for the existence of these two components was not further investigated.

The selective μ-receptor agonist, EM (3 μM), induced an outward current in 8 of 11 neurons tested at a HP of −70 mV. Five of five neurons that responded to EM also responded to ME. In four opioid-sensitive neurons, we have compared the magnitude of response to ME and EM. The mean response to EM (3 μM) was 38 ± 19 pA, which was significantly larger than the response to ME (3 μM) 29 ± 15 pA. Three neurons that were not affected by EM (3 μM) were also unaffected by ME (3–10 μM).

Opioid-activated potassium conductance

The voltage dependence of the conductance activated by ME was studied by subtracting the current obtained in the presence of ME from that in the absence of ME at a given voltage. This was accomplished by using slow ramp voltage clamp protocols (−130 to −40 mV in 1.5 s). The resulting current was plotted as a function of membrane potential. The ME-induced current reversed in polarity at −100 ± 3.8 mV (n = 9). The reversal potential was slightly more negative (−104 mV) in RVL neurons that exhibited higher sensitivity to ME than in less sensitive neurons (−97 mV). The latter value is very close to the value of −96.5 mV predicted by the Nernst equation in our experimental conditions where the intracellular and extracellular K+ ion concentrations ([K+]i) were 130 and 3 mM, respectively. In two cells, we tested the effect of ME in 3 and 9 mM [K+]o. The reversal potential of the ME current shifted from −96 mV ([K+]o = 3 mM) to −68 mV when [K+]o was raised to 9 mM (Fig. 3A, 1 and 2). This shift also was predicted by the Nernst equation for a current predominantly carried by K+ ions (calculated ER = −96 and −68 mV, in 3 and 9 mM [K+]o, respectively). In all cells tested, opioid-induced
current exhibited inward rectification. On average, the opioid conductance at −60 mV was 1.4 ± 0.6 nS and increased progressively with hyperpolarization to −2.3 ± 0.9 nS at −120 mV (n = 7).

In five RVL bulbospinal neurons, we examined the effect of the nonselective K⁺ channel blocker BaCl₂ on the magnitude of the outward current produced by 3 μM ME (n = 3) and 3 μM EM (n = 2). In these experiments, opioids were applied first to test for the sensitivity of the neurons and then reapplied for the same amount of time after 5-10 min preincubation with BaCl₂ (1 mM). On average, BaCl₂ attenuated the current induced by opioids by 90% (n = 5, Fig. 3A). The nonselective μ opioid receptor antagonist naloxone (1 μM) almost completely blocked the ME-induced outward current in three cells tested (Fig. 3B) and the EM-induced outward current in another cell.

Properties of spontaneous postsynaptic currents

The spontaneous PSCs were studied at a HP of −70 mV and were recorded as inward currents. The inhibitory PSCs were directed inwardly because the equilibrium potential for the chloride ions in our recording conditions was −38 mV. The mean frequency of PSCs in control conditions was 3.1 ± 2.2 Hz (n = 23 including 9 TH-ir neurons, range 0.9–11 Hz). Because no significant difference was found between TH-ir and other neurons, the following results with both cell types were pooled. In 13 of 16 neurons tested, the frequency of PSCs did not differ in control (2.23 ± 1.0 Hz) and in TTX (2.18 ± 0.9 Hz). Only in three cells did we observe a large decrease in the frequency of synaptic events (~80% reduction) after perfusion with TTX (1 μM). This would indicate that most of the spontaneously occurring PSCs recorded in RVL neurons were action potential-independent miniature PSCs (mPSCs).

We first sought to determine whether glutamate, γ-aminobutyric acid (GABA), or glycine mediated the mPSCs. The mPSCs could be separated clearly in two groups according to their kinetics and pharmacological properties. In control conditions, the mPSCs consisted primarily of fast synaptic currents (decay time 2–5 ms) intermixed with relatively slower mPSCs (decay time 15–27 ms) that were 5–30% of the total number of PSCs. The α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor antagonist CNQX (10 μM) eliminated all fast mPSCs within 2 min (n = 15, Fig. 4) and decreased the baseline frequency of mPSCs by 74% (from 2.52 ± 1.5 to 0.65 ± 0.65, n = 9), indicating that excitatory mPSCs (mEPSCs) are approximately three times more frequent than inhibitory ones (mIPSCs). The application of the competitive and specific GABAₐ receptor antagonist gabazine (SR95531, 3 μM) (Michaud et al. 1986; Mienville and Vicini 1987) eliminated all mPSCs with slow kinetics within 3 min (n = 12, Fig. 4). The glycine receptor antagonist strychnine (3–10 μM) had little effect on the mPSCs recorded in the presence of TTX and CNQX (n = 4, not shown). Gabazine alone was sufficient to abolish CNQX-insensitive mPSCs in eight of eight neurons tested (Fig. 5), indicating that in most cells, mIPSCs consisted exclusively of GABA-mediated postsynaptic currents.

The properties of mIPSCs and mEPSCs are compared in Table 2. In all cases, the amplitude distributions of both mEPSCs and mIPSCs (range 5–100 pA) were skewed toward larger amplitudes, without displaying clear peaks (not shown). The mean amplitudes of mIPSCs and mEPSCs were 19.2 and 14.5 pA, respectively. At the HP of −70 mV, this would correspond to a mean peak conductance of 0.6 nS for mIPSCs and 0.2 nS for mEPSCs, assuming a reversal potential of −38 and 0 mV for GABA and glutamate currents, respectively. Moreover, the mIPSCs had relatively longer time to peak, rise time and decay time. As a result, the total amount of current (area of a mPSC) was six times larger for an average mIPSC than for a mEPSC. This would indicate that even though...
the other neuron were reduced first in frequency and then inhibited completely (not illustrated). Assuming that the regular frequency of the EPSCs can be taken as a measure of the firing rate of the antecedent interneuron, this result suggests that ME could reduce or inhibit the firing of excitatory interneurons projecting to at least some RVL bulbospinal neurons.

Because the occurrence of action-potential dependent PSCs was rare in our conditions, probably because of using the thin slice preparation, we used focal electrical stimulation to evoke EPSCs in the presence of gabazine (3 μM) and strychnine (10 μM). Four cells in which we could evoke EPSCs that have a mean amplitude >50 pA were analyzed for the effect of EM (3 μM). The mean amplitude of EPSCs in those four cells was reduced from 99 ± 22 pA in control to 58 ± 13.5 pA (41% reduction) after EM application (Fig. 6). On the other hand, the decay time of the EPSCs was unchanged by EM (6.8 ± 1.2 ms in control and 6.8 ± 1.8 ms after EM application).

Inhibition of mEPSCs and mIPSCs by opioids

We isolated mEPSCs by applying TTX and gabazine. ME markedly reduced the frequency of mEPSCs in eight of nine neurons including four TH-ir cells. In the remaining cell, the effect of ME on mEPSCs was ambiguous due to a large variation in baseline frequency of mEPSCs. The average reduction in the eight cells with stable mEPSCs was 69% (from 3.0 to 0.92 Hz, range of inhibition: 15–90%). A cell showing a large decrease in the frequency of mEPSCs after application of ME is shown in Fig. 7A. The onset of the presynaptic effect (reduction in mEPSC frequency) coincided with the peak of the postsynaptic response. This difference in latency was observed in all cells tested and is one indication that the presynaptic and postsynaptic effects may

Inhibition of spontaneous and evoked EPSCs by opioids

As mentioned above, most of the PSCs recorded in RVL bulbospinal neurons were TTX insensitive. In the absence of TTX and in the presence of gabazine, we could distinguish in two rare cases relatively high-amplitude regularly occurring EPSCs. These events were presumed to be monosynaptic action potential-dependent EPSCs that were evoked by the tonic action potential discharge of a single interneuron. In one of these cells, application of ME reduced the mean frequency of EPSCs from 4 to 1 Hz, whereas the EPSCs of

FIG. 4. Tetrodotoxin (TTX)-resistant miniature excitatory and inhibitory postsynaptic currents (mEPSCs and mIPSCs) could be distinguished by their pharmacological properties and their kinetics. Right and left: data from 2 different cells. Top 3 traces: consecutive segments recorded in the presence of TTX (1 μM). Note the presence of both fast and slow PSCs (*) with a predominance of fast PSCs. Application of the specific γ-aminobutyric acid (GABA A) receptor antagonist gabazine (3 μM) eliminates all the PSCs with slow kinetics as shown in the 3 consecutive traces (middle, left). Application of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 μM) eliminates all PSCs with fast kinetics. Bottom: histograms showing the distribution (bin = 1 ms) of the decay time of the events (recorded in periods of 180 s) in TTX and after additional application of gabazine (left) and CNQX (right). Relatively few events with decay time between 8 and 27 ms were sensitive to gabazine (left), whereas the large peak of events in the right histogram (decay time 2–7 ms) was sensitive to CNQX.

FIG. 5. PSCs in a typical RVL neuron are mediated by glutamatergic and GABAergic receptors. A voltage-clamp recording (top, HP = −70 mV) from a RVL bulbospinal neuron displayed fast inward currents (<80 pA). Application of the AMPA receptor antagonist CNQX (10 μM) decreased the frequency of baseline PSCs from 6 to ~2 Hz as shown by the histogram (bin = 30 s), indicating that ~7% of the baseline PSCs are excitatory events mediated by glutamate receptors. Further application of the GABA A receptor antagonist gabazine (3 μM) abolished all the remaining PSCs, indicating that they were inhibitory events mediated by GABA A receptors.
TABLE 2. Properties of mIPSCs and mEPSCs

<table>
<thead>
<tr>
<th></th>
<th>mIPSCs</th>
<th>mEPSCs</th>
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<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Range</td>
</tr>
<tr>
<td>n</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Time to peak, ms</td>
<td>3.9 ± 0.42</td>
<td>3.2–4.5</td>
</tr>
<tr>
<td>Rise time, ms</td>
<td>1.8 ± 0.23</td>
<td>1.4–2.1</td>
</tr>
<tr>
<td>Decay time, ms</td>
<td>19.7 ± 3.2</td>
<td>15–22</td>
</tr>
<tr>
<td>Amplitude, pA</td>
<td>19.2 ± 6.0</td>
<td>9–26</td>
</tr>
<tr>
<td>Area, pA × ms</td>
<td>509 ± 160</td>
<td>342–740</td>
</tr>
</tbody>
</table>

Numbers are means ± SD of averages (>100 events) from n different cells. mIPSCs and mEPSCs, miniature inhibitory and excitatory postsynaptic currents.

OPIOID INHIBITORY ACTIONS IN RVL

involve distinct mechanisms that operate with different kinetics.

The decrease in glutamatergic synaptic transmission by ME was mimicked by the highly specific µ opioid receptor agonist EM. In three cells tested, EM (3 µM) decreased the baseline mEPSCs frequency by 62% (from 0.83 to 0.33 Hz). The reduction in mEPSCs frequency by ME was reversed by the addition of naloxone (1 µM) in two of two cells tested. This result suggests that the presynaptic effect is predominantly mediated by µ opioid receptors.

Because there was no evidence for the presence of glycinergic mIPSCs (see previous section), all mIPSCs recorded in the presence of TTX and CNQX were considered GABAergic. On average, application of ME reduced the frequency of mIPSCs by 43% (n = 5 including 2 TH-ir cells, range 25–60%, 0.46–0.31 Hz; Fig. 7B). Application of EM (3 µM) decreased the frequency of mIPSCs by an average of 25% (n = 2). There was no apparent change in the amplitude of mIPSCs after ME or EM application. However, we could not support this latter statement by a quantitative analysis, which was difficult to perform because of the relatively low frequency of mIPSCs.

To show that ME reduced the frequency of mEPSCs by a direct effect on the neuronal terminals and not as a consequence of modulation of postsynaptic glutamate receptors or a loss in event detectability, we constructed amplitude histograms of mEPSCs using data from fixed periods of time. ME did not cause a significant change in the mean amplitude of mEPSCs (Fig. 8), whereas there was an increase in the mean of the interevent time interval. On average, opioid peptides (ME, n = 4; EM, n = 2) did not change the mean amplitude of mEPSCs (13.8 ± 5.2 pA in control to 13.1 ± 4.3 pA in drug, P = 0.49), whereas the mean interevent interval was increased significantly by 198% in the same cells (759 ± 484 ms in control to 1,504 ± 923 ms in drug). Therefore, the decrease of mEPSC frequency was

FIG. 6. Effect of EM on evoked EPSCs. EM reversibly reduced the amplitude of evoked EPSCs in presence of gabazine (3 µM) and strychnine (10 µM). Each trace is an average of 5 consecutive EPSCs evoked with a frequency of 0.2 Hz. EPSCs were evoked by electrically stimulating the region of the nucleus ambiguus.

FIG. 7. Decrease of mPSCs by ME. A: spontaneous mEPSCs were recorded in the presence of TTX (1 µM), gabazine (3 µM), and strychnine (10 µM). ME produced an outward current in voltage clamp (HP = −70 mV, top) associated with a simultaneous decrease in the mean frequency of mEPSCs binned at 10 s (histogram). B: decrease of mIPSCs by ME in another neuron recorded in the presence of TTX (1 µM) and CNQX (10 µM). ME produced an outward current in voltage clamp (HP = −70 mV, top) associated with a simultaneous decrease in the mean frequency of mIPSCs binned at 60 s (histogram).
FIG. 8. ME did not affect the amplitude of mEPSCs. Normal and cumulative distribution plots of amplitude (A and B) and interevent interval (C and D) of mEPSCs in control and after application of ME. ME had no significant effect on the amplitude distribution but markedly shifted the frequency distribution to longer interevent intervals. All data are from the same neuron and represent mEPSCs detected in the presence of TTX (1 μM), gabazine (3 μM), and strychnine (10 μM) in 135-s epochs in control (n = 216 events) and after addition of ME (3 μM, n = 92 events).

unlikely due to a change in amplitude. Moreover, the reduction by ME of the frequency of mEPSCs appeared to be independent of the postsynaptic response because in two opioid-insensitive cells an average reduction of 55% (from 1 to 0.45 Hz) of control mPSCs was observed. Finally, the decrease in mEPSCs frequency by opioids (ME, n = 1, and EM, n = 2) persisted in the presence of Ba²⁺ (1 mM, Fig. 9), which abolished the postsynaptic effect. This also indicates that the mechanism of presynaptic inhibition did not seem to involve K⁺ channels that are sensitive to Ba²⁺.

DISCUSSION

The present study provides evidence that the majority of C1 and non-C1 RVL bulbospinal neurons have postsynaptic opioid receptors coupled to a potassium conductance. Furthermore, the pharmacological characterization of synaptic currents in these cells suggests the presence of predominantly excitatory inputs mediated by glutamate and inhibitory inputs mediated by GABA. In addition, our results indicate that opioid receptors also are located on presynaptic excitatory and inhibitory inputs to these cells and that their activation can reduce transmitter release. Finally, the highly selective endogenous μ opioid receptor agonist EM mimicked both the presynaptic and postsynaptic inhibitory actions of ME.

Postsynaptic inhibition by opioids of C1 and non-C1 bulbospinal neurons

Our results indicate that opioids exert a postsynaptic inhibitory effect on a large fraction of RVL bulbospinal neurons (65–70%). The outward current induced by opioids persisted in the presence of TTX and was due to the opening of a barium-sensitive and inwardly rectifying potassium conductance. A similar potassium conductance also is activated in most bulbospinal RVL neurons by GABAᵦ (Li and Guyenet 1996) or α₂-adrenergic receptors (Li et al. 1995). Because opioid responses were reproduced over prolonged periods of time, unaffected by intracellular dialysis, this would indicate that ME is coupled to inwardly rectifying K⁺ channels via a membrane delimited mechanism (Grigg et al. 1996; Miyake et al. 1989). Our results suggest that RVL neurons exhibit a much higher sensitivity to opioids than to α₂-adrenoceptor agonists. A relatively small outward current (12 pA) was found to be evoked by the α₂-receptor agonist α-methyl-norepinephrine (30 μM) at a HP of −50 to −60 mV (Li et al. 1995) compared with a mean of 37-pA current produced by ME (3 μM) at a HP of −70 mV in the present study.

The opioid reversal potentials were generally very close to the K⁺ equilibrium. However, a group of neurons displayed a slightly more negative opioid reversal potential that was correlated with a relatively larger ME-induced current. It is possible
FIG. 9. BaCl₂ abolished the postsynaptic effect of EM but preserved the presynaptic effect. EM (3 μM) induced an outward current in voltage clamp (HP = −70 mV, top left) associated with a decrease in the frequency of mEPSCs (bottom left histogram, bin = 30 s) recorded in the presence of TTX (1 μM), gabazine (3 μM), and strychnine (10 μM). A subsequent application of EM (delay of 15 min) to the same neuron after addition of BaCl₂ did not produce any postsynaptic effect (top right), whereas it still decreased the frequency of mEPSCs (bottom right histogram). Note that the reduction of the frequency of mEPSCs was long lasting and extended beyond the recovery of the postsynaptic response on wash.

that this group may have conserved a larger dendritic field that contributed to an additional opioid current that impaired our ability to adequately clamp the ME current as has been shown in the locus coeruleus (Travagli et al. 1996).

A similar percentage (~70%) of catecholaminergic and noncatecholaminergic neurons were found to be responsive to opioids. This is in contrast to other brain regions, where ME appears to induce a direct inhibitory effect only on presumed nonaminergic neurons such as the dorsal raphe (Jolas and Aghajanian 1997), the substantia nigra (Lacey et al. 1989), and the ventral tegmental area (Cameron et al. 1997; Johnson and North 1992a,b). It should be noted that a lower percentage (35%) of the recorded bulbospinal neurons were found to be catecholaminergic compared with 60–70% in previous studies performed at room temperature (Li and Guyenet 1996; Li et al. 1995). It is possible that whole cell recording at 32°C and the long duration of the recordings (1–4 h) could have dialyzed some of the TH in the neurons. Alternatively, because, unlike the previous reports, we recovered a higher percentage of neurons histologically, this might represent the actual percentage of C1 neurons in the RVL bulbospinal projection. Even though not all bulbospinal RVL neurons are catecholaminergic, they appear to be similar to locus coeruleus noradrenergic neurons in showing high degree of sensitivity to opioids (Pepper and Henderson 1980).

**Synaptic inputs on identified RVL bulbospinal neurons**

With few exceptions the PSCs recorded in RVL bulbospinal neurons were TTX-insensitive mPSCs. RVL neurons had both CNQX-sensitive PSCs, i.e., glutamatergic mEPSCs with relatively fast kinetics, and mIPSCs with slower kinetics that were blocked by the selective GABA<sub>A</sub>-receptor antagonist gabazine. The average ratio between glutamatergic and GABAergic PSCs was 3:1. The combination of CNQX and gabazine eliminated all PSCs, and CNQX-resistant PSCs were insensitive to strychnine, indicating that RVL bulbospinal neurons may not receive glycinergic inputs. This result is consistent with the absence of blood pressure change after microinjection of strychnine into the RVL in vivo (e.g., Guyenet et al. 1990). The predominance of glutamatergic PSCs and the absence of glycinergic PSCs is in contrast to a prior study performed with penetrating electrodes on randomly sampled RVL neurons in juvenile rats (Hayar et al. 1997). In the latter study, inhibitory inputs mediated by both glycine and GABA were largely predominant. The brevity of the mEPSCs demonstrated in the present study suggests that these events might not be readily detectable in recordings made with sharp high-resistance intracellular electrodes. In addition, because the reticulospinal projection is a small percentage of the total RVL neurons (~200 in the rat) (Ruggiero et al. 1994), it is probable that most of the neurons recorded by Hayar et al. (1997) were in fact propriobulbar interneurons and not bulbospinal neurons. Further investigation is required to determine whether RVL bulbospinal neurons have unique properties or whether the differences between this study and the previous one (Hayar et al. 1997) could be attributed to developmental changes or to the use of thinner slices.

**Presynaptic inhibition by opioids**

According to the present data, opioids exert presynaptic inhibitory effects on both GABAergic and glutamatergic inputs of RVL bulbospinal neurons. Reductions of both inhibitory and excitatory synaptic transmission by opioids also have been reported in nucleus accumbens (Yuan et al. 1992), periaqueductal gray (Chieng and Christie 1994; Vaughan and Christie 1997), and dorsal raphe nucleus (Jolas and Aghajanian 1997). In other structures, selective effects of opioids on either inhibitory (Capogna et al. 1993) or excitatory (Pan et al. 1990) neurotransmission have been reported.
The reduction in synaptic release is probably due to a presynaptic mechanism because of the ability of opioids to reduce miniature EPSCs frequency without affecting their amplitude. Moreover, EM reduced the amplitude of the evoked EPSCs without altering their decay time, suggesting that postsynaptic glutamate receptors were not affected by opioids. In physiological conditions, it would be predicted that synaptic currents are a mixture of miniature PSCs and PSCs evoked by action potentials. Therefore in addition to reducing the frequency of spontaneous quantal release, opioids could also reduce the amount of neurotransmitter released by action potentials, thus decreasing the amplitude of synaptic currents. Alternatively, opioids could inhibit the activity of presynaptic excitatory interneurons, as suggested by this study. Finally, the present results confirm previous reports that barium-sensitive K+ channels are not involved in the presynaptic effect of µ opioid receptors (Copogna et al. 1993; Vaughan and Christie 1997).

Interestingly, our observation that the presynaptic effect of ME is slower in onset than its postsynaptic effect (Fig. 7B) suggests that the transduction mechanism used by opioids to decrease neurotransmitter release may involve second messengers rather than a direct modulation of membrane conductances. The inhibition of the adenosine 3’,5’-cyclic monophosphate (cAMP) pathway may account for the opioid modulation of the secretory machinery. Opioids inhibit adenylate cyclase activity via G-protein-coupled receptors. A decrease in the level of cAMP leads to a reduction in the cAMP-dependent protein kinase activity, which in turn inhibits the phosphorylation of synapsin I and II (Childers et al. 1992), two proteins that regulate the vesicle release process (Greengard et al. 1993).

The pre- and postsynaptic inhibitory actions of ME probably involve µ opioid receptors because they were reproduced by EM, a recently discovered endogenous peptide shown to be >4,000-fold selective for µ over kappa and delta opioid sites (Zadina et al. 1997). When injected intravenously in the rat, endomorphin 1 and 2 induced significant decrease in systemic arterial pressure and cardiac output (Champion et al. 1997). Moreover, both peptides were found to be 10-fold more potent than ME with respect to vasodepressor activity (Czapla et al. 1998). Our results indicate that EM induced a larger postsynaptic response than ME at the concentration of 3 µM. The reasons for this difference remain to be determined in future studies. We do not exclude the presence of other types of opioid receptors in the RVL because there is evidence from in vivo study in the rabbit of a tonically active opioid input to RVL interacting predominantly with delta receptors (Morilak et al. 1990). However, the latter study did not exclude the presence of µ opioid receptors because injection of the specific µ receptor agonist DAMGO in the RVL also elicited hypotensive effects. Furthermore, immunocytochemical studies indicated that both µ and delta receptors could be involved in direct and indirect inhibition of bulbospinal rostroventral medullary neurons (Kalyuzhnly et al. 1996).

Physiological consequences

Glutamate and GABA appeared to be the only transmitters mediating fast synaptic transmission in identified RVL bulbospinal neurons of neonatal rats. Because, as shown previously (Li et al. 1995), the spontaneous pacemaker-like activity present in these cells was unaffected by blocking these two types of transmission, the present results reinforce the view that the discharge of these cells in slices is mostly due to intrinsic membrane properties (Kanggra and Loewy 1994). Our study showed that neuromodulators such as endogenous opioids could suppress the autoactivity of these cells and decrease in addition the excitatory input. The presynaptic inhibition will be more effective in particular when the discharge of RVL neurons is driven by a neuronal network as suggested by some in vivo studies (Ito and Sved 1997; Lipski et al. 1996).

The presynaptic reduction of glutamate release and the postsynaptic activation of a potassium conductance constitute two cooperative mechanisms used by opioids to reduce the activity of the majority of RVL bulbospinal neurons. Because these cells provide the major excitatory drive to sympathetic preganglionic neurons (Sun 1995, 1996), these mechanisms may account for the large sympathoinhibition and hypotension observed when opioid agonists are microinjected into the RVL in vivo (Punnen and Sapru 1986). Yet, the functional significance of the simultaneous decrease of GABA release onto these neurons remains to be elucidated. It is possible that glutamate and GABA synaptic currents are modulated by different neuronal sources of endogenous opioids because both intrinsic and extrinsic sources of enkephalinergic terminals have been described in the RVL (Morilak et al. 1989). A disinhibitory mechanism by opioids leading to an increase in neuronal excitability has been reported in the hippocampus (Ziegglansberger et al. 1979) and ventral tegmental area (Johnson and North 1992a). Therefore, it is likely that opioids could exert a net excitatory effect on some RVL neurons, in particular those that receive an important inhibitory input and exhibit low postsynaptic sensitivity to these neuropeptides. This hypothesis is consistent with the finding that some RVL bulbospinal neurons were excited by intravenous administration of morphine (Baraban et al. 1995). The general depression by opioids of synaptic transmission may protect neurons against the deleterious effect of CNS hypoperfusion and ischemia in stressful physiological conditions, such as severe hypotensive hemorrhage (Sandor et al. 1987).

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