OPIATE SLOWING OF FELINE RESPIRATORY RHYTHM AND EFFECTS ON PUTATIVE MEDULLARY PHASE-REGULATING NEURONS.

Peter M. Lalley¹, Department of Physiology, The University of Wisconsin, Madison, Wisconsin 53706.

Running title:
Opioid-mediated respiratory rhythm slowing

Key words and phrases
Fentanyl
medullary respiratory neurons
phrenic nerve activity
Naloxonazine
Naltrindole

¹Address for correspondence
University of Wisconsin, Madison. Medical Sciences Center, Department of Physiology, 1300 University Avenue, Madison, WI, 53706.

Tel: 608-263-4697
Fax: 608-265-5512
E-mail: pmlalley@facstaff.wisc.edu

ABSTRACT

Opiates have effects on respiratory neurons that depress tidal volume and air exchange, reduce chest wall compliance and slow rhythm. The most dose-sensitive opioid effect is slowing of the respiratory rhythm through mechanisms that have not been thoroughly investigated. An in vivo dose-response analysis was performed on medullary respiratory neurons of adult cats to investigate two untested hypotheses related to mechanisms of opioid-mediated rhythm slowing: (1) Opiates suppress intrinsic conductances that limit discharge duration in medullary Inspiratory and Expiratory neurons, (2) Opiates delay the onset and lengthen the duration of discharges postsynaptically in phase-regulating Post-Inspiratory (Post-I) and Late-Inspiratory (Late-I) neurons. In anesthetized and unanesthetized decerebrate cats, a threshold dose (3 µg/kg) of the µ-opioid receptor agonist fentanyl slowed respiratory rhythm by prolonging discharges of Inspiratory and Expiratory bulbo-spinal neurons. An additional 2-4 µg/kg also lengthened the inter-burst silent periods in each type of neuron and delayed the rate of membrane depolarization to firing threshold. These changes took place without altering synaptic drive potential amplitude, input resistance, peak action potential frequency, action potential shape or afterhyperpolarization. Fentanyl also prolonged discharges of Post-I and Late-I neurons in doses that slowed the rhythm of inspiratory and expiratory neurons, without altering

Copyright © 2005 by the American Physiological Society.
peak membrane depolarization and hyperpolarization, input resistance or action potential properties. The temporal changes evoked in the tested neurons can explain the slowing of network respiratory rhythm, but the lack of significant, direct opioid-mediated membrane effects predicts actions emanating from other types of upstream bulbar respiratory neurons to account for rhythm slowing.

**INTRODUCTION**

Opiates are well known for their ability to depress ventilation by slowing breathing frequency and reducing tidal volume, gas exchange, upper airway patency and chest wall compliance (3, 4, 36, 39,). They also blunt respiratory network responsiveness to hypoxia and carbon dioxide/acidosis (11, 25, 51, 60).

Opiate slowing of respiratory rhythm, leading to arrest of breathing after highest doses, has been the topic of many experimental investigations (2, 10, 14, 16, 22, 24, 30, 34, 37, 48, 52, 60). According to most recent studies, rhythm slowing seems to be principally related to depression of Inspiratory interneurons in the preBoetzinger Complex (PBC, 15, 34, 37), a region within the ventrolateral respiratory column (VRC) that seems to be critical for the generation of the respiratory rhythm (40, 52). However, endogenous peptides are distributed throughout the bulbar respiratory network, and opioid receptors are found on many types of respiratory neurons (19, 32-35, 38, 47, 51). Thus, complimentary opioid actions on other types of respiratory neurons that contribute to rhythm slowing are also possible. This study was undertaken to investigate two of them.

In the present study, two previously untested hypotheses related to opioid mechanisms of rhythm slowing were investigated: (1) Opiates depress intrinsic membrane conductances that limit discharge length in VRC Inspiratory and Expiratory neurons. (2) Opiates slow the onset and prolong the duration of discharges postsynaptically in VRC Late-Inspiratory and Post-Inspiratory neurons, whose proposed functions are to terminate the inspiratory phase and delay the onset of the expiratory phase (12, 42, 43).

A dose-response analysis of the effects of intravenously administered fentanyl, a phenylpiperidine opiate and μ–opioid receptor agonist, on rhythm slowing was carried out in adult anesthetized and unanesthetized decerebrate cats. To estimate whether direct opiate effects on membrane conductances in VRC Inspiratory, Late-Inspiratory, Post-Inspiratory and Expiratory neurons contribute to respiratory network rhythm slowing, attention was given to how fentanyl affected membrane potential, input resistance and action potential threshold, shape and afterhyperpolarization.

**METHODS**

**Animal preparation**

Data were obtained from 10 pentobarbital-anaesthetized and 3 midcollicular decerebrated, unanaesthetized adult male cats. Care and use of animals were in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated.
by the U.S. National Institutes of Health, and approved by the University of Wisconsin Medical School Animal Care and Utilization Committee. The procedures used in this study were previously described in detail (29). The animals were initially anesthetized with 5% halothane in 100% O₂, administered while the animals were in an anesthesia chamber. During halothane anesthesia, they were removed from the chamber and given atropine methyl nitrate, 0.2-mg/kg i.p., to minimize airway fluid secretion. Ten of the cats were then given 40 mg/kg pentobarbital sodium i.p. to produce deep anesthesia. Ten to fifteen minutes were allowed for the development of satisfactory anesthesia for surgical procedures and opioid testing. At least four hours elapsed between the termination of halothane administration and the beginning of experimentation. Supplemental doses of pentobarbital (4-8 mg/kg i.v.) were administered if symptoms indicating significant lightening of anesthesia occurred: (1) spontaneous increases of arterial blood pressure and heart rate; (2) irregular breathing or discharges of phrenic nerve activity that decreased in duration and increased in frequency; (3) shivering; (4) movement and cardio-respiratory changes evoked by surgical procedures. The other 3 cats were maintained under halothane (3 – 3.5% in oxygen) anesthesia and decerebrated at the midcollicular level, with removal of brain tissue rostral to the transection, using the method of Kirsten and St. John (27). Halothane anesthesia was maintained until all surgical procedures were completed. Decerebrate rigidity without evidence of pain or discomfort was evident within 20 minutes after discontinuing anesthesia.

Surgical procedures performed on both anesthetized and decerebrate cats included placement of catheters in one femoral artery to monitor blood pressure and in both femoral veins to administer drugs and infuse Ringer lactate solution for tissue hydration. A cannula was also inserted in the trachea below the larynx to maintain airway ventilation. Animals were mounted in a stereotaxic head holder and suspended by thoracic and lumbar spinal clamps. Blood pressure, tracheal pressure, rate of breathing, body temperature, end-tidal CO₂ and inspired oxygen were monitored continuously and recorded on chart paper. Temperature was measured rectally and maintained at 36-38°C by external heating. Animals were paralyzed with gallamine triethiodide (4 mg/kg i.v. to start, 4 – 8 mg per hour thereafter) and mechanically ventilated with oxygen-enriched (60% O₂) room air. Stroke volume of the ventilator was set at 10 ml/kg body weight and by adjusting ventilation rate, end-tidal CO₂ was maintained at 4.6 – 5.3 vol.%, which was within the range recorded during spontaneous breathing after production of surgical anesthesia.

Nerve and brain preparation, recording procedures and measurements

To monitor respiratory network rhythm, phrenic nerves (C₄-C₅) were exposed bilaterally through a dorsal cervical approach and prepared for recording from their central ends with bipolar silver hook electrodes. Phrenic nerve activity was recorded with an AC preamplifier (Grass Instruments, Quincy, MA; 2,000-10,000 X amplification; band-pass filter, 100 – 3000 Hz).

Intracellular recordings were obtained from respiratory neurons of the ventrolateral medulla. The head was ventroflexed, the dorsal medulla was exposed by occipital
craniotomy, the dura and arachnoid membranes were reflected and a patch of pia membrane was removed to allow insertion of a glass microelectrode. To provide recording stability, bilateral pneumothorax was performed and pressure feet were placed gently on the surface of the medulla over and near the site of microelectrode insertion. Following pneumothorax, application of 1-2 cm H$_2$O pressure to the expiratory outflow prevented atelectasis. Neurons were impaled with sharp microelectrodes filled with 2M K-methylsulfate (DC resistance, 60 – 80 MΩ). Membrane potential was recorded in discontinuous single electrode current clamp mode (SEC-05XL amplifier, npi, Tamm, Germany; bandwidth, D.C.- 10 KHz; switching frequency, 25 KHz). To test the effects of fentanyl on intrinsic membrane conductances, neuron input resistance, action potential threshold, shape and afterhyperpolarization were measured. Input resistance measurements were made by injecting 60 mSec negative-going constant current pulses through the microelectrode and measuring the resulting hyperpolarizing voltage drop across the cell membrane.

Phrenic nerve activity and neuron membrane potential were displayed on an oscilloscope (Tektronix Instruments, Beaverton OR) and registered on magnetic tape (Vetron Technology, Rebersburg, PA; D.C.-5 KHz), as well as on a computer-controlled data acquisition system (PowerLab, AD Instruments, Castle Hill, NSW, Australia; D.C.-10 KHz) and a paper chart recorder (Gould, Cleveland OH: D.C.-10 KHz).

**Identification of bulbospinal and vagal respiratory neurons**

To identify bulbospinal inspiratory and expiratory neurons, two concentric coaxial stimulating electrodes (SNEX-100, AM Systems, Everett WA) were positioned bilaterally in the cervical reticulospinal tracts at the C3 level. Stimulation with single bilateral shocks (5 – 15 V, 0.1 mSec pulse duration) antidromically activated bulbospinal neurons as verified by collision with spontaneous action potentials. Axons of vagal respiratory motoneurons were antidromically activated by single shocks (1.5 – 3 V, 0.1 mSec) applied to the central end of the ipsilateral cervical vagus nerve.

**Opioid agonist and antagonist administration**

Fentanyl citrate (Sigma-Aldrich Chemical Co., St. Louis, MO) was dissolved in Ringer’s solution (40 or 80 µg/ml) and administered intravenously in increments of 1 µg/kg at 1 minute intervals until a cumulative dose of 10 µg/kg was reached, then additional increments of 2 – 5 µg/kg were given to determine how large a dose was required to produce additional effects other than on rhythm, such as depression of discharge intensity (e.g., Fig. 3E).

The µ-opioid receptor antagonist naloxonazine hydrochloride and the δ-opioid receptor antagonist naltrindole hydrochloride (Sigma-Aldrich) were each dissolved in Ringer’s Solution (0.2 mg/kg) and injected intravenously in a dose of 100 µg/kg.
**Euthanasia**

Experiments were terminated by intravenous injection of sodium pentobarbital in sufficient quantity (> 100 mg/kg) to produce permanent cardiac arrest.

**Statistics**

Control and test values were evaluated for significance of difference by a paired student t-test using SigmaPlot version 4.11 software (Jandel Scientific, USA). Differences were accepted as significant if p < 0.05. SigmaPlot was also used to derive means and standard errors.

**RESULTS**

The effects of fentanyl on respiratory rhythm, membrane potential and discharge properties were measured along with phrenic nerve activity in twenty-seven neurons of the medulla that were located within the VRC: six bulbospinal augmenting Inspiratory neurons, three non-vagal, non-bulbospinal Inspiratory neurons, nine bulbospinal augmenting Expiratory neurons, three vagal Post-Inspiratory motoneurons, three Post-Inspiratory neurons that were synaptically but not antidromically activated by vagus nerve stimulation and three late-discharging Inspiratory neurons.

The Expiratory neurons were located 1 – 2 mm caudal to the obex, 2.5 – 3.5 mm lateral to the midline and 2.0 –3.0 mm below the dorsal surface of the medulla (Figure 1). The other neurons were located 1 – 3 mm rostral, 3.4 – 3.8 mm lateral, 3.4 – 3.8 mm below the dorsal surface. The vagal Post-I motoneurons were within Nucleus Ambiguus, since they were recorded amongst antidromically activated Inspiratory and Expiratory vagal motoneurons that exhibited an augmenting membrane potential and discharge pattern (8). The non-vagal Inspiratory neurons and the Post-I and Late-I neurons were medial to this region and therefore are assumed to be separate from the more laterally located PBC respiratory neurons (50).

Figure 1 Here

All of the neurons exhibited dose-related changes of respiratory rhythm or discharge intensity in less than one minute after each effective dose of fentanyl, without change of heart rate or blood pressure.

**Dose-related effects of fentanyl on bulbospinal Inspiratory neurons.**

To test the hypothesis that one of the mechanism that opioids employ to slow breathing is to prolong discharges of VRC Inspiratory and Expiratory neurones through depression of intrinsic membrane conductances, effects of fentanyl on temporal properties, neuron input resistance and action potential properties were measured.
Bulbospinal Inspiratory neurons under control conditions exhibited augmenting patterns of membrane depolarization and robust bursts of action potentials that began shortly after the onset of phrenic nerve discharges and continued until phrenic nerve activity entered its declining post-inspiratory phase. Thereafter, membrane hyperpolarization and cessation of firing occurred during the post-inspiratory and expiratory phases (Fig. 2A, left panel). Under control conditions, burst frequency was \(20 \pm 0.7 \text{ min}^{-1}\) (mean \(\pm\) SE); burst duration, \(1.1 \pm 0.04 \text{ sec}\); peak action potential frequency, \(42 \pm 2 \text{ action potentials sec}^{-1}\).

Fentanyl evoked progressive, dose-dependent rhythm slowing. The effects of a greater-than-threshold dose of fentanyl on a bulbospinal inspiratory neuron are shown in Fig. 2A. Effects of three cumulative doses of fentanyl (3, 6, 10 \(\mu\)g/kg) on burst frequency, peak action potential frequency and discharge duration in the six inspiratory neurons tested are summarized graphically in figure 2C.

**Figure 2 Here**

The most sensitive dose-related changes evoked by fentanyl occurred after a cumulative 3 \(\mu\)g/kg dose in all of the neurons. Discharge duration lengthened to \(1.6 \pm 0.07 \text{ sec}\) (145 \% increase, \(p < 0.001\)) in parallel with prolongation of the phrenic nerve inspiratory phase discharge. This effect reduced burst frequency to \(15 \pm 0.6 \text{ min}^{-1}\) (27 \% decrease, \(p < 0.05\)). Doses of 3 – 5 \(\mu\)g/kg of fentanyl also slowed the rates of membrane depolarization and action potential frequency augmentation (Fig 2A). Peak action potential frequency at the end of the inspiratory phase decreased, but not significantly, to \(38 \pm 1.5 \text{ action potentials sec}^{-1}\) (\(p > 0.05\)).

After a total of 6 \(\mu\)g/kg, discharge duration was lengthened to \(1.8 \pm 0.1 \text{ sec}\) (145 \% increase, \(p < 0.001\)) and the rates of membrane depolarization and spike frequency augmentation were further slowed, whereas peak action potential frequency remained unaltered. Another change that contributed to rhythm slowing after this larger cumulative dose was prolongation of the silent period. The prolongation of the inspiratory discharge and the expiratory silent period together decreased burst frequency to \(11 \pm 1.4 \text{ min}^{-1}\) (46 \% decrease, \(p < 0.001\)).

A cumulative 10 \(\mu\)g/kg dose slowed cycle frequency to \(9 \pm 1.2 \text{ min}^{-1}\) (54 \% decrease, \(p < .001\), due to further prolongation of both inspiratory discharges (increased to \(2.5 \pm 0.32 \text{ sec}\), 227 \% increase, \(p < 0.001\)) and expiratory silent periods.

The prolongation of inspiratory phase discharge duration and expiratory silent period occurred without affecting neuron input resistance, firing threshold, action potential shape, duration or afterhyperpolarization (Fig. 2B).

After doses of 10 \(\mu\)g/kg and greater, effects indicative of postsynaptic depression appeared, as previously described (29). In the six neurons, fentanyl produced hyperpolarization of membrane potential and depression of depolarizing synaptic drive potentials. Peak action potential frequency declined significantly after the 10 \(\mu\)g/kg dose
(to $22 \pm 2.8$ action potentials sec$^{-1}$, 48 % reduction, $p < 0.001$), and neuron input resistance was reduced.

**Dose-dependent changes in non-vagal, non-bulbospinal inspiratory neurons.**

In the VRC, rostral to the bulbospinal inspiratory neurons, Inspiratory neurons were encountered that exhibited plateau-like depolarizing synaptic drive potentials and action potential frequencies that were maximal with the onset of phrenic nerve activity and maintained throughout the inspiratory phase. They were not activated antidromically by spinal cord or vagus nerve stimulation. An example of dose-dependent fentanyl effects on one of the neurons is illustrated in figure 3.

![Figure 3 Here](image)

The threshold dose was $3 \mu g/kg$, which slowed rhythm by lengthening discharge duration (Fig 3B). Further lengthening of discharge and prolongation of the silent period occurred after larger ($6 - 15 \mu g/kg$) doses (Fig. 3C, D), without affecting synaptic drive potential amplitude and without altering action potential shape, threshold or afterhyperpolarization.

After cumulative $18 - 20 \mu g/kg$ doses, slowing of rhythm was accompanied by reduced action potential frequency, increased action potential afterhyperpolarization and reduced synaptic noise during the postinspiratory and expiratory phases (Fig. 3E).

**Fentanyl effects on bulbospinal Expiratory neurons.**

The Expiratory neurons of the caudal VRC that were recorded in anesthetized (6 cells) and decerebrate unanesthetized cats (3 cells) exhibited periodic membrane hyperpolarization and absence of firing in parallel with the phrenic nerve discharge during the inspiratory phase. They depolarized when the phrenic nerve activity declined during the postinspiratory phase, and discharged action potentials during the late expiratory phase in conjunction with the phrenic nerve silent period, as previously described (1).

The dose-related slowing of rhythm by fentanyl recorded in two neurons is illustrated in Figures 4 and 6, and results from six neurons are summarized graphically in figure 5. Discharge duration before giving fentanyl was $1.95 \pm 0.19$ sec; burst frequency, $16.1 \pm 1.7$ min$^{-1}$; peak action potential frequency, $56 \pm 10.1$ APs sec$^{-1}$.

![Figure 4 Here](image)

The threshold dose of fentanyl in all of the neurons, whether anesthetized or decerebrate, was $3 \mu g/kg$. It increased discharge duration to $2.51 \pm 0.25$ sec$^{-1}$ (29 % increase, $p < 0.05$), prolonged the silent period in conjunction with longer phrenic nerve inspiratory discharges and reduced neuron burst frequency to $11.2 \pm 1.5$ min$^{-1}$ (30.4 % decrease,
Although peak action potential frequency was on average increased, the change was not statistically significant.

Figure 5 Here

Increasing the dose further prolonged the discharge duration and the silent period. After a cumulative 6 µg/kg dose, discharge duration lengthened to 4.19 ± 0.85 sec (115% increase, p < 0.01) and burst frequency slowed to 8.6 ± 1.7 min⁻¹ (45% decrease, p < 0.001). An additional opioid effect after the total dose reached 9-12 µg/kg was a more gradual depolarization of membrane potential during the postinspiratory phase of phrenic nerve activity, as seen in fig. 4C,D and previously reported (29). Identical dose-related opioid effects were observed in expiratory neurons of unanesthetized decerebrate cats.

Rhythm slowed over the range of 3–12 µg/kg without changing synaptic drive potential amplitude or action potential threshold, spike properties and after-hyperpolarization (6D). Input resistance also remained unchanged. Effects on inward rectification, another opiate postsynaptic outcome (58), was also not evident; steady membrane hyperpolarization beyond the reversal level for the inhibitory synaptic drive potential failed to alter neuron input resistance (Fig 6C).

Doses of fentanyl larger than 12 µg/kg had other effects, including depression of excitatory and inhibitory synaptic drive potential amplitude and marked reduction of action potential frequency (29).

Figure 6 Here

Effects on Post-Inspiratory and Late-Inspiratory neurons.

Measurements were made on Late-I and Post-I neurons to test the second hypothesis: That an additional mechanism through which opiates slow breathing is to delay the onset and prolong the duration of discharges in VRC Late-Inspiratory and Post-Inspiratory neurons postsynaptically. There is evidence that the neurons modulate the rate of respiration by terminating Inspiratory neuron discharges, and that Post-I neurons can prolong the expiratory phase silent period and delay the onset of Expiratory neuron discharges (12, 18, 21, 43). Thus, slowing and prolongation of their discharge properties would implicate their contribution to opioid rhythm slowing, and alteration of action potential properties and input resistance would suggest postsynaptic effects.

It was possible to maintain stable recordings from only a few neurons of each type long enough to test several doses of fentanyl on each cell. Fentanyl was tested on two types of Post-I neurons, none of which responded to spinal cord stimulation. One type, the vagal Post-I motoneuron (n = 3 cells), was activated synaptically as well as antidromically by vagus nerve stimulation. The neurons, in the absence of stimulation, hyperpolarized during the phrenic nerve inspiratory discharge then depolarized to firing threshold just as phrenic nerve inspiratory activity was terminated and evolved into the postinspiratory discharge phase. Membrane potential then dropped below firing threshold during the
expiratory phase and depolarized to threshold again just before the next cycle of inspiratory phrenic nerve activity (Fig. 7B1). Single shocks (1.5 - 3.0 V, 0.1 ms,) applied to the ipsilateral vagus nerve evoked constant-latency antidromic action potentials that were followed by excitatory postsynaptic potentials with onset latencies of 5 - 7 mSec and bursts of 3 – 4 action potentials (Fig. 7A). Several shocks applied at the end of phrenic nerve inspiratory activity at a rate of 3 Hz produced robust, prolonged Post-I neuron discharges. In association with the neuron effects, there was prolongation of the phrenic nerve postinspiratory discharge and lengthening of the silent period that resulted in slowing of the phrenic nerve rhythm (Fig. 7B2).

**Figure 7 Here**

Fentanyl administration prolonged membrane potential depolarization and discharge, and increased discharge intensity in all three cells (Fig 8A, B). Threshold dose was 3 µg/kg. A cumulative 6 µg/kg dose prolonged depolarization from 3.6 sec (control average) to 6.8 sec, increased the post-inspiratory discharge duration from 1.2 seconds to 4.4 sec and lengthened the pre-inspiratory discharge time from 110 mSec to 223 mSec. Action potential frequency was increased from 5 sec<sup>-1</sup> to 19 sec<sup>-1</sup> during the post-inspiratory phase and from 6 sec<sup>-1</sup> to 10 sec<sup>-1</sup> during the pre-inspiratory phase. In conjunction with these effects, phrenic nerve inspiratory discharges were prolonged from 1.7 sec (control average) to 3.2 sec and expiratory silent periods were lengthened from 2.1 sec to 5.7 sec.

**Figure 8 Here**

The other type of Post-I neuron was non-vagal and non-bulbospinal, but was synaptically activated by vagus nerve stimulation. These neurons (n = 3 cells) were located medial to vagal respiratory motoneurons, and were therefore not part of the PBC. Ipsilateral single shocks evoked EPSPs (5.2 ± 0.7 mSec onset latency) and action potentials (17.5 ± 5.3 mSec) that were accompanied by depression of phrenic nerve discharges (Fig. 9A). Continuous vagus nerve stimulation (not shown) prolonged MP depolarization and discharge duration. Phrenic nerve discharges were shortened and the silent periods lengthened to a degree that cycle frequency slowed. The effects of vagus nerve stimulation confirm findings of Hayashi et al. in adult rats *in vivo* (21) and support their conclusion that the Post-I neurons contribute to inspiratory termination.

Responses to fentanyl were similar to those measured in the other type of Post-I neuron. The threshold dose (3 µg/kg) prolonged hyperpolarizing and depolarizing synaptic drive potentials, and increased discharge intensity and duration (Fig 9C). These effects increased with dose. After 6 µg/kg, the depolarizing synaptic drive potential duration was lengthened from 1.4 sec (control average) to 3.2 sec, discharge duration from 0.6 sec to 3.2 sec, action potential frequency increased from 8.3 sec<sup>-1</sup> to 40 sec<sup>-1</sup> and the duration of the hyperpolarizing synaptic drive potential increased from 2.1 sec to 3.3 sec. In association with the neuronal effects, phrenic nerve inspiratory discharges were lengthened from 1.5 sec (control average) to 3.3 sec and the silent period from 2.1 sec to 10.8 sec.
In either type of Post-I neuron, input resistance, action potential shape, threshold and afterhyperpolarization were not altered.

**Figure 9 Here**

The Late-I neurons (n = 3), located in the same VRC region where non-vagal Post-I neurons were found, exhibited membrane potential and discharge properties similar to those described by Richter (42) and Haji et al. (18). Under control conditions (Fig. 10A), they depolarized at the onset of phrenic nerve activity and discharged briskly late in the inspiratory phase. When the Late-I neurons reached peak action potential frequency, phrenic nerve inspiratory activity terminated and transited into the postinspiratory discharge phase. They then hyperpolarized until the next cycle of phrenic nerve activity began.

Fentanyl (threshold dose, 3 µg/kg) slowed the rate of membrane depolarization, prolonged its duration, lengthened the late inspiratory discharge without altering peak action potential frequency and increased neuron input resistance (Fig. 10 B). These changes increased with dose. After 6 µg/kg, rate of MP depolarization decreased from 14.2 mV/sec (control average) to 5.1 mV/sec, discharge duration lengthened from 0.4 sec to 0.8 sec, peak action potential frequency did not change appreciably from the control average of 43 sec⁻¹ and input resistance increased from 25 MΩ to 33 MΩ. In conjunction with slowing of Late-I neuron rhythm, phrenic nerve inspiratory phase discharge duration increased from 1.1 sec to 1.8 sec and the expiratory silent period increased from 1.7 sec to 3.4 sec. Action potential threshold, shape and afterhyperpolarization in the Late-I neurons were unchanged.

**Figure 10 Here**

**Effects of µ- and δ-opioid receptor antagonists.**

Fentanyl and opioid receptor antagonists were tested on phrenic nerve activity to verify that slowing of the respiratory network rhythm was attributable to µ-opioid receptor-mediated modulation. Given in increments of 1 µg/kg up to a total of 6 µg/kg, fentanyl evoked the usual dose-related effects on phrenic nerve activity. During slowing produced by the 6 µg/kg cumulative dose, the δ-opioid receptor antagonist naltrindole (100 µg/kg) and the µ-opioid receptor antagonist naloxonazine (100 µg/kg) were sequentially administered. Naltrindole failed to alter the effects of fentanyl, whereas subsequent administration of naloxonazine reversed the slowing of rhythm (n = 4 experiments; results not illustrated).

**DISCUSSION**

The issue of how opioids slow breathing has received considerable attention, not only because of the clinical consequences related to opiate overdose, but also because of the presence of opioid receptors in areas that are deemed critical for rhythm generation. Opioid mechanisms of slowing in neonatal in vitro preparations have been reported and
applied to theories of respiratory rhythm generation (37, 56). From the in vitro studies, it now seems clear that a major site where opiate substances slow rhythm is in the preBoetzinger complex (34, 37). However, opiates are known to have actions at other sites in the adult, intact respiratory network that contribute to slowing of breathing (20, 23, 24, 55).

The main findings and conclusions derived from this study are: (1) Lengthening of Inspiratory and Expiratory neuron discharges, effects that cause slowing of respiratory rhythm, are not related to opioid postsynaptic actions on any of the VRC types of neurons tested. (2) Other candidate sites of opiate-mediated rhythm slowing, in particular the PBC and the rostral pons, are implicated from the pattern of slowing.

Arguments to support the conclusions are presented in the following paragraphs, along with a discussion of the candidate sites, types of neurons and putative mechanisms that can and cannot account for the opioid-mediated slowing of the respiratory rhythm.

**Opioid receptors are present on VRC Inspiratory and Expiratory neurons, but do not appear to contribute to network rhythm slowing.**

The evident lack of direct effects on Inspiratory and Expiratory neurons that could contribute to slowing was surprising: first, because i.v doses of morphine and of fentanyl have postsynaptic effects on the neurons (19, 29); second, there is evidence that calcium-activated potassium currents shorten discharge duration in VRC Inspiratory and Expiratory neurons (40, 44) and third, because opioids are known to decrease calcium current through voltage-gated calcium channels in many types of CNS neurons (53). Taken together, these findings raise the possibility that opiates lengthen discharge duration in Inspiratory and Expiratory neurons by depressing intrinsic calcium-activated potassium conductances. Instead, there was no evidence from measurements of input resistance, action potential threshold, shape and afterhyperpolarization that the doses of fentanyl that slowed rhythm acted on membrane conductances. Slowing is evidently generated through effects on other neurons that control the onset and termination of Inspiratory and Expiratory neuron discharges.

**Medullary Late-Inspiratory and Post-Inspiratory neurons do not appear to be directly responsible for opioid slowing of rhythm.**

Late-I and Post-I neurons, according to the network concept of rhythm control, are responsible off-switching Inspiratory neurons. Post-I neurons, in addition, slow the onset of Expiratory neuron discharges and prolong the silent period of phrenic nerve activity (21, 42, 43). A delay in firing of Late-I and Post-I neurons and prolongation of Post-I neuron discharges might thus lead to lengthening of the inspiratory and expiratory phases. Fentanyl did indeed delay and prolong late-I and Post-I firing in association with slowing of cycle frequency in Inspiratory and Expiratory neurons and in phrenic nerve activity. However input resistance and action potential properties in Post-I neurons were unaltered. In Late-Inspiratory neurons, resistance increased throughout all phases of the respiratory cycle, an opiate effect that might have occurred through reduction of afferent
tonic excitatory drive from a number of sources (42, 46). The lengthening of discharge duration can also be explained by an upstream effect: delayed inhibitory synaptic input from Post-I neurons (42). Overall, the measurements do not implicate postsynaptic opiate effects on Late-I and Post-I neurons in network rhythm slowing.

**The properties of rhythm slowing eliminate a few other brain sites of opioid action but implicate the rostral pons and the PBC**

The lengthening of both inspiratory and expiratory phases of the respiratory cycle eliminates a number of potential opioid-mediated mechanisms. For example, opioids enhance Hering-Breuer reflex-mediated slowing of respiration (5, 7, 9, 12, 21, 61), but the effect is due solely to prolongation of the expiratory phase and in the present study, the reflex was eliminated before testing fentanyl by bilateral cervical vagotomy. Respiration is also slowed when N-methyl-D-aspartate (NMDA) receptors are pharmacologically blocked and vagal pulmonary feedback to the central nervous system is prevented (13, 57). Although opioids can suppress NMDA-mediated synaptic transmission (26), NMDA receptor blockade selectively prolongs the inspiratory phase. (13, 31). Anesthetics, particularly pentobarbital, produce rhythm slowing (57) and enhance slowing by opioids (48). However, opioid-mediated reductions of cycle frequency occurred within the same range of doses and to similar dose-dependent degrees in both anesthetized and unanesthetized decerebrate preparations in the present study.

The pattern of rhythm slowing suggests two potential sites of opiate action in the bulbary respiratory network. One is the Pons in the regions of the Kolliker-Fuse nucleus (KFN) and medial Parabrachial nucleus (NPBM). The other site is the preBotzinger Complex.

Direct administration of morphine into the KFN and NPBM regions slows respiratory rhythm by prolonging both the inspiratory and expiratory phases (23, 24, 55). There are functional synaptic connections between KFN neurons and VRG inspiratory and expiratory neurons (49), and computer modeling of the ponto-medullary respiratory network suggests that the timing and patterns of discharge of all of the neurons tested for opiate responsiveness in this study can be influenced by excitatory synaptic inputs from pontine neurons (46).

Inspiratory and Pre-inspiratory neurons of preBotzinger Complex are also potential targets. Opioids slow discharge frequency of Inspiratory neurons in the neonatal brainstem preparation, and the neurons project to other areas of the VRC (35, 38). In the cat a subset, the type-2 Pre-I neuron, exhibits a biphasic pattern of discharge that consists of a brief burst at the onset of phrenic nerve activity and one at the termination of the phrenic nerve inspiratory discharge (50, fig. 7). It was suggested that they play a role in phase transition between expiration and inspiration (6, 28, 51). Lengthening of the interval between the biphasic discharges of type-2 neurons fits with the lengthening of both inspiration and expiration by opioids. Opiate effects on feline Pre-I neurons have not, however, been reported, and in the more rostral (Para-Facial) region of the neonatal rodent preparation, Pre-I neurons that discharge before and immediately after inspiratory
motor nerve discharges do not respond to application of μ-opioid receptor agonists (37, 54).

In summary, the study eliminates several sites in the medullary VRC that might have contributed to slowing of respiratory rhythm by fentanyl. The pattern of slowing suggests that an important site may be the pons. The latter, along with neurons of the PBC, could be the primary targets for opiate slowing in the adult respiratory network, although additional sites such as the Boetzinger Complex, a more rostral component of the VRC, should also be considered (43).

ACKNOWLEDGEMENTS

Research supported by National Institutes of Health grant no. HL 65526.

REFERENCES


**FIGURE LEGENDS**

*Figure 1. Locations in the ventrolateral respiratory column (VRC) of the medulla oblongata where tested neurons were located.*

Enclosed (boxed) areas on the left side in each of the three medullary cross sections define the locations of the Inspiratory, Late-Inspiratory, Post-Inspiratory and Expiratory neurons tested. Anatomical abbreviations on the right side: Amb, nucleus ambiguus; C, caudal cuneate nucleus; CST, corticospinal tract; CUR, cuneate nucleus, rostral division; CX, cuneate nucleus, external division; G, gracile nucleus; IO, inferior olive; LRN, lateral reticular nucleus; P, pyramidal tract; PBC, preBoetzinger Complex; TS, solitary tract; 5SP, spinal trigeminal nucleus, parvocellular division; 5ST, spinal trigeminal tract.

*Figure 2. Dose-related effects of fentanyl responsible for the slowing of rhythm in bulbospinal Inspiratory neurons of the VRC.*

Panel A. Slowing of respiratory rhythm in a bulbospinal inspiratory neuron by the µ-opioid receptor agonist fentanyl. Upper traces in left and right panels are membrane potential (MP). Upper and lower dashed lines are references that denote action potential threshold and maximum membrane hyperpolarization, respectively. Lower traces are electroneurograms of phrenic nerve activity (PNA). Panel B. Expanded time records of action potentials. Dashed lines denote action potential threshold (upper lines) and maximum afterhyperpolarization (lower). Panel C. Graphical summary of the dose-related effects of fentanyl on discharge duration, peak action potential frequency (APs/sec) and discharge frequency (bursts/min) in 6 bulbospinal inspiratory neurons. Lines are meant only to connect the 3, 6 and 10 µg/kg dose-related data points; other (1, 2, 4, ..., 9 µg/kg) data points are left out to simplify the graphical presentation. Threshold
dose in all experiments was 3 µg/kg. Values on the ordinate scale are represented as percents of control responses. Actual control values (mean ± SE) are listed in the upper left corner. Abscissa, cumulative doses of fentanyl given intravenously.

**Figure 3. Dose-related slowing of respiratory rhythm by fentanyl in a non-vagal, non-bulbospinal Inspiratory neuron.**

In panels A – E, the upper traces are recordings of membrane potential and discharge properties. Lower traces, electroneurograms of phrenic nerve activity (PNA). The time scale in panel A applies to all other panels. Doses of fentanyl shown are cumulative.

**Figure 4. Progressive lengthening of discharges and slowing of their rate with increasing doses of fentanyl in bulbospinal Expiratory neurons of the caudal VRC.**

Time scale in panel A applies to all other panels. Increasing cumulative doses of fentanyl lengthen discharge duration, prolong inspiratory phase membrane hyperpolarization and slow the rate of depolarization to firing threshold. Maximum membrane hyperpolarization and depolarization were unchanged by fentanyl. The largest cumulative dose of fentanyl (panel D) also depressed the intensity of phrenic nerve discharges.

**Figure 5. Graphical summary of the dose-relayed slowing of rhythm in bulbospinal expiratory neurons.**

Results obtained from 6 neurons tested in pentobarbital-anesthetized cats. Format is identical to Fig. 1B. Absolute control values are listed in the upper left corner,

**Figure 6. Input resistance and action potential properties are unchanged in bulbospinal Expiratory neurons during rhythm slowing by fentanyl.**

In the upper traces of panels A – C, Brief, regularly spaced downward deflections of membrane potential to measure of neuron input resistance were produced by 60 mSec hyperpolarizing constant current pulses. In panel C, membrane potential was hyperpolarized beyond the reversal level for the inhibitory synaptic drive potential by applied anionic DC current (1 nA). Time scale in panel A also applies to panels B and C. Panel D, traces are action potentials recorded at a faster time scale. Discharges in the upper and lower panels were recorded during peak late-expiratory phase intensities under control conditions (upper panel) and after 6 µg/kg fentanyl (lower), in the absence of DC current and hyperpolarizing current pulses. Dashed lines denote spike threshold (upper) and afterhyperpolarization (lower).
**Figure 7. Vagus nerve stimulation produces prolongation of discharge in Post-I vagal motoneurons, accompanied by respiratory network slowing.**

Panel A, superimposed traces on an expanded time scale to illustrate antidromic followed by synaptic responses of a vagal Post-Inspiratory neuron (MP; upper traces) to 3 Hz single shocks applied to the ipsilateral vagus nerve. Panel B, responses recorded on a compressed time scale without (1.) and with (2.) vagus nerve stimulation. Note that vagus nerve stimulation lengthens the silent period of PNA in conjunction with robust, sustained Post-I neuron firing. Both the silent period and the neuron discharge outlast vagus nerve stimulation and terminate simultaneously.

**Figure 8. Fentanyl prolongs and intensifies vagal Post-I neuron discharges and slows respiratory network rhythm.**

Time scale in panel A also applies to panel B. Recordings are from the same neuron and phrenic nerve shown in Figure 8.

**Figure 9. Fentanyl also prolongs non-vagal Post-I neuron discharges in conjunction with slowing of respiratory network rhythm.**

Panel A, upper trace, synaptic activation of the Post-I neuron by a single shock applied to the ipsilateral vagus nerve. Lower trace, phrenic nerve reflex depression, as denoted by dashed reference line, by the vagus nerve single shock. Panels B and C, effects of fentanyl on membrane potential and discharge properties of the same Post-I neuron and on phrenic nerve activity. Records in B and C were taken on a more compressed time scale. Brief, regularly space downward deflections of membrane potential were produced by 60 mSec hyperpolarizing constant current pulses, applied for the purpose of measuring neuron input resistance. Middle traces, rate meter records as moving averages of phrenic nerve compound action potential frequency (time constant, 0.1 Sec). Bottom traces, electroneurograms of phrenic nerve activity.

**Figure 10. Fentanyl slows rhythm in a Late-Inspiratory neuron in conjunction with slowing of respiratory network rhythm.**

Time scale in panel A also applies to panel B. Rhythmic membrane depolarization and hyperpolarization in the neuron are slowed along with phrenic nerve activity by fentanyl, and neuron input resistance is increased during all phases of the respiratory cycle.
FIGURES

3.0 - 3.6 mm Rostral to Obex

Non-vagal Inspiratory Neurons

Vagal Inspiratory Neurons

Late Inspiratory Neurons

2.5 - 3.0 mm Rostral to Obex

Bulbospin. Inspiratory Neurons & Post-I Neurons

1 mm Caudal to Obex

Bulbospin. Expiratory Neurons

Figure 1
Figure 2
Figure 3
Figure 4

Figure 5

Raw Control Data

\[ n = 6 \text{ cells} \]

- 16.1 ± 1.0 bursts/min
- 56 ± 10.1 APs/sec
- 1.95 ± 0.19 sec (discharge duration)

\[
\begin{align*}
\text{Dose, } \mu\text{g/kg} & \quad \text{APs/sec} \\
0 & \quad 117 \pm 20.3 \% \\
3 & \quad 100 \%
\end{align*}
\]

\[
\begin{align*}
\text{Bursts/min} & \quad \text{Discharge duration} \\
0 & \quad 69.6 \pm 13.3 \%** \\
6 & \quad 54.6 \pm 19.3 \%**
\end{align*}
\]

\[
\begin{align*}
\text{Dose, } \mu\text{g/kg} & \quad 215 \pm 20.2 \% \\
6 & \quad 97.6 \pm 22.8 \%
\end{align*}
\]

\*, p < 0.05

**, p < 0.001
Figure 8
A. Vagal Reflex Response

B. Control
C. Fentanyl 6μg/kg

Figure 9