Fentanyl and Morphine, but not Remifentanil, Inhibit Acetylcholine Release in Pontine Regions Modulating Arousal

Steven Mortazavi, M.D.,* Janel Thompson, B.S.,† Helen A. Baghdoyan, Ph.D.,‡ Ralph Lydic, Ph.D.‡

Background: Opioids inhibit the rapid eye movement (REM) phase of sleep and decrease acetylcholine (ACH) release in medial pontine reticular formation (mPRF) regions contributing to REM sleep generation. It is not known whether opioids decrease ACH release by acting on cholinergic cell bodies or on cholinergic axon terminals. This study used in vivo microdialysis to test the hypothesis that opioids decrease ACH release at cholinergic neurons in the laterodorsal tegmental nuclei (LDT) and LDT axon terminals in the mPRF.

Methods: Nine male cats were anesthetized with halothane, and ACH levels within the mPRF or LDT were assayed using microdialysis and high-pressure liquid chromatography (HPLC). ACH levels were analyzed in response to dialysis of the mPRF and LDT with Ringer's solution (control), followed by dialysis with Ringer's solution containing morphine sulfate (MSO₄) or naloxone. ACH in the mPRF also was measured during either dialysis delivery or intravenous infusion of remifentanil and during dialysis delivery of fentanyl.

Results: Compared with dialysis of Ringer's solution, microdialysis with MSO₄ decreased ACH by 23% in the mPRF and by 30% in the LDT. This significant decrease in ACH was antagonized by naloxone. MSO₄ and fentanyl each caused a dose-dependent decrease in mPRF ACH when delivered by dialysis. Remifentanil delivered by continuous intravenous infusion or by dialysis into the mPRF did not alter mPRF ACH.

Conclusions: Morphine inhibits ACH at the cholinergic cell body region (LDT) and the terminal field in the mPRF. ACH in the mPRF was not altered by remifentanil and was significantly decreased by fentanyl. Thus, MSO₄ and fentanyl disrupt cholinergic neurotransmission in the LDT–mPRF network known to modulate REM sleep and cortical electroencephalographic activity. These data are consistent with the possibility that inhibition of pontine cholinergic neurotransmission contributes to arousal state disruption by opioids. (Key words: Arousal state control; cholinergic neurotransmission; laterodorsal tegmental nucleus; microdialysis.)

MORPHINE sulfate (MSO₄) is known to decrease cholinergic neurotransmission in many brain regions. Opioid inhibition of acetylcholine (ACH) release is directly relevant to anesthesia because ACH plays a key role in the central nervous system regulation of arousal, respiratory control, and perception of painful stimuli. Although opioids can contribute to clinically significant disruptions of arousal state and cardiopulmonary control, the cellular and molecular mechanisms underlying these opioid side effects remain incompletely understood. These diverse effects are unified, however, by the identification of a neuronal network in the pontine brain stem through which opioids and ACH modulate arousal state, breathing, and antinociceptive behavior.

The medial pontine reticular formation (mPRF) is a cholinergic region that receives its cholinergic input from the more rostral laterodorsal tegmental nuclei (LDT) and pedunculopontine tegmental nuclei (PPT). Cholinomimetics microinjected into the mPRF of conscious animals enhance rapid eye movement (REM) sleep, whereas MSO₄ microinjected into the mPRF inhibits REM sleep and concurrently increases apneic breathing. This MSO₄-induced REM sleep inhibition is mediated by μ-opioid receptors in the mPRF. ACH release in the mPRF is significantly increased during REM sleep, and systemically administered MSO₄ inhibits ACH release in the mPRF. The forgoing basic data are consistent with the clinical observation that opioids contribute to the sleep deprivation, delirium, and parasomnias comprising intensive care unit (ICU) syndrome.

The degree to which mPRF ACH is inhibited by opioids at cell bodies in the cholinergic LDT and cholinergic terminals projecting from LDT to the mPRF has not been quantified. Therefore, this study examined the hypothe-
sis that dialysis delivery of MSO₄, fentanyl, or remifentanil would decrease ACh in the mPRF and LDT regions of the pons. ACh in the mPRF also was measured during intravenous delivery of remifentanil.

Methods and Materials

Experimental Model and Microdialysis Procedure

Adult male cats (n = 9) were implanted with a permanent, plastic cranioplast designed to provide subsequent access to the pontine brain stem. Cats were allowed to recover for 4 weeks before beginning the microdialysis experiments. For each experiment an animal was anesthetized by mask induction with halothane. The trachea was sprayed with lidocaine, 0.5%, and then intubated. An Ohmeda Rascal II monitor (Ohmeda, Salt Lake City, UT) was used to measure end tidal CO₂ and halothane. End-tidal CO₂ was maintained at 30 mmHg by adjusting ventilation. Halothane was maintained at 1.4% by adjusting inspired concentration (1 MAC for cat = 1.2%). Blood pressure and oxygen saturation were measured noninvasively throughout each experiment using a Dinamap (Critikon, Tampa, FL) and an Ohmeda Biox 5700 Pulse Oximeter (Ohmeda, Boulder, CO). A T/Pump Heat Therapy System (Gaymar, TP100 Series, Orchard Park, NY) and a rectal thermometer (Model 43TA, Yellow Springs Instrument Co., Yellow Springs, OH) were used to maintain core body temperature at 37°C. Repeated microdialysis experiments in the same animal were separated by a minimum of 1 week.

Microdialysis studies of unanesthetized cats have demonstrated that ACh levels in the pontine brain stem vary significantly across the sleep-wake cycle (reviewed in reference 3). Fluctuating levels of arousal, therefore, will significantly alter measures of ACh release in the mPRF and LDT. Thus, halothane anesthesia was used in the present study to eliminate spontaneous oscillations during sleep and wakefulness. Holding arousal state constant with halothane made it possible to test the hypothesis that ACh levels in the LDT and mPRF were altered by opioids, independent of fluctuating states of arousal. ACh levels in the mPRF are slightly reduced below waking levels by halothane, but halothane does not alter high-pressure liquid chromatography (HPLC) column sensitivity for ACh detection. The use of halothane anesthesia as an experimental tool for studies of pontine cholinergic neurotransmission has been demonstrated.

As described elsewhere, in vivo microdialysis was accomplished with a polycarbonate membrane (20 kDa pore, 2 mm length, 0.5 mm diameter). Before the in vivo portion of each experiment, preexperimental probe recoveries were determined by dialyzing a vial containing a known concentration of ACh. At the termination of the in vivo dialysis experiment, postexperimental probe recoveries again were collected using a known concentration of ACh. Pre- and postexperimental probe recoveries were compared to ensure that measures of ACh in pmol/10 min of dialysis reflected neurochemical changes rather than dialysis probe damage. Quantification of ACh with these techniques is widely regarded as measuring neurotransmitter release rather than neurotransmitter turnover because ACh release is blocked by dialysis delivery of tetrodotoxin and by dialysis with Ca²⁺-free Ringer's solution.

For each experiment, the microdialysis probe was placed in the mPRF or LDT using the stereotaxic coordinates of Berman. The large size of the feline brain made it possible to perform repeated dialysis sampling from different locations within the mPRF of each animal. After stereotaxically inserting the probe, pontine dialysis began at a rate of 5 µl/min, and every 10 min sequential 30-µl dialysate samples were collected for quantifying ACh (pmol/10 min of dialysis). The dialysis probe was linked via a liquid switch to three different syringes, each containing different drug solutions. Control data were provided by measures of ACh during 50-80 min (5-8 samples) of dialysis with Ringer's solution. The liquid switch then was turned to the syringe containing 8.8 µM MSO₄ dissolved in Ringer's solution, and another 50-80 min of dialysis samples were obtained. During the third portion of these experiments, the liquid switch was again activated to permit dialysis delivery of Ringer's solution containing naloxone (88 nM). Additional experiments simultaneously delivered these same concentrations of MSO₄ and naloxone while measuring mPRF ACh.

Acetylcholine in the mPRF also was measured during continuous intravenous administration of remifentanil (0.5 µg · kg⁻¹ · min⁻¹) (Ultiva, Glaxo Wellcome, Research Triangle Park, NC) and during mPRF microdialysis delivery of Ringer's solution (control) versus Ringer's solution containing remifentanil (8.8 µM). The dose-dependent effects of MSO₄ or fentanyl on mPRF ACh were examined by dialyzing the mPRF with three concentrations of MSO₄ or fentanyl (0.088, 0.88, and 8.8 µM).

Acetylcholine Assay

At the end of each 10-min dialysis interval, the 30-µl sample immediately was injected into the HPLC coupled
with an electrochemical detection system (Bioanalytical Systems, West Lafayette, IN). Samples were carried in a
50 mM NaHPO₄ mobile phase, pH 8.5, at 1 ml/min. Samples initially passed through a polymeric analytical
column, permitting separation of ACh and choline. Next, the samples passed through an immobilized enzyme
reactor wherein immobilized acetylcholinesterase and choline oxidase converted ACh into H₂O₂ and betaine.
H₂O₂ was produced from ACh and choline in stoichiometric amounts and measured at a 0.5-V applied potential
on a platinum electrode relative to a Ag⁺/AgCl reference electrode. The area under the chromatogram
peaks was proportional to the amount of ACh and choline in the samples. Chromatogram peaks were recorded
on a flatbed recorder and simultaneously digitized and stored on disk using ChromGraph (Bioanalytical Sys-
tems) software. The area under the chromatogram peak from each injection was referenced to a standard curve
to calculate the amount of ACh in each dialysis sample, expressed as pmol/10 min of dialysis.

Statistical Analysis
Pre- and postexperimtal dialysis probe recoveries were compared by independent t test to ensure that the
ACh release data were not confounded by any alterations in dialysis probe recovery. Descriptive statistics pro-
vided mean ± SD values for all dependent measures. Repeated measures analysis of variance (ANOVA) was
used to evaluate the presence of a statistically significant effect of microdialysis drug delivery on ACh release.
When ANOVA indicated a statistically significant drug main-effect on ACh, the different drug conditions were
compared using Tukey and Dunnett multiple comparison tests. Systolic and mean arterial blood pressure and
heart rate were analyzed for all experiments. For all inferential statistics, the probability (P) value for statisti-
cal significance was P < 0.05.

Results
Morphine sulfate suppressed ACh release from micro-
dialysis sites within the mPRF and LDT nuclei. Figure 1
illustrates the anatomic relationship between the LDT
and mPRF (fig. 1A) and unilateral dialysis probe place-
ment in the LDT (fig. 1B) and mPRF (fig. 1C) regions of
the pontine brain stem. All LDT ACh measures were
obtained from animals in which no probe lesions had
been created in the mPRF terminal field. Likewise, all
mPRF dialysis measures were obtained from animals in

Anesthesiology, Vol. 90, No. 4, Apr 1999

Fig. 1. Sagittal views of the pontine brain stem. (A) Schematic
diagram indicating that cholinergic LDT neurons send projec-
tions to the cholinergic mPRF. The LDT–mPRF relationship
is simplified here for illustrative purposes. LDT connectivity is
complicated, including LDT reciprocal connections and projec-
tions to the thalamus and to the contralateral mPRF (reviewed in
reference 9). Cresyl violet-stained sections of the brain stem
illustrate lesions used to confirm dialysis probe placement in
the LDT (B) and mPRF (C). In B and C, the black arrow marks
the tip of the dialysis probe lesion in the LDT and mPRF, respec-
tively; calibration bars at lower right = 1 mm. Approximate
lateraltities are 1.2 mm for LDT (B) and 1.6 mm for mPRF (C).
6N = abducens nerve; 7G = genu of facial nerve; PAG = peri-
aqueductal gray; TB = trapezoid body.

which no microdialysis probes had been placed in the
LDT cell body region. ACh levels in the mPRF were
Fig. 2. Time course of ACh in pmol/10 min of LDT dialysis during a representative experiment from one animal. LDT ACh is plotted as a function of dialysis time in min. Time bins 10–50 plot ACh during Ringer’s solution (control) dialysis. Time bins 60–100 indicate ACh during dialysis delivery of morphine to the LDT, and bins 110–150 show dialysis delivery of naloxone (88 ns). There are no indications of variance here because each bar represents the pmol value of ACh measured during one 10-min dialysis sample. In contrast, the data summarized by figures 3–7 represent mean change in ACh release averaged by condition for multiple experiments conducted in multiple animals.

significantly decreased (−23%) to 0.23 pmol/10 min. During dialysis delivery of naloxone, ACh in the mPRF averaged 0.29 pmol/10 min, which was not significantly different from the Ringer’s solution control group. To confirm naloxone blocking (fig. 4A) and rule out the possibility of non-specific wash out of morphine during microdialysis with naloxone, the two compounds also were coadministered by microdialysis. Figure 4B shows that mPRF ACh was not altered by MSO₄ when it was coadministered with naloxone.

Dialysis of the mPRF with increasing concentrations of MSO₄ caused a dose-dependent decrease in mPRF ACh (figure 5). Average mPRF ACh (pmol/10 min) by dialysis condition was Ringer’s solution, 0.26; 0.088 μM MSO₄, 0.28; 0.88 μM MSO₄, 0.22; 8.8 μM MSO₄, 0.11. It was not possible—with the electrical sensitivity necessary to measure ACh in fractions of a pmol—to deliver morphine in concentrations greater than 8.8 μM. For example, at a concentration of 88.0 μM, MSO₄ passing over the electrochemical detector produced a chromatographic peak of sufficient amplitude and duration to occlude the ACh chromatogram.

The forgoing results with MSO₄ encouraged quantification of mPRF ACh during dialysis delivery of synthetic opioids. In three animals, mPRF ACh was measured during mPRF dialysis delivery of 8.8 μM remifentanil followed by naloxone (fig. 6A) and during continuous intravenous delivery of remifentanil (fig. 6B). In contrast to MSO₄, remifentanil had no effect on mPRF ACh during dialysis delivery into the mPRF (fig. 6A) or during intravenous administration (fig. 6B).

Morphine Inhibits Pontine Cholinergic Neurotransmission

The time course for a typical experiment in which microdialysis was used to deliver Ringer’s solution (control), Ringer’s solution containing MSO₄ (8.8 μM), and Ringer’s solution containing naloxone (88 ns) to the cholinergic LDT is shown in figure 2. These time-course data illustrate that individual experiments yielded multiple dialysis measures of ACh per treatment condition (Ringer’s solution, morphine, naloxone). Figure 3 summarizes the group data from all experiments in which the LDT was dialyzed with morphine followed by naloxone. Mean ACh in the LDT was 0.36 pmol/10 min with Ringer’s solution and 0.25 pmol/10 min during dialysis delivery of morphine. Morphine caused a significant decrease (−30%) in LDT ACh, and this decrease was reversed by LDT administration of naloxone.

Figure 4A summarizes the effects of mPRF morphine and naloxone on mPRF ACh levels. During dialysis of the mPRF with Ringer’s solution, mean mPRF ACh was 0.30 pmol/10 min of dialysis. When dialysis was switched to Ringer’s solution containing MSO₄, ACh in the mPRF was decreased by dialysis delivery of 1 μM tetrodotoxin, a finding consistent with the well-accepted view that these microdialysis data reflect measures of ACh release. The blood pressure and heart rate data revealed no anesthesia-induced hypotension or bradycardia during pontine microdialysis.
mPRF ACh release was measured in response to mPRF dialysis delivery of fentanyl. Fentanyl caused a dose-dependent decrease in mPRF ACh (fig. 7). Average mPRF ACh (pmol/10 min) by dialysis condition was Ringer's solution, 0.29; 0.088 μm fentanyl, 0.25; 0.88 μm fentanyl, 0.20; 8.8 μm fentanyl, 0.17.

**Discussion**

**Morphine Depresses ACh at Pontine Cholinergic Cell Bodies (LDT) and Cholinergic Terminals (mPRF)**

Intravenous administration of morphine previously was shown to decrease cholinergic neurotransmission in mPRF regions known to regulate electroencephalographic (EEG) and behavioral arousal. It was not clear, however, whether morphine decreased ACh release by actions at cholinergic cell bodies or by actions at cholinergic terminals (fig. 1A). The present data resolve this question by showing that dialysis delivery of morphine decreased ACh release in the cholinergic LDT (figs. 1B and 5) and in the LDT terminal projection field within the mPRF (figs. 1C and 4). At both the cholinergic LDT and the mPRF regions receiving LDT cholinergic efferents, the statistically significant reductions in ACh caused by morphine were reversed by naloxone (figs. 3 and 4).

A statistically significant, dose-dependent decrease in mPRF ACh was observed during microdialysis delivery of morphine (fig. 5) or fentanyl (fig. 7) to the mPRF. These data parallel the previous discovery that microinjection of morphine into the mPRF of intact, unanesthetized cats caused a naloxone-reversible, dose-dependent inhibition of REM sleep. Morphine inhibition of REM sleep and mPRF ACh (fig. 5) also is consistent with converse lines of evidence demonstrating REM sleep-specific enhancement of cholinergic neurotransmission. For example, mPRF ACh release is significantly increased during REM sleep, and electrically stimulating pontine cholinergic neurons significantly increases mPRF ACh release and REM sleep.

**Morphine Differentially Alters Supraspinal and Spinal ACh**

The present finding that morphine decreased pontine ACh is consistent with other data indicating that morphine inhibits cholinergic neurotransmission. Although

**Fig. 5.** Mean ± SD mPRF ACh as a function of dialysis delivery of increasing concentrations of MSO₄ to the mPRF. Six dialysis experiments in four animals revealed a statistically significant concentration main-effect of MSO₄ on ACh (F = 7.1; d.f. = 3, 151; P < 0.0002). For the group data and the representative experiment shown here, only 8.8 μmol MSO₄, was significantly different (*) by multiple comparisons test from all other morphine concentrations in altering mPRF ACh.
OPIOID INHIBITION OF PONTINE ACh

Fig. 6. Mean + SD mPRF ACh during administration of remifentanil. In three animals, mPRF ACh was not significantly altered during dialysis delivery of Ringer's solution, 8.8 μM remifentanil, or naloxone (88 mg) to the mPRF (A). There was no significant difference in mPRF ACh comparing mPRF Ringer's solution dialysis to mPRF dialysis during 50 min of continuous intravenous administration (B) of remifentanil (0.5 μg · kg⁻¹ · min⁻¹).

opiods have been shown to decrease ACh release in many brain regions, it should be clear that opioid effects on ACh release vary as a function of the tissue in which ACh is measured. Morphine decreases ACh in the pons (figs. 3-5), but opioids stimulate ACh release in spinal cord. In ewes and in one human volunteer, intravenous morphine increased release of ACh in spinal cord. Opioid receptors are differentially distributed throughout the brain, and the effects of activating even a particular opioid receptor subtype can be site-dependent within the nervous system.

In addition to the forgoing site-specific differences in the effects of opioids on ACh, there also are some remarkable similarities between spinal and pontine cholinergic neurotransmission. These similarities are demonstrated by four congruent lines of evidence. First, muscarinic cholinergic receptors play a role in regulat-

ing ACh release in spinal cord, and ACh release in the mPRF also is modulated by muscarinic autoreceptors of the M2 subtype. Second, the ubiquitous transmembrane signaling molecule nitric oxide has been associated with cholinergic neurotransmission in the mPRF and spinal cord. Inhibition of mPRF nitric oxide synthase decreases mPRF ACh release, and in spinal cord ACh stimulates the production of nitric oxide. Third, long-standing (reviewed in reference 24) and recent evidence shows that spinal application of cholinomimetics is powerfully antinociceptive. In the same regions of the mPRF illustrated by figure 1, microinjection of cholinergic agonists and acetylcholinesterase inhibitors enhanced antinociceptive behavior. Finally, remifentanil has been observed not to alter neurotransmission at pontine and spinal levels. The inability of remifentanil to alter mPRF ACh (fig. 6) is similar to the finding that continuous spinal remifentanil infusion failed to alter glutamate release. The variety of cellular cascades by which opioids differentially alter supraspinal and spinal ACh release remains to be specified. The forgoing similarities and differences suggest research opportunities aiming to characterize the supraspinal and spinal mechanisms of opioid action on cholinergic neurotransmission and arousal state control.

Potential Clinical Relevance

Although patients who receive opioids report feeling sleepy, morphine actually increases wakefulness and inhibits the REM phase of sleep. At the beginning of

Fig. 7. Mean + SD mPRF ACh during mPRF dialysis delivery of fentanyl. There was a statistically significant concentration main-effect (F = 4.35; d.f. = 3, 59; P < 0.008) of fentanyl on mPRF ACh. Dunnett statistic indicated that both 0.88 μM and 8.8 μM fentanyl significantly decreased mPRF ACh compared with the Ringer's solution control group.
this decade, pioneering studies documented significant changes in REM sleep and cardiopulmonary control caused by the perioperative use of opioids. The first night after surgery is characterized by REM sleep inhibition, followed by intense REM sleep rebound on the second and third postoperative nights.\textsuperscript{28} Postoperative patients treated with morphine display a dose-related decrement in REM sleep.\textsuperscript{28} Additionally, hypoxic episodes during REM sleep rebound on postoperative nights 2 and 3 are 300% more frequent than on the preoperative night.\textsuperscript{30} It now is clear from many clinical studies that cardiopulmonary complications are correlated with REM sleep rebound occurring postoperatively.\textsuperscript{4,30–35} Disruption of cortical cholinergic neurotransmission also is known to cause delirium and impaired memory function.\textsuperscript{36} Transient postoperative delirium after anesthesia has an incidence of 10–60% and contributes to an increase in patient morbidity, delayed functional recovery, and prolonged hospital stays.\textsuperscript{36} Thus, multiple lines of evidence demonstrate the potential clinical significance of understanding the mechanisms by which opioids disrupt arousal state control.

**Limitations and Conclusions**

One limitation of the present study is the inability to account for the observation that remifentanil did not decrease mPRF ACh levels. Two factors contributed to the expectation that remifentanil would depress mPRF ACh. First, mPRF ACh was decreased by morphine (figs. 4 and 5). Second, remifentanil behaves as a µ-opioid agonist with an analgesic intrathecal potency that is 17 times greater than morphine.\textsuperscript{37} Remifentanil hydrochloride is formulated to contain glycine, a potent inhibitory neurotransmitter.\textsuperscript{38} It is not clear from these data whether the inability of remifentanil to alter pontine ACh levels is a result of the presence of glycine or of the fact that remifentanil is rapidly hydrolyzed.

Acetylcholine modulates cortical activation and behavioral arousal, but no single neurotransmitter regulates arousal state control. Monoamines are also known to alter sleep,\textsuperscript{29} sedation, and nociception.\textsuperscript{39} Therefore, another limitation of the present study is the possibility that monoamines or some other neurotransmitter system contributed to opioid inhibition of ACh. This possibility is open to future experimental tests.

In conclusion, the present data show for the first time that diazys delivery of opioids decreased ACh levels in the cholinergic LDT (figs. 2 and 3) and in the LDT terminal projection field within the mPRF (figs. 4, 5, and 7). The naloxone-blocking data (fig. 4) and opioid dose-dependent reduction of ACh levels (figs. 5 and 7) suggest µ-receptor modulation of cholinergic neurotransmission. The finding that centrally administered opioids decreased ACh levels in the mPRF agrees with previous data showing that microinjection of opioids into the mPRF disrupts REM sleep.\textsuperscript{10,11} In the human brain, pontine reticular sites homologous to those described here (fig. 1) also regulate arousal and REM sleep.\textsuperscript{41} Thus, the present results are consistent with the possibility that inhibition of pontine cholinergic neurotransmission contributes to arousal state disruption by opioids. Finally, cholinergic stimulation of the mPRF has been shown to reverse halothane-induced depression of cortical excitability.\textsuperscript{18,42} Taken together, these findings support the working hypothesis that pontine cholinergic mechanisms known to generate naturally occurring states of consciousness preferentially modulate the altered states of consciousness produced by analgesic and anesthetic molecules.\textsuperscript{3,6}

The authors thank Dr. Garfield Russell for his critical reading of the manuscript; for expert assistance, the authors also thank N. Parisi, J. L. DiVittore, W. A. Martin, J. Graybal, and P. Myers.

**References**


10. Keifer JC, Baghdoyan HA, Lydic R. Sleep disruption and in-
creased apneas after pontine microinjection of morphine. Anesthesiology 1992; 77:973–82.