The Effects of Morphine-Induced Increases in Extracellular Acetylcholine Levels in the Rostral Ventrolateral Medulla of Rat

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Accepted for publication February 3, 1999. This paper is available online at http://www.jpet.org

ABSTRACT

The present study examined the role of the rostral ventrolateral medulla (RVLM) in the modulation of acetylcholine (ACh) release by morphine. We examined the effect of morphine on the release of ACh in the RVLM of freely moving rats using the in vivo microdialysis method. The basal level of ACh was 303.0 ± 28.2 fmol/20 µl/15 min in the presence of neostigmine (10 μM). Morphine at a low dose of 5 mg/kg (i.p.) increased ACh release by the RVLM by 42.4%. A higher morphine dose (10 mg/kg i.p.) significantly increased the release of ACh by 75.4%, with a maximal effect (86.4%) at 75 min. This enhancement following i.p. administration of morphine was reversed by naloxone (1 mg/kg i.p.). Addition of morphine (10^{-4} M) to the perfusion medium increased the ACh release by 85.8% of the predrug values. The increased ACh release induced by local application of morphine was reversed by pretreatment with naloxone (1 mg/kg i.p.). The antinociceptive effect of locally applied morphine into the RVLM was assessed using the hot-plate test and tail immersion test in unanesthetized rats. Local application of morphine (10^{-4} M) via a microdialysis probe induced an increase in both tail withdrawal and hot-plate response. These findings suggest that morphine seems to exert a direct stimulatory effect on ACh release by the RVLM and that morphine-induced nociception is, in part, activated by the release of ACh in freely moving rats.

The rostral ventrolateral medulla (RVLM), which includes the nucleus reticularis gigantocellularis (NRGC)/nucleus reticularis gigantocellularis α (NRGCa) and the lateral reticular nucleus, regulates part of the physiological function of antinociceptive and cardiovascular control via the cholinergic system (Ossipov and Gebhart, 1986; Zhuo and Gebhart, 1992; Kubo et al., 1997). Anatomical evidence has demonstrated that the pedunculopontine tegmental nucleus in the brainstem is a major source of cholinergic afferents to the RVLM (Mitani et al., 1988; Yasaki et al., 1990; Sherriff and Hender- son, 1994). In addition, other studies have shown the existence of a descending cholinergic system from the RVLM to the spinal cord and the presence of small to medium-sized cholinergic neurons and choline acetyltransferase mRNA in small cells of the NRGC/nucleus reticularis paragigantocellularis (NRPG) (Bowker et al., 1983; Jones et al., 1986; Tago et al., 1989; Ruggiero et al., 1990; Lauterborn et al., 1993). These observations suggest that the cholinergic system plays an important role in the RVLM.

The interaction of morphine with cholinergic neurons in the central nervous system is well known. Using in vivo microdialysis, morphine has been shown to depress the release of acetylcholine (ACh) in the brain of rat (Rada et al., 1991; Taguchi et al., 1993). On the other hand, also using in vivo microdialysis, i.v. administration of morphine has been shown to produce a dose-dependent increase in ACh in human cerebrospinal fluid and sheep dorsal horn (Bouaziz et al., 1996). Furthermore, the cholinergic system in the central nervous system is considered part of an endogenous pain control system, activation of which can produce antinociception and analgesia in a variety of animals including humans (Christensen and Gross, 1948; Gillberg et al., 1990; Zhuo and Gebhart, 1990, 1991; Iwamoto and Marion, 1993; Hood et al., 1995). Pharmacological studies provide evidence that acetylcholine or carbachol administered into particular brainstem nuclei can produce pronounced antinociception that is reversed by muscarinic antagonists (Brodie and Proudfit, 1984; Yaksh et al., 1985). In addition, there is evidence that the descending cholinergic system, spinal cholinergic receptors, and anticholinesterase are involved in the mechanisms of opioid analgesia (Dirksen and Nijhuis, 1983; Naguib and Yaksh, 1994; Fang and Proudfit, 1996; Hood et al., 1997). For example, the antinociception produced by systemic administration of morphine is antagonized by intrathecal administration of the cholinergic muscarinic receptor antagonist, atropine (Chiang and Zhuo, 1989). Moreover, various combinations of morphine with anticholinesterase have been demonstrated to result in an increased antinociceptive effect.

ABBREVIATIONS: ACh, acetylcholine; RVLM, rostral ventrolateral medulla; NRGC, nuclei reticularis gigantocellularis; TTX, tetrodotoxin.
Male adult Wistar rats (300–350 g) were anesthetized with pentobarbital sodium (50 mg/kg i.p.) and positioned in a stereotaxic apparatus. In each rat, the skull was exposed, and a hole was drilled to barbital sodium (50 mg/kg i.p.) and positioned in a stereotaxic apparatus. In each rat, the skull was exposed, and a hole was drilled to barbital sodium (50 mg/kg i.p.) and positioned in a stereotaxic apparatus. In each rat, the skull was exposed, and a hole was drilled to barbital sodium (50 mg/kg i.p.) and positioned in a stereotaxic apparatus. In each rat, the skull was exposed, and a hole was drilled to barbital sodium (50 mg/kg i.p.) and positioned in a stereotaxic apparatus. In each rat, the skull was exposed, and a hole was drilled to barbital sodium (50 mg/kg i.p.) and positioned in a stereotaxic apparatus. In each rat, the skull was exposed, and a hole was drilled to barbital sodium (50 mg/kg i.p.) and positioned in a stereotaxic apparatus. In each rat, the skull was exposed, and a hole was drilled to barbital sodium (50 mg/kg i.p.) and positioned in a stereotaxic apparatus. In each rat, the skull was exposed, and a hole was drilled to barbital sodium (50 mg/kg i.p.) and positioned in a stereotaxic apparatus. In each rat, the skull was exposed, and a hole was drilled to barbital sodium (50 mg/kg i.p.) and positioned in a stereotaxic apparatus. In each rat, the skull was exposed, and a hole was drilled
decreased the output of ACh in the RVLM by 50.4 ± 3.0% at 15 min and by 61.4 ± 3.4% at 30 min [F(1,120) = 64.57, P < .001; n = 5]. ACh release returned to baseline levels 45 min after addition of calcium ions (Fig. 2B).

**Potassium Depolarization.** We next assessed the extent to which depolarizing concentrations of high potassium ion (30 mM for 30 min) affect the extracellular concentration of ACh in the RVLM. As shown in Fig. 2C, high potassium rapidly increased the release of ACh by 33.5 ± 1.4% at 15 min and by 37.2 ± 3.8% at 30 min [F(1,120) = 12.92, P < .001; n = 5]. ACh release returned to predrug levels within 30 min after termination of this treatment.

**Effects of Systemic-Administered Morphine on the Release of ACh in the RVLM.** Figure 3 shows the effects of morphine (5 and 10 mg/kg i.p.) on the release of ACh in the RVLM. At a dose of 5 mg/kg, morphine induced a significant increase in ACh release (42.4 ± 7.2% at 75 min) compared with the saline group [F(1,170) = 107.79, P < .001; n = 6] (Fig. 3). Recovery was observed 175 min later. At a dose of 10 mg/kg, morphine significantly increased the release of ACh by 39.1 ± 8.1% at 30 min, with a peak of 86.4 ± 4.9% at 75 min [F(1,170) = 314.75, P < .001; n = 6]. Naloxone (1.0 mg/kg i.p.; n = 5), administered i.p. 30 min before administration of morphine (5 mg/kg), attenuated the morphine-induced increase in ACh release in the RVLM [F(1,170) = 135.01, P < .001; n = 6] (Fig. 3).

**Effects of Locally Applied Morphine on the Release of ACh in the RVLM.** The effects of local application of morphine (10⁻⁵ and 10⁻⁴ M) on ACh release into the RVLM is shown in Fig. 4. Morphine (10⁻⁵ M) had no effect on the release of ACh in the RVLM [F(1,170) = 1.39, P = .241; n = 5]. Local application of morphine (10⁻⁴ M) significantly increased the release of ACh by 52.9 ± 8.2% at 30 min, with a peak of 85.8 ± 2.5% at 45 min [F(1,170) = 46.59, P < .001; n = 5]. A significant increase in ACh release was observed between 30 and 60 min during local application of morphine; this increase recovered after drug removal. Pretreatment

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**Fig. 1.** Locations of the microdialysis probe in the rostral ventrolateral medulla and mean quantity of ACh (in femtomoles) recovered in 20-μl samples from brain sites (1–5). The values are basal release of ACh in the rostral ventrolateral medulla and not following systemic administration of morphine. Sites are illustrated on representative coronal brainstem sections. The rectangles show the estimated positions of the tips of the dialysis membranes. NRM, nucleus raphe magnus, Pyr, pyramidal tract; sp5, spinal trigeminal tract (Paxinos and Watson, 1986).

**Fig. 2.** A, effects of TTX perfusion on ACh release in the RVLM. TTX (1 μM) significantly decreased the release of ACh (n = 5). The shaded area depicts the time of TTX perfusion through the microdialysis probe. Each point represents the mean ± S.E.M. B, effects of calcium-free perfusion solution on ACh release into the RVLM. Perfusion of calcium-free solution (shaded area) significantly decreased the release of ACh (n = 5). Each point represents the mean ± S.E.M. C, effects of potassium depolarization on the ACh release in the RVLM. A high concentration of potassium ions (30 mM) in the solution (shaded area) significantly increased ACh release (n = 5). Each point represents the mean ± S.E.M.
with systemic administration of naloxone (1.0 mg/kg i.p.) 30 min before local application of morphine (10^{-4} M) significantly attenuated the morphine-induced increase in ACh release in the RVLM \( [F_{(1,170)} = 61.67, P < .001; n = 5] \) (Fig. 4).

**Effects of Local Application of Morphine into the RVLM on the Hot-Plate Response.** Figure 5A shows the effects of morphine (10^{-5} and 10^{-4} M) on the hot-plate nociceptive response. The hot-plate control latency was 8.7 ± 0.3 s. At a concentration of 10^{-5} M, morphine did not affect the hot-plate response compared with the control group \( [F_{(1,54)} = 2.03, P = .160; n = 4] \) (Fig. 5A). At a concentration of 10^{-4} M, morphine significantly increased the hot-plate response by 50.6 ± 6.1% at 60 min \( [F_{(1,54)} = 83.71, P < .001; n = 4] \). A significant increase in the hot-plate response was observed between 30 and 90 min. Systemic administration of naloxone (1.0 mg/kg i.p.), 30 min before local application of morphine (10^{-4} M), completely blocked the morphine-induced antinociceptive response. Each point represents the mean ± S.E.M.

**Effects of Local Application of Morphine into the RVLM on the Tail Immersion Response.** Figure 5B shows the effects of morphine (10^{-5} and 10^{-4} M) on the tail immersion nociceptive response. The tail immersion withdrawal control latency was 1.9 ± 0.1 s. At a concentration of 10^{-5} M, morphine did not affect the tail immersion response, whereas morphine at 10^{-4} M significantly increased the tail immersion response. The shaded area depicts the time of morphine perfusion (60 min) via the microdialysis probe. Systemic administration of naloxone (1.0 mg/kg i.p.), 30 min before local application of morphine (10^{-4} M), completely blocked the morphine-induced antinociceptive response. Each point represents the mean ± S.E.M.
NRGC concentrated in the more ventral part of the NRGC and the RVLM, including the NRGC. In addition, in situ hybridization studies have found that in four medulla regions as assessed using in vivo microdialysis. Anatomical studies have shown that a dense plexus of retrogradely labeled cholinergic fibers and terminals exists in the gigantocellular reticular field following injection of Fluoro-gold into the pedunculopontine tegmental nucleus (Yasui et al., 1990). Large- and giant-sized cells in the NRGC/NRGCc are retrogradely labeled by wheat germ agglutinin-horseradish peroxidase from the spinal cord and stain positively for acetylcholinesterase (Bowker et al., 1983). Jones et al. (1986) found that most neurons labeled following injection of [3H]choline into the upper cervical spinal cord of rat were concentrated in the more ventral part of the NRGC and NRGCc. In addition, in situ hybridization studies have found the presence of choline acetyltransferase mRNA in small cells in the NRGC (Lauterborn et al., 1993). The present study’s mapping of the medulla showed that the cholinergic terminal lies in the RVLM. In combination, these results support the suggestion that ACh plays an important role in the RVLM, including the NRGC.

Discussion

The present findings demonstrate that the basal ACh concentration in the RVLM, including the NRGC, is higher than that in four medulla regions as assessed using in vivo microdialysis. Anatomical studies have shown that a dense plexus of retrogradely labeled cholinergic fibers and terminals exists in the gigantocellular reticular field following injection of Fluoro-gold into the pedunculopontine tegmental nucleus (Yasui et al., 1990). Large- and giant-sized cells in the NRGC/NRGCc are retrogradely labeled by wheat germ agglutinin-horseradish peroxidase from the spinal cord and stain positively for acetylcholinesterase (Bowker et al., 1983). Jones et al. (1986) found that most neurons labeled following injection of [3H]chocline into the upper cervical spinal cord of rat were concentrated in the more ventral part of the NRGC and NRGCc. In addition, in situ hybridization studies have found the presence of choline acetyltransferase mRNA in small cells in the NRGC (Lauterborn et al., 1993). The present study’s mapping of the medulla showed that the cholinergic terminal lies in the RVLM. In combination, these results support the suggestion that ACh plays an important role in the RVLM, including the NRGC.

We found that perfusion, via a dialysis probe, of solution containing TTX (a voltage-dependent Na+ channels blocker) decreased output of ACh within the RVLM, similar to our previous report in the striatum (Taguchi et al., 1993). Similarly, removal of calcium ions produced a decrease in ACh output in the RVLM. In addition, potassium depolarization produced an increase in ACh output. Thus, the dialysate ACh collected from the RVLM resulted from spontaneous, sodium channel- and calcium channel-dependent neuronal release.

In the present study, i.p. administration of morphine increased the release of ACh in the RVLM. In addition, local application of morphine was shown to enhance ACh release in the RVLM by in vivo microdialysis. Chiang and Zhuo (1989) have suggested that a descending cholinergic system is involved in antinociception produced following the systemic administration of morphine. In addition, microdialysis experiments suggest that increased ACh levels in cerebrospinal fluid result from systemic morphine-induced activation of bulbar/spinal pathways (Bouaziz et al., 1996). Recently, systemic morphine increased ACh and norepinephrine in dorsal horn microdialysates, and these increases were attenuated by naloxone or cervical spinal cord transection (Xu et al., 1997). These observations lead to the conclusion that morphine activates descending cholinergic neurons from the brainstem. In contrast, cat spinal cord transection does not reduce the amount of choline acetyltransferase in the spinal cord (Kanazawa et al., 1979). The antinociceptive effect of morphine microinjected into the ventrolateral periaqueductal gray is reduced by intrathecal administration of atropine (Fang and Proudfit, 1996). This antinociceptive effect of microinjected morphine into the ventrolateral periaqueductal gray is considered to be the result of activation of the spinal cholinergic muscarinic receptors. Thus, there is no direct evidence that activation of the pathway from the brainstem including the RVLM to the spinal cord may induce antinociception that is mediated by the descending cholinergic system. Consequently, morphine may act on both spinal cholinergic neurons and descending cholinergic neurons from the brainstem to cause ACh release. The present data demonstrate that systemic administration of morphine increases the concentration of ACh in the extracellular space in the RVLM. In addition, morphine was applied via a dialysis membrane directly into the RVLM with a resultant increase in ACh release. The release of ACh induced by systemic administration and local application of morphine was attenuated by naloxone, an antagonist for the opiate receptor. Our observations suggest that the increase in ACh release induced by morphine activated the cholinergic neurons via an opiate receptor located in the RVLM. However, an important question to address is whether the effect of morphine in increasing ACh release is mediated directly by cholinergic terminals or cholinergic cell bodies. In our experiment, the microdialysis probe measured the release of ACh from cholinergic terminals within the RVLM region of the brainstem. Therefore, the present results encourage further experiments designed to determine whether the actions of morphine are exerted on the cholinergic terminals or the cholinergic cell bodies within the RVLM. Furthermore, the terminals responsible for ACh release within the RVLM may arise from cholinergic interneurons intrinsic to the region or from other nuclei-containing cholinergic neurons such as pedunculopontine tegmental nucleus. The exact origin of the cholinergic innervation responsible for the morphine-induced ACh release in the RVLM remains to be determined.

With respect to the antinociceptive effect of morphine, microinjection of morphine or glutamate into, and electrical stimulation of, the RVLM including the NRGC/NRGCc has been shown to inhibit the spinal nociceptive reflex and spinal dorsal horn neuron response to peripheral stimulation (Satoh et al., 1979; Azami et al., 1982; Sandkuhler and Gebhart, 1984; Zhuo and Gebhart, 1990, 1997). Furthermore, central administration of morphine inhibits the tail-flick reflex and response to the hot-plate test, suggesting that morphine induces activation of the descending inhibitory system, primarily the serotonergic and noradrenergic pathways (Satoh et al., 1983; Taing and Tang, 1989). In the present experiments, local application of morphine into the RVLM via a microdialysis probe produced increases in the hot-plate response and tail immersion withdrawal response. These antinociceptive effects were reduced by naloxone. These findings support the notion that the antinociceptive effects of morphine are mediated by release of ACh in the RVLM. Cholinergic activation in the RVLM is important in the relay of the descending inhibitory system from midbrain stem to spinal cord. Therefore, the morphine-induced release of ACh in the RVLM most likely results in activation of supraspinal antinociceptive mechanisms as well as the descending inhibitory system. Recently, systemic or intrathecal administration of cholinergic drugs, particularly anticholinesterase, has been shown to enhance antinociception of opioid administration in animals including humans (Beilin et al., 1997; Hood et al., 1997). In addition, electrical stimulation or glutamate microinjec-
tion in the NRGC and NRGCs produce antinociception that is attenuated by intrathecal administration of atropine (Zhuo and Gebhart, 1990). Thus, the activation of the cholinergic system in the RVL is, in part, involved in antinociception. However, the possibility that descending monoaminergic neurons mediate the antinociceptive response to noxious stimulation cannot be excluded, as intrathecal injection of either serotonergic or noradrenergic agonists can reduce the nociceptive responses of the tail-flick test and hot-plate test (Jensen and Yakah, 1984; Fang and Proudftit, 1996). These observations lead to the suggestion that local application of morphine via a microdialysis probe into the RVLML causes the activation of a descending serotonergic system and a descending noradrenergic system.

In conclusion, our results show that antinociceptive morphine treatments increase the release of acetylcholine in the RVL. Because other studies have indirectly suggested a role for cholinergic systems in morphine analgesia, these studies support the hypothesis that activation of brain stem cholinergic mechanisms plays an important role in morphine antinociception in the rat.

References


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