Alpha 2-Adrenoceptor Modulates the Release of Acetylcholine from the Rostral Ventrolateral Medulla in Response to Morphine

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The present study examined the role of the noradrenergic system in the modulation of acetylcholine (ACh) release in the rostral ventrolateral medulla (RVLM) using in vivo microdialysis of morphine. The basal level of ACh was 325.0 ± 21.1 fmol/20 μl/15 min in the presence of neostigmine (10 μM). Intraperitoneal (i.p.) administration of 5 and 10 mg/kg morphine significantly increased ACh release by the RVLM. This enhancement was reversed by naloxone (1 mg/kg, i.p.). In addition, pretreatment with yohimbine (0.5 mg/kg, i.p.) or prazosin (0.2 mg/kg, i.p.) attenuated the systemic morphine-induced release of ACh in the RVLM. However, propranolol (0.2 mg/kg, i.p.) did not affect the morphine-induced ACh release. The addition of morphine (10⁻⁴ M) to the perfusion medium increased the ACh release by 72.4% of the predrug values. The increased ACh release induced by local application of morphine was attenuated by pretreatment with yohimbine, but not prazosin. These findings suggest that morphine exerts an indirect stimulatory effect on the release of ACh by the RVLM and that the morphine-induced increase in ACh release is modulated by α₂-adrenoceptors in freely moving rats.

Key words acetylcholine (ACh); morphine; α₂-adrenoceptor; rostral ventrolateral medulla (RVLM); in vivo microdialysis

Electrophysiological and behavioral studies have identified the rostral ventrolateral medulla (RVLM), including the nucleus reticularis gigantocellularis (NRGC)/nucleus reticularis gigantocellularis alpha (NRGCA), as part of a descending system involved in the modulation of nociception at the spinal cord dorsal horn. For example, microinjection of morphine or glutamate into the RVLM and its electrical stimulation have been shown to inhibit the spinal nociceptive reflex and spinal dorsal horn neuron response to peripheral stimulation.¹—⁵

We recently demonstrated that microinjecting carbachol into the NRGC/NRGCA induced antinociceptive effects via cholinergic muscarinic receptors.⁶ Moreover, systemic administration and microinjection of morphine increased the release of acetylcholine (ACh) into the extracellular space of the RVLM including the NRGC/NRGCA.⁷ Anatomically, the RVLM receives cholinergic projections primarily from the pedunculopontine tegmental nucleus.⁸,⁹ In addition, other studies have shown the existence of a descending cholinergic system from the RVLM to the spinal cord, and demonstrated the presence of small to medium-sized cholinergic neurons and choline acetyltransferase mRNA in small cells of the NRGC/NRGCA.¹⁰,¹¹ A descending cholinergic system is involved in antinociception produced following the systemic administration of morphine.¹² These observations suggest that the cholinergic system plays an important role in antinociception in the RVLM, including the NRGC/NRGCA.

Catecholaminergic fibers are found throughout the reticular formation including the NRGC/NRGCA.¹³ Nerve terminals that show positive immunoreactivity for dopamine-β-hydroxylase¹³ and binding sites for the α₂-adrenoceptor agonist, clonidine, are present in the NRGC.¹⁴,¹⁵ Arterial pressure-related neurons in the NRGC respond to clonidine.¹⁶ Furthermore, using in vivo microdialysis, Tsou et al.¹⁷ demonstrated that the extracellular concentration of norepinephrine (NE) in the NRGC was approximately 10 nm. These findings led to the suggestion that interactions between cholinergic and noradrenergic neurons may participate in morphine-induced ACh release in the RVLM. In the present study, we investigated whether the adrenergic receptors alter the effects of systemically or locally applied morphine on the release of ACh in the RVLM, including the NRGC/NRGCA, in freely moving rats.

MATERIALS AND METHODS

Animals Male Wistar rats, weighing 270 to 340 g at the time of the experiments, were purchased from Charles River Japan, Inc. All animals were housed individually under automatically controlled environmental conditions and 12 h light–dark cycles, and were handled in accordance with the guidelines for animal care and use, published by the US National Institute of Health. The rats had free access to food and water. All animals were quarantined in centralized animal facilities for at least seven days after arrival from the supplier.

Surgical Procedures After an acclimation period, animals were anesthetized with pentobarbital-Na (50 mg/kg, i.p.) and positioned in a stereotaxic apparatus. The skull was exposed and drilled in order to implant a guide cannula into the upper part (3.0 mm) of the rostral ventrolateral medulla: bregma: –11.5 mm, lateral: 0.9 mm, depth: –10.5 mm.¹⁸ The guide cannula was held firmly in place by dental acrylic cement and anchored to the skull using stainless steel screws. All experiments were performed 3 d after surgery.

In Vivo Microdialysis Microdialysis probes (dialysis membrane: length 2.0 mm, diameter 0.5 mm, AF-02, Eicom) were inserted into the RVLM using a stereotaxic apparatus. The skull was exposed and drilled in order to implant a guide cannula into the upper part (3.0 mm) of the rostral ventrolateral medulla: bregma: –11.5 mm, lateral: 0.9 mm, depth: –10.5 mm. The guide cannula was held firmly in place by dental acrylic cement and anchored to the skull using stainless steel screws. All experiments were performed 3 d after surgery.
morphine induced a significant increase in ACh release on the release of ACh in the RVLM. At a dose of 5 mg/kg, Figure 1A shows the effects of morphine (5, 10 mg/kg, i.p.) perfusion solution containing 10 mM neostigmine (125 mM NaCl, 3 mM KCl, 1.3 mM CaCl₂, 1 mM MgCl₂, 23 mM NaHCO₃) in aqueous potassium phosphate buffer (1 mM, pH 7.4) containing neostigmine (10 mM) was perfused into the dialysis probe at a rate of 2 μl/min. The perfusate was collected at 15 min intervals. ACh levels in each perfusate were quantitatively measured using high-performance liquid chromatography with electrochemical detection (HPLC-ECD), as described previously. The system consisted of a pump (EP-300, Eicom), separating column (Eicompak AC-GEL; 2.0×150 mm), enzymatic reactor (AC-Enzypak, Eicom), and an electrochemical detector (ECD-300, Eicom). Acetylcholine was converted into choline and then hydrogen peroxide by an enzyme reactor containing acetylcholine esterase and choline oxidase, which was detected electrochemically. The mobile phase consisted of 0.05 M phosphate buffer (pH 8.2) containing 300 mg/l sodium 1-decanesulfonate and 5 mg/l EDTA-2Na delivered at a constant flow rate of 0.15 ml/min using an HPLC pump. The column temperature was maintained at 33 °C.

Histological Procedures At the end of each experiment the animals were sacrificed by a pentobarbital-Na overdose. The brain was fixed in 10% formalin, frozen, and then 60 μm thick sections were cut using a freezing microtome. The tracks of the dialysis cannula were verified microscopically in the histological sections.

Data Analysis Dialysis data are shown as the mean±S.E.M. of the percentage of baseline obtained from each rat before drug treatment. Data were analyzed by repeated measurement two-way analysis of variance, followed by the Tukey–Kramer HSD test. Differences were considered significant when p values were less than 0.05.

Drugs The drugs used were morphine hydrochloride (Sankyo), naloxone hydrochloride (Endo Laboratories), prazosin hydrochloride (Sigma), yohimbine hydrochloride (Sigma), and propranolol hydrochloride (Sigma). All drugs were dissolved in sterile saline. All solutions were sterilized by filtering through a Millipore filter (0.2 μm). The control group received physiological saline only.

RESULTS

Effects of Systemically Administered Morphine on the Release of ACh in the RVLM The amount of extracellular ACh recovered from the RVLM by chronically implanted microdialysis probes was 325.0±21.1 fmol/20 μl sample. Basal release of ACh was stable over 3 h after administration of perfusion solution containing 10 μM neostigmine (Fig. 1A). Figure 1A shows the effects of morphine (5, 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 (36.3±4.3% at 75 min) compared with the saline group (F(1170)=3.90, p<0.001; n=6) (Fig. 1A). Recovery was observed 165 min later. At a dose of 10 mg/kg, morphine significantly increased the release of ACh by 50.0% at 30 min, with a peak of 86.8±20.8% at 75 min (F(1170)=172.4, p<0.001; n=6) (Fig. 1A). Naloxone (1 mg/kg), administered intraperitoneally 15 min before administration of morphine (5 mg/kg), attenuated the morphine-induced increase in ACh release in the RVLM (F(1170)=3.90, p<0.001; n=4) (Fig. 1B).

Effects of α- and β-Adrenoceptor Antagonists on the

ACh Release Induced by Systemic Morphine Administration in the RVLM Prazosin (0.2 mg/kg), administered intraperitoneally 15 min before administration of morphine (5 mg/kg), completely attenuated the morphine-induced increase in ACh release in the RVLM (F(1170)=48.39, p<0.001; n=5) (Fig. 2A). Similarly, yohimbine (0.5 mg/kg, i.p.) attenuated morphine-induced release of ACh in the RVLM (F(1170)=32.42, p<0.001; n=6) (Fig. 2B). In contrast, pretreatment with propranolol (0.2 mg/kg, i.p.) did not affect morphine-induced release of ACh (F(1170)=1.64, p=0.203; n=6) (Fig. 2C).

Effects of Locally Applied Morphine on the Release of ACh in the RVLM The effects of local application of morphine (10⁻³, 10⁻⁴ M) on ACh release in the RVLM are shown in Fig. 3A. Morphine (10⁻³ M) had no effect on the release of ACh in the RVLM (F(1136)=0.90, p=0.345; n=5). Local application of morphine (10⁻⁴ M) significantly increased the release of ACh by 48.6±5.7% at 15 min with a peak of 72.4±15.0% at 45 min (F(1136)=40.54, p<0.001; n=5). An increase in ACh release was observed between 30 and 60 min during local application of morphine, although the levels recovered after drug removal. Systemic administration of naloxone (1 mg/kg, i.p.) 15 min before local application of morphine (10⁻⁴ M) significantly attenuated the morphine-induced increase in ACh release in the RVLM (F(1136)=

![Figure 1](image-url)
45.85, \( p<0.001; n=5 \) (Fig. 3B).

**Effects of Systemic Administration of \( \alpha_1 \)- and \( \alpha_2 \)-Adrenoceptor Antagonists on the Release of ACh in the RVLM in Response to Locally Applied Morphine**

Prazosin (0.2 mg/kg), administered intraperitoneally 15 min before local administration of morphine (10^{-4} M), did not affect the increase in ACh release in the RVLM (Fig. 3C). However, pretreatment with systemically administered yohimbine (0.5 mg/kg, i.p.) 15 min before local application of morphine (10^{-4} M) significantly attenuated the morphine-induced increase in ACh release in the RVLM (\( F(1136)=22.35, p<0.001; n=5 \) (Fig. 3C).

**DISCUSSION**

Our in vivo microdialysis findings indicate that the morphine-induced increase in ACh release is regulated by \( \alpha_2 \)-adrenoceptors in the RVLM including NRGC/NRGC\( \alpha \). Our
previous study using in vivo microdialysis, which has indicated that either systemically administered or locally infused morphine enhanced the release of ACh from the RVLM and that morphine locally injected into the RVLM induced an antinociceptive effect, supports a potential relationship between cholinergic neurons and morphine antinociception. Likewise, other microdialysis studies suggest that increased ACh levels in cerebrospinal fluid result from systemic morphine-induced activation of bulbospinal pathways. Local application of morphine into the RVLM via a microdialysis probe increases the hot-plate and tail immersion withdrawal responses. In addition, morphine or carbachol produces antinociception when microinjected into the NRGC, and the increases in ACh were attenuated by prazocin and yohimbine, which blocks the increase in microdialysate concentrations of NE and ACh in response to systemic administration and local infusion of morphine into the RVLM. These results confirmed that activation of cholinergic neurons in the RVLM is involved in antinociception and analgesia.

Anatomical evidence has demonstrated that dopamine-β-hydroxylase-containing fibers and nerve terminals are present in the reticular formation including the NRGC. Pharmacologically, selective destruction of giant multipolar neurons in the NRGC by kainic acid significantly attenuates the analgesic efficacy of clonidine and morphine on the hot-plate test. These findings suggest possible interactions between ACh release and noradrenergic neurotransmission in nociception of the RVLM.

In the present study, we used α- or β-adrenoceptor antagonists to determine whether or not morphine-induced ACh release was mediated by noradrenergic neurotransmission. Although the effects of systemic morphine on ACh release were not mitigated by propranolol, α-adrenoceptor antagonists, prazocin, and yohimbine, attenuated this response. Similarly, systemic administration of morphine was previously shown to increase microdialysate concentrations of NE and ACh in lumbar cerebrospinal fluid, and the increases in ACh were blocked by idazoxan, an α2-adrenoceptor antagonist. Furthermore, activation of opioid receptors increases extracellular levels of NE in the stria terminals of the rat red nucleus. Thus, these data suggest that systemic morphine-induced increases in ACh release are mediated by α2-adrenoceptors in the RVLM.

We next investigated whether the increase in ACh in response to locally applied morphine was dependent on the α1- or α2-adrenoceptors in the RVLM. In fact, local application of morphine into the RVLM via a microdialysis probe increased the release of ACh. The increase induced by local application of morphine was attenuated by yohimbine, an α2-adrenoceptor antagonist. In contrast, prazocin, an α1-adrenoceptor antagonist, had no effect on ACh release. The failure of prazocin to attenuate the morphine-induced ACh release leads us to conclude such morphine-induced ACh release is not mediated by the α1-adrenoceptor in the RVLM. In the present study, systemic administration of prazocin did not block the increase in ACh release induced by local application of morphine, but did block the increase produced by systemic morphine administration. Thus, a comparison blockade of prazocin, in response to systemic administration and local application of morphine, suggested that the action of the α2-adrenoceptor antagonist was not mediated at the level of the RVLM, but through another system at a site outside the RVLM. Our results suggest that the increase in ACh release induced by local application of morphine is mediated by α2-adrenoceptors in the RVLM. Although α2-adrenoceptors regulate the interaction of noradrenergic neurotransmission and ACh release in the RVLM, the inhibitory mechanisms affecting ACh release in response to yohimbine are unclear. Previously, presynaptic α2-adrenoceptors were shown to inhibit the release of NE, and NE spillover was observed to increase in response to α2-adrenoceptor antagonists and diminish in response to agonists. In addition, yohimbine blocks the increase in spinal NE release after systemic administration of morphine. Based on these results, we hypothesized that NE released in the RVLM may act on presynaptic α2-adrenoceptors of cholinergic neurons. Further investigation is required to clarify these issues.

In summary, morphine stimulated the release of ACh in the RVLM, and the morphine-induced increase in ACh release was regulated by α2-adrenoceptors.

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