The Dorsal Raphe Nucleus as a Site of Action of the Antinociceptive and Behavioral Effects of the \( \alpha4 \) Nicotinic Receptor Agonist Epibatidine

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Received October 15, 2004; accepted December 13, 2004

ABSTRACT

The mechanisms and sites of action of epibatidine-induced antinociception and side effects are poorly understood. The present study tested the hypothesis that the serotoninergic dorsal raphe nucleus is a site of action of epibatidine. Behavioral responses of rats to hindpaw formalin injection were compared after direct administration of epibatidine into the dorsal raphe and after subcutaneous administration. Different groups of rats were injected with formalin into the rear paw after administration of either epibatidine (0.01, 0.015, 0.03, and 0.06 \( \mu \)g) in the dorsal raphe or epibatidine (2.5–5 \( \mu \)g/kg) subcutaneously. Assessment of pain related behavior was done evaluating the incidence of favoring, lifting, and licking of the injected paw in the different groups. Abnormal behavior (freezing) was also recorded. Epibatidine was at least 100 times more potent when administered into the dorsal raphe nucleus versus systemically, implicating this nucleus as a site of action of the analgesic effects of epibatidine. Thus, epibatidine (0.015, 0.03, and 0.06 \( \mu \)g) in the dorsal raphe resulted in a significant lower pain score in the second phase of the formalin test compared with control rats and was as effective as subcutaneous epibatidine. The analgesic effects of epibatidine were regionally selective in that administration of epibatidine within the periaqueductal gray area but outside the dorsal raphe area was not analgesic. The highest doses of intraraphe epibatidine (i.e., 0.03–0.06 \( \mu \)g) also produced “freezing” behavior immediately after injection, which was relatively short-lived compared with the analgesic effect. Together, the results implicate the dorsal raphe nucleus as a target for the analgesic and perhaps anxiogenic effects of epibatidine.

Nicotine and nicotinic agonists have been known for many years to have analgesic properties. However, the high incidence and severity of side effects associated with these drugs has limited their clinical use. Recent studies on the analgesic effects of epibatidine, a nicotinic acetylcholine receptor (nAChR) ligand (Qian et al., 1993; Sullivan et al., 1994; Bannon et al., 1998), and other epibatidine derivatives such as ABT-594 (Bannon et al., 1998) have triggered a new interest on the mechanism of antinociception produced by nicotinic agonists. It has been postulated that nicotinic acetylcholine receptor agonists produce their antinoceptive effects predominantly via activation of descending inhibitory pain pathways originating in the brainstem regions, including the nucleus raphe magnus (Bitner et al., 1998).

Central modulation of pain involves both the nucleus raphe magnus (NRM) and dorsal raphe (DR) nucleus. The NRM can directly control pain transmission in the dorsal horn of the spinal cord via descending projections. The effects of the DR on the spinal cord are most likely mediated by its interconnection with the NRM (Wang and Nakai, 1994). Although there is clear experimental evidence that the NRM mediates the antinociception produced by epibatidine and the nicotinic agonist ABT-594 (Bitner et al., 1998, Curzon et al., 1998), the participation of other brain areas has never been investigated. The DR is another potential site of action, because it is an area where both electric stimulation and morphine application have antinociceptive effects. The DR contains the largest pool of serotoninergic neurons in the brain (Dahlstrom and Fuxe, 1964). These neurons express nAChr containing the \( \alpha4 \) subunit (Cucchiaro and Commons, 2003), which is thought to be a primary receptor site for epibatidine. There are multiple data showing that the systemic administration of nicotine modifies neural activity in the dorsal ra-
phe, with different subgroups of neurons exhibiting different responses. Thus, systemic nicotine inhibits approximately 60 to 70% of cells recorded in the DR, while increasing the firing of the remaining one-third of DR neurons (Engberg et al., 2000; Mihaiescu et al., 2002). Nicotine can also induce a concentration-dependent increase in serotonin release from rat midbrain slices (Mihaiescu et al., 1998).

Together, these findings suggest that the DR could be an important contributor to the positive effects of nicotinic ligands on antinociception. A few studies have suggested that serotonergic neurons localized in the DR mediate the anxiolytic effects of low doses of nicotine (File, 2000; Cheeta et al., 2001), and this is mediated by increased 5-HT release in the DR (Seth et al., 2002). However, there are no data on the interplay between serotonergic neurons localized in the dorsal raphe, nicotine agonists, and antinociception. The purpose of the present study was to test whether the DR is a target for epibatidine-induced antinociception or side effects. The effect of local administration of epibatidine in the DR on nociceptive response and motor behavior was measured. These data have then been compared with those observed in rats that received systemic epibatidine.

Materials and Methods

Male Sprague-Dawley rats (250–300 g) were housed in pairs under a 12:12-h light/dark cycle with water and food available ad libitum. For all experiments that used implanted cannulas, rats were singly housed. The protocols were in accordance with the animal care guidelines at the University of Pennsylvania and The Children’s Hospital of Philadelphia and followed the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health.

Surgical Procedure. Rats were anesthetized with halothane and placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA) with the skull on a horizontal plane. A hole was drilled to accept a skull screw. Coordinates for the placement of the intracranial cannula guides were from intra-aural, 0; antero-posterior, –0.5 mm; mediolateral, +0.27 mm; dorsoventral, –0.49 mm. The entry angle was –25° from the vertical. Coordinates were chosen according to the atlas of Paxinos and Watson (1998). Cannula guides (26-gauge, Plastics One Inc., Wallingford, CT) were positioned, and cranioplastic cement was used to affix the cannula guide to the skull and skull screw. A dummy cannula was inserted into the guide to keep it clear. Rats were allowed to recover for 3 days before the behavioral studies.

Peripheral Epibatidine Injection. Three groups of rats received subcutaneous saline (control group, n = 9), 2.5 μg/kg epibatidine (n = 9), or 5 μg/kg epibatidine (n = 9). The study drugs were injected in the back of the rats, in the lumbar area. Then 5% formalin (50 μl) was injected subcutaneously into the plantar surface of one rear paw, using a 27-gauge needle and an insulin syringe.
Results

Intracerebral cannulas were placed in 46 rats. We could histologically confirm the correct placement of the cannulas within the DR in 31 rats, and we considered the other 15 rats with the cannula outside the DR as control group (OOA group). The placement of the cannulas is shown in Fig. 1.

Pain Behavior: Peripheral Epibatidine. Intraplantar administration of formalin induces a biphasic pattern of pain-related behavior, with an early acute period (phase 1; 0–9 min), which corresponds to an acute pain response, a brief quiescent period, and a second phase of sustained “tonic” pain behavior (phase 2; 10–60 min), which represents a chronic inflammatory (Abbott et al., 1995).

Rats that received subcutaneous saline (control group) exhibited this typical biphasic time course (Fig. 2). Pain behavior decreased after the initial 5-min peak (phase 1), to rise again after about 10 min and peak at 25 to 30 min (phase 2). The pain score of rats that received subcutaneous epibatidine (2.5 and 5 μg/kg) showed an initial peak, which was significantly lower than that observed in the control group ($p < 0.001$) (Fig. 2). During phase 2, the pain score after the formalin injection was also attenuated from minute 15 through 40 compared with control rats ($p < 0.001$) (Fig. 2). The pain behavior equalized to that of control rats after 40 min. The global pain score during phase 2 (AUC) was significantly lower in rats that received 2.5 and 5 μg/kg epibatidine compared with the AUC observed in control rats ($p < 0.02$) (Fig. 3).

Pain Behavior: Intra-DR Epibatidine. Differences in pain scores in animals that received intra-DR epibatidine were analyzed at every time point after the formalin injection. However, in all time points before 30 min, there is a...
possible interference of the “freezing” behavior, which was seen after higher doses of epibatidine (see below), on the pain response. Consistent with the appearance of freezing, the initial response to the formalin administration (phase 1) was significantly lower after intracranial administration of 0.01, 0.015, 0.03, or 0.06 μg of epibatidine (Fig. 4) compared with that observed after intracranial ACSF \((p = 0.0002)\). When analyzing phase 2, we observed that the intra-DR administration of 0.01 μg of epibatidine \((n = 5)\) resulted in similar pain behaviors compared with those observed after ACSF \((n = 6)\) (Fig. 3), with similar AUC (Fig. 3). However, the AUC after intra-DR administration of higher doses epibatidine, 0.015 μg \((n = 6)\), 0.03 μg \((n = 7)\), and 0.06 μg \((n = 7)\), was significantly lower compared with that seen after the intra-DR administration of ACSF or 0.01 μg of epibatidine \((p < 0.02)\) (Fig. 3). The analysis of pain scores at individual time points after 30 min, when freezing scores are at baseline values, we found significant differences in pain scores between rats injected with ACSF and 0.01 μg of epibatidine versus rats injected with the higher doses of epibatidine \((0.015, 0.03, \text{and } 0.06 \text{ μg})\). At this time point, the curves of rats treated with higher doses of epibatidine plateau (Fig. 4) whereas those of rats injected with ACSF and 0.01 μg of epibatidine continued to rise, showing a typical second phase of sustained ‘tonic’ pain behavior. Pain scores were still significantly different at 35 and 40 min after administration of 0.015, 0.03, or 0.06 μg of epibatidine and became similar to those of rats injected with ACSF and 0.01 μg of epibatidine 45 min after the drug administration.

Pain behavior score of rats in which epibatidine was injected in areas outside the DR was analyzed as a placement control. Two of these rats received 0.01 μg of epibatidine, three rats received 0.015 μg of epibatidine, seven rats received 0.03 μg of epibatidine, and three rats received 0.06 μg of epibatidine. The pain scores in this group of rats were similar to those observed in rats injected with intra-DR ACSF or 0.01 μg of epibatidine (Fig. 5) and significantly higher than those observed in rats in which the cannulas were correctly placed and that received higher doses of epibatidine \((p < 0.01)\). The difference was significant at time 30, 35, and 40 min after administration of epibatidine (Fig. 5).

When analyzing the pain behavior in rats that received mepamylamine before the infusion of the study drug \((n = 7)\), we did not observed any analgesic effect of epibatidine, and the pain score in this group of rats was similar to that of rats infused with ACSF (Fig. 6). In addition, freezing behavior was not observed (data not shown).
The administration of subcutaneous epibatidine (2.5 and 5 μg/kg) did not affect motor behavior. However, injection of epibatidine directly into the DR led to substantial freezing. The duration and intensity of freezing was dose-dependent. Postinjection freezing was greater in rats that received 0.03 and 0.06 μg of epibatidine, compared with rats injected with the lower doses (0.015 and 0.01 μg) as well as rats injected with subcutaneous epibatidine or saline (p < 0.001) (Fig. 7). Freezing extinguished within 15 min in the lower dose experiments and within 25 to 30 min after 0.03 and 0.06 μg of epibatidine, respectively (Fig. 7), when there was a rapid transition to a normal motor behavior. During this period of time, rats were immobile, with an increased muscular tone and fixed gaze. No evident signs of tonic and/or clonic convulsion were noticed.

**Discussion**

The original studies on the antinociceptive properties of nicotine implicated the midbrain and descending antinociceptive pathways in mediating nicotine induced antinociception (Iwamoto, 1991). More recent data showed that the direct administration into the nucleus raphe magnus of epibatidine and ABT-594, a novel nAChR agonist, produces antinociception in acute pain models (Curzon et al., 1998). In the present study, the administration of epibatidine directly into the DR produced antinociception in a dose-dependent manner. Moreover, epibatidine was at least 100 times more potent when administered into the DR compared with systemically. The minimum effective dose was 0.015 μg/kg. The administration of higher doses (0.03 and 0.06 μg/kg) did not increase the intensity of antinociception. To confirm the regional specificity of epibatidine, we quantified the pain score of rats in which the intracranial cannula was located outside of the DR. In contrast to intra-DR infusions, epibatidine administration in sites located outside the DR, in the PAG, or in areas adjacent to the DR had no antinociceptive effects even at the highest doses studied. Together, these findings support the conclusion that epibatidine acts specifically in the DR to produce the antinociceptive effects, at least in a chronic pain model.

Our data cannot be compared with those of previous studies where epibatidine was infused into the nucleus raphe magnus (Curzon et al., 1998). These studies were done using acute pain models (i.e., hot box test), and even though the formalin test consists of two different phases (initial acute phase followed by a tonic response), we could not properly analyze rats’ behavior in the initial phase because of freezing. Freezing probably interfered with the rats’ ability to respond to the acute nociceptive stimulus. However, we could determine that antinociception was still present at 30 min, when the freezing behavior was no longer evident.

Motor inhibition (freezing) was observed after infusion of epibatidine in the DR. The duration of the freezing behavior was dependent on the dose administered and was significantly prolonged in rats injected with the highest dose. Freezing behavior could reflect anxiogenic activity. Consistent with this possibility, other studies have shown that the electrical stimulation of the DR induces a transient inhibitory reaction similar to that observed in our study (Graeff and Silveira Filho, 1978) and that the administration of high-dose nicotine in the DR has anxiogenic effects (Cheeta et al., 2001). These effects seem to be mediated by serotonin because they can be blocked by the selective 5-HT₁A receptor antagonist WAY 100635 (Cheeta et al., 2000; Kenny et al., 2000). Previously, we have shown that serotonergic cells in the DR express α4 nicotinic receptors (Cucchiaro and Commons, 2003). Therefore, it is conceivable that the activation of DR serotonergic cells by epibatidine induces this specific behavior.

A reduced locomotor activity has been reported after the systemic administration and direct infusion into the nucleus raphe magnus of both epibatidine and ABT-594. The motor effects of systemic epibatidine have been attributed in the past to the affinity of epibatidine for neuromuscular nicotinic receptors. ABT-594 has the theoretical advantage of inducing fewer side effects relative to epibatidine because of a preferential selectivity for neuronal α4β2 nAChR. This explanation seems to be an oversimplification since a reduced locomotor activity has also been observed after infusion of ABT-594 directly into the nucleus raphe magnus and in our studies where epibatidine was infused into the DR and not systemically.

It should be noted that we observed freezing only in rats in...
which epibatidine was infused in the DR and not in rats where the cannula was located in the PAG, even at the highest doses. Freezing is a behavior commonly found also after electrical manipulations of the dorsal PAG area of the midbrain (Borelli et al., 2004). This difference can be explained by the fact that none of the injections made in the PAG area in our study were localized in the dorsal PAG (Fig. 1), which is the area leading to defensive reactions and freezing (Brandao et al., 1999; Vianna et al., 2003).

The results of this study suggest, as it has been previously shown for the PAG (Helmstetter and Landeira-Fernandez, 1990), that the DR could integrate the mechanisms of fear-anxiety and analgesia. However, the sensitivity to epibatidine of the two neuronal processes is different because the antinociceptive effect could be dissociated from freezing behavior at a low dose (0.015 μg) of epibatidine.

The analgesic and motor effects of epibatidine were blocked by the prior administration of mecamylamine into the DR, suggesting that both effects were secondary to stimulation of nAChR and specific for activation of DR neurons.

In conclusion, data from the present study provide evidence consistent with the possibility that the antinociceptive action of epibatidine and henceforth other nicotinic agonists may in part be mediated by activation of the dorsal raphe. The major limitation to the clinical use of nicotinic agonists is their toxicity, convulsions being one of the most severe. We have shown that the intra-DR administration of antinociceptive doses of epibatidine does not cause tonic-clonic convulsions. However, it does result in behavior manifestations that resemble a fear or anxiogenic response.

References


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