0022-3565/94/2702-0702$03.00/0
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Vol. 270, No. 2
Printed in U.S.A.

Epibatidine, An Alkaloid From the Poison Frog *Epipedobates tricolor*, Is a Powerful Ganglionic Depolarizing Agent[1]

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Accepted for publication April 18, 1994

ABSTRACT

Epibatidine, a newly discovered alkaloid from the skin of *Dendrobatidae* frogs, has structural similarities to nicotine. We examined the effects of epibatidine on cardiorespiratory function and ganglionic synaptic transmission. Superior cervical or splanchnic sympathetic nerve discharge (sSND) and phrenic nerve discharge (PND) were recorded along with arterial pressure (AP) in urethane-anesthetized, paralyzed and artificially ventilated rats. Epibatidine administered i.v. at low doses (0.5–2 μg/kg) produced a transient increase in AP and sSND, followed by a decrease and return to baseline; this low dose of epibatidine also produced a dose-dependent increase in PND. At high doses (cumulative dose of 8–16 μg/kg), epibatidine produced bradycardia, a profound depression in sSND and a transient elimination of PND. After i.v. administration of the ganglionic blocker chlorisondamine (5 mg/kg), AP was still increased by 1 μg/kg epibatidine (+39 ± 11 mm Hg). This pressor effect was not altered by pretreatment with the *alpha*-1 adrenergic antagonist phentolamine (+40 ± 10 mm Hg); however, it was blocked by additional pretreatment with the vasopressin antagonist [β-mercapto-β,β-cyclopentamethylenepropiony[1],O-ET-Tyr[2],Val[4],Arg[8]]vasopressin (50 μg/kg i.v.; +2 ± 0.4 mm Hg). Low doses of epibatidine (0.5–2 μg/kg) produced firing of postganglionic neurons in a decentralized ganglion preparation and potentiated synaptic transmission; at high doses (cumulative dose of 8–16 μg/kg), the alkaloid blocked ganglionic synaptic transmission. These results suggest that epibatidine is a potent agonist of ganglionic nicotinic receptors and that the alkaloid elicits cardiorespiratory effects similar to those of nicotine.

Epibatidine [exo-2-(6-chloro-3-pyridyl)-7-azabicyclo[2.2.1]heptane] is a minor alkaloid present in the skin of the South American poison frog *Epipedobates tricolor* (genus *Dendrobatidae*; Daly *et al.*, 1992). This compound, whose structure was determined only recently (Spande *et al.*, 1992), produces a Straub tail response in mice and a positive response to the hotplate analgesia test but has no affinity for opiate receptors (Spande *et al.*, 1992). Based on the outcome of these behavioral tests, it has been proposed that epibatidine might be an analgesic agent of a heretofore unknown class with a potency close to three orders of magnitude higher than that of morphine (Bradley, 1993; Spande *et al.*, 1992).

The total chemical synthesis of epibatidine has been performed recently by three different groups (Broka, 1993; Fletcher *et al.*, 1993; Huang and Shen, 1993). The structural similarity between epibatidine and the *Solanaceae* alkaloid anabasine [neonicotine; 2-(3-pyridyl)piperidine] was noted by the authors who originally determined the structure of the frog alkaloid (Spande *et al.*, 1992). A side-by-side representation of the structures of epibatidine and nicotine [1-methyl-2-(3-pyridyl)pyrrolidine] reveals striking similarities (fig. 1), which suggested to us that these drugs may have closely related pharmacological properties. The purpose of the present study was to test the possibility that epibatidine has depolarizing ganglionic stimulating properties analogous to those of nicotine.

Received for publication December 13, 1993.
[1] This work was supported by National Institutes of Health grants HL-28752 and HL-39841 (P.G.G.).

Methods

Physiological experiments *in vivo*. Twenty-nine Sprague-Dawley male rats (330–375 g) were anesthetized with halothane during surgery; then, this anesthetic was withdrawn and replaced by urethane administered i.v. in a dose of 1.2 g/kg (Huangfu *et al.*, 1992; Koshiya *et al.*, 1993). Femoral AP was measured, and MAP was computer averaged (Koshiya *et al.*, 1993). The rats were artificially ventilated with 100% O_2 and, except when specified in "Results," were paralyzed with pancuronium bromide (1 mg/kg) after a stable level of anesthesia with urethane had been achieved. End-tidal CO_2 was maintained around 4.5%, and body temperature was maintained at 37°C. Drugs were injected i.v. *via* a femoral vein.

PND was recorded with bipolar electrodes (bandpass 50–3000 Hz), rectified, integrated and averaged over 2-sec periods as previously described (Koshiya *et al.*, 1993). This integrated activity is a function of both PND amplitude and rate and thus measures the overall strength of the central respiratory drive. The mass sSND was measured distal

ABBREVIATIONS: sSND, splanchnic sympathetic nerve discharge; PND, phrenic nerve discharge; AP, arterial pressure; MAP, mean arterial pressure; AVP, vasopressin; O-E-AVP, [β-mercapto-β,β-cyclopentamethylenepropionyl[1], O-ET-Tyr[2], Val[4], Arg[8]]vasopressin; HR, heart rate.

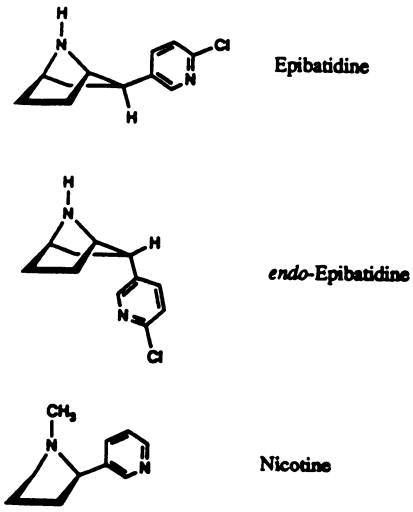

Fig. 1. Structure of epibatidine, its inactive diastereoisomer endo-epibatidine and nicotine.

to the suprarenal ganglia, where it consists mostly of postganglionic fibers (Huangfu *et al.*, 1992). sSND was rectified, integrated and measured in arbitrary units as previously described (Huangfu *et al.*, 1992; Koshiya *et al.*, 1993). For quantitation, 100 sSND units was defined as sSND at rest before administration of any drug, and 0 units was defined as the electrical recording noise measured after administration of a large dose of the sympatholytic drug clonidine (Koshiya *et al.*, 1993).

In some experiments, the suprarenal or the cervical sympathetic ganglia were decentralized by sectioning their preganglionic inputs (splanchnic nerves and cervical sympathetic chain, respectively). Postganglionic neuronal activity was recorded with a bipolar electrode placed on a postganglionic connective (splanchnic nerve distal to suprarenal ganglion or cephalic sympathetic trunk adjacent to internal carotid artery, bandpass 10–3000 Hz). Drug-induced postganglionic activity was rectified and quantified as in intact preparations (Koshiya *et al.*, 1993). To stimulate postganglionic neurons synaptically, the preganglionic connective was stimulated with a bipolar electrode (intensity 150–500 μA, duration 0.1 msec, frequency 0.5 Hz). Before determining the effect of epibatidine on ganglionic transmission, stimulus intensity was adjusted to the level that produced a half-maximal response. Synaptically evoked responses were usually multiphasic. The shortest latency peak was the largest and had a latency of 5 to 7 msec. All peaks were blocked completely and reversibly by i.v. administration of the ganglionic blocker trimethaphan camsylate (6–8 mg/kg). Five consecutive responses were computer averaged, and the effect of drugs was quantitated by their effect on the amplitude of the largest synaptically evoked peak.

Drugs and chemicals. Epibatidine and its inactive enantiomer [endo-2-(6-chloro-3-pyridyl)-7-azabicyclo[2.2.1]heptane] were synthesized as described previously (Huang and Shen, 1993). Other drugs that were used were trimethaphan camsylate (reversible ganglionic blocker; Roche Laboratories, Nutley, NJ), chlorisondamine (long-lasting ganglionic blocker; CIBA-GEIGY Corp., Summit, NJ), phenylephrine (*alpha* adrenergic agonist; Sigma Chemical Co., St. Louis, MO), pancuronium bromide (neuromuscular blocker; Astra Pharmaceuticals, Worcester, MA), phentolamine (*alpha*-1 adrenergic antagonist; CIBA-GEIGY Corp.) and O-E-AVP (vasopressin V_1-receptor antagonist; Sigma Chemical Co.).

Statistical analysis. A one-way analysis of variance followed by Dunnett's *t* test or Tukey's Studentized range (HSD) test (as appropriate) was used to analyze the effect of drugs. Significance level was set at .05. All results are expressed as mean ± S.E.M.

Results

Effects of epibatidine on AP, HR and sympathetic and phrenic nerve discharge. The threshold i.v. dose of epibatidine for production of cardiorespiratory effects was 0.5 μg/kg. The drug produced the spectrum of effects depicted in figure 2 (representative case). At low doses (cumulative less than 2 μg/kg), the drug produced a transient drop in HR that preceded a rise in AP. As AP rose, HR returned to and exceeded control values. Postganglionic sympathetic nerve activity (sSND) was transiently increased by the drug and then dipped below control level as AP rose and finally returned toward control level. PND was increased. These effects were qualitatively similar but of gradually larger amplitude with successive injections of the drug. At doses in excess of 2 to 4 μg/kg (cumulative), the drug produced profound depression of sSND following an early burst of activity; sustained bradycardia was also observed. sSND was essentially eliminated at the highest dose (cumulative 8–16 μg/kg). PND was generally further stimulated at doses in excess of 2 μg/kg but could be transiently suppressed for a few seconds to 1 min after the injection. The effects of the drug on PND and sSND were maintained without recovery for at least 1 to 2 hr, whereas AP tended to slowly recover toward control. One animal was maintained anesthetized for 12 hr after the highest dose of drug, and recovery was observed after 6 to 7 hr. The diastereoisomer endo-epibatidine (structure in fig. 1) produced no detectable effects in doses up to 8 to 16 μg/kg. In two cases, the experiment was performed in the absence of the muscular relaxant pancuronium. No evidence of muscle fasciculation was observed during i.v. injection of epibatidine. Identical cardiovascular effects were observed. The pooled results of six experiments like the one depicted in figure 2 are summarized in figure 3.

The same experiment was repeated in one additional rat in which both vagus nerves, cervical sympathetic chain, superior laryngeal nerves and carotid sinus nerves had been sectioned (for surgical details, see Haselton and Guyenet, 1989). The drug produced qualitatively similar effects in this debuffered preparation except that the transient increases in sSND and in AP produced by low doses were of a slightly longer duration. In this deafferented preparation, PND was still dose-dependently increased by low doses despite the absence of carotid chemoreceptor afferents, and PND was still transiently inhibited at high doses despite the absence of cardiopulmonary afferents.

Effects of sympatholytic drugs and vasopressin antagonists on the effect of epibatidine on AP. The series of three experiments illustrated in figure 4 was conducted in quadruplicate to clarify the mechanism by which epibatidine raises AP. In a first set of four rats (group A), epibatidine (1 μg/kg) was injected i.v. after administration of the long-lasting ganglionic blocker chlorisondamine (5 mg/kg i.v.). As illustrated in fig. 4A, the drug still produced a substantial increase in AP (+39 ± 11 mm Hg). In the second group of four rats (group B; typical case illustrated in fig. 4B), epibatidine (1 μg/kg) was given after i.v. administration of the ganglionic blocker chlorisondamine plus the *alpha*-1 adrenergic antagonist phentolamine (10 mg/kg). Note that the dose of phentolamine administered abolished the pressor effect of phenylephrine (0.15–0.18 mg/kg i.v.), but the effect of epibatidine on MAP was unchanged (+40 ± 10 mm Hg; P = NS compared with group A). In a third group of four rats (group C; typical example illustrated in fig. 4), epibatidine was given after injection of chlorisondamine plus phentolamine plus the vasopressin V_1-receptor antagonist O-E-AVP (50 μg/kg i.v.). This combination of drugs abolished the pressor effect of epibatidine (+2 ± 0.4 mm Hg; P < .05 compared with groups A and B). Note that

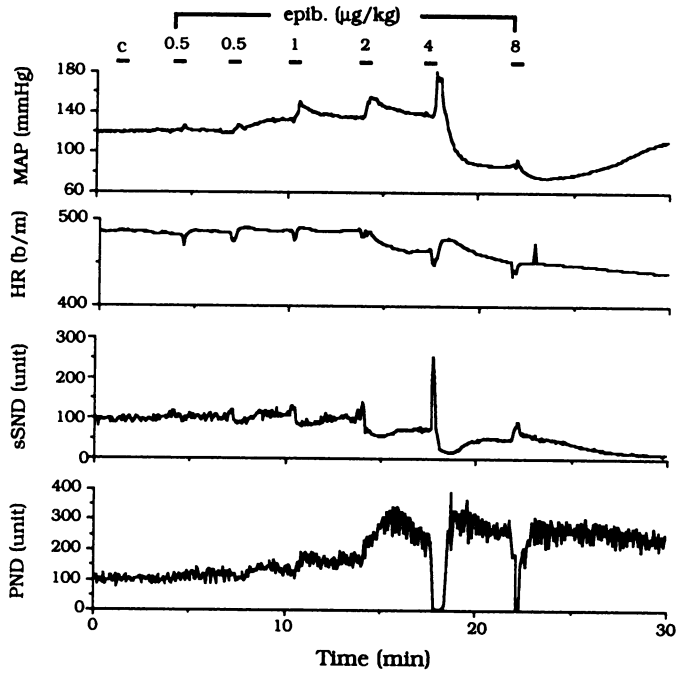

Fig. 2. Effect of epibatidine on cardiorespiratory variables *in vivo*. MAP, HR, sSND and PND were simultaneously measured. Intravenous injections of the inactive analog endo-epibatidine were done in quick succession at bar under c (cumulative dose of 16 μg/kg). Epibatidine (epib) was then administered every 200 sec in the doses indicated.

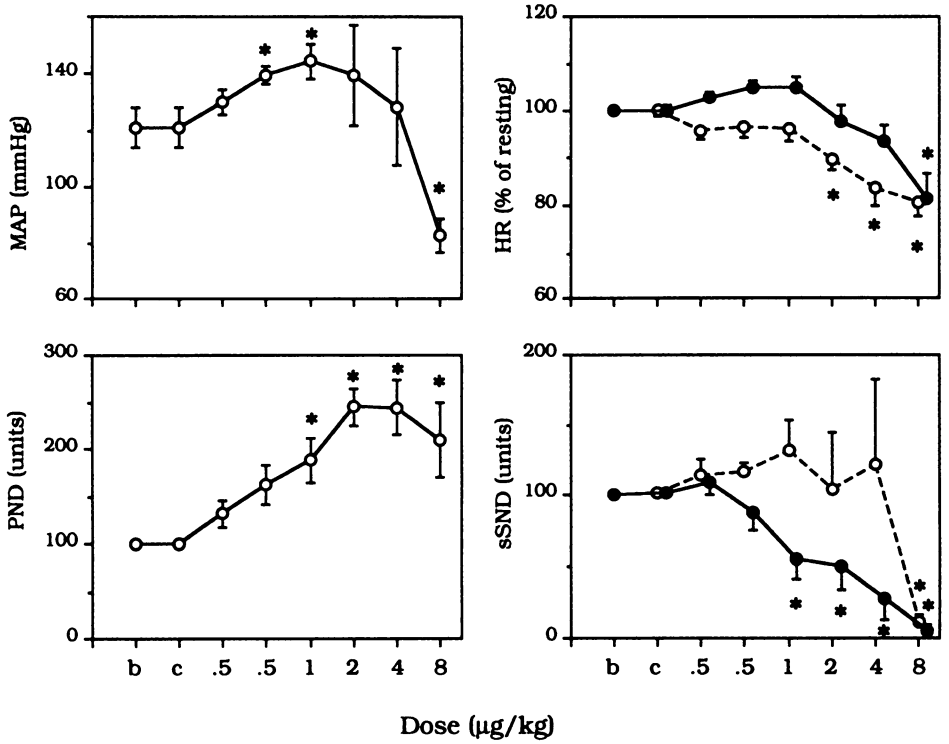

Fig. 3. Summary of the effect of epibatidine on cardiorespiratory parameters. Based on six experiments like that shown in the previous figure. Cumulative doses of epibatidine are indicated. b indicates baseline; c, values after i.v. injection of 16 μg/kg of endo-epibatidine; open symbols, immediate effects of epibatidine; closed symbols, delayed effects of the drug (1 min after bolus). * $P < .05$ relative to control values (c).

further administration of epibatidine still produced no pressor effect.

Effects of epibatidine on synaptic transmission in decentralized sympathetic ganglia. In a first series of experiments (n = 3), postganglionic sympathetic activity was recorded from the splanchnic nerve distal to the decentralized suprarenal ganglion (after section of the preganglionic splanchnic nerve). Intravenous injection of epibatidine (2 μg/kg) produced an intense but transient burst of activity in the splanchnic nerve (fig. 5) accompanied by the usual rise in AP. A second administration of the same dose of epibatidine immediately after i.v. administration of the reversible ganglionic blocker trimethaphan was completely devoid of effect on sSND. Administration of 2 μg/kg epibatidine after recovery from the action of the ganglionic blocker produced a response similar to that of the first dose. The outcome of the two other cases was identical.

In a second series of experiments, we examined the effects of

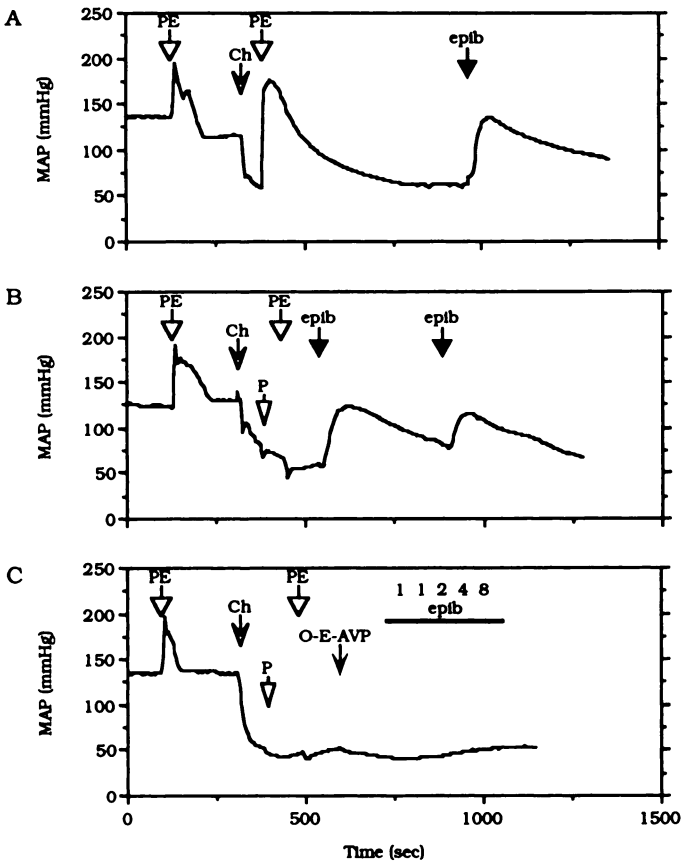

Fig. 4. Effects of chlorisondamine (Ch), phentolamine (P) and *d*-AVP on pressor effect of epibatidine (epib). A, Administration of epib (1 μg/kg i.v.) after ganglionic blockade with Ch (5 mg/kg i.v.). Phenylephrine (PE; 0.15–0.18 mg/kg i.v.) was given to demonstrate potentiation of pressor agent after ganglionic blockade. B, Administration of epib (epib, 1 μg/kg twice) after administration of Ch and P (10 mg/kg i.v.). PE was administered twice to demonstrate completeness of *alpha* adrenergic blockade. C, Effect of epib after injection of Ch plus P plus the V_1-vasopressin receptor O-E-AVP (50 μg/kg i.v.). PE was administered twice to demonstrate *alpha* adrenergic blockade.

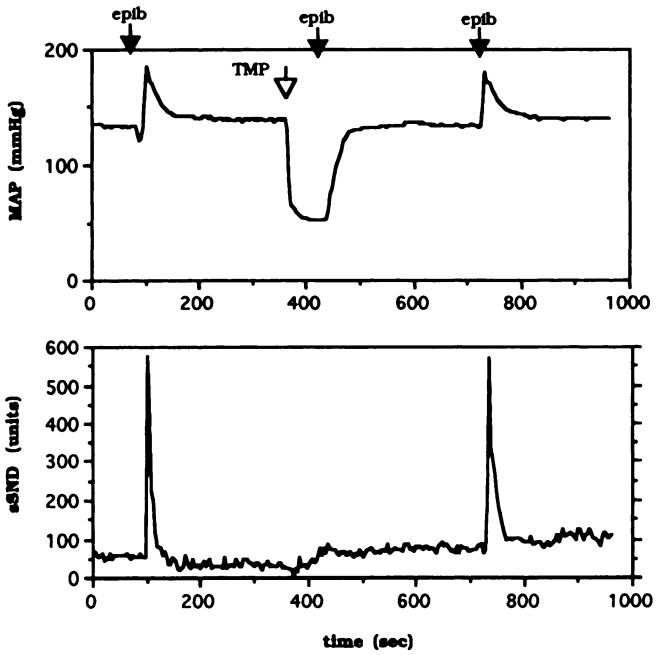

Fig. 5. Effect of epibatidine (epib) on decentralized suprarenal ganglion. sSND is expressed in arbitrary units. No spontaneous activity was present at rest as the ganglion was decentralized. Epib was administered three times in the same dose of 2 μg/kg. Trimethaphan (TMP) was given i.v. in a bolus dose of 8.6 mg/kg.

epibatidine on potentials evoked in a postganglionic nerve by single-pulse stimulation of the preganglionic nerve. Two experiments done with the suprarenal ganglion and two done with the superior cervical ganglion had identical outcomes. Figure 6 depicts an example of one experiment in which transmission through the superior cervical ganglion was examined. Maximal evoked responses were determined (left of diagram); then, the stimulation intensity was reduced to produce a half-maximal response (a), and this stimulation intensity was maintained for the remainder of the experiment. As shown in insets a and b, the evoked response was totally eliminated by i.v. injection of the ganglionic blocker trimethaphan, indicating that it was mediated synaptically. After recovery from the ganglionic blocker, endo-epibatidine was administered i.v. in doses up to 8 μg/kg (cumulative). The diastereoisomer endo-epibatidine produced no effect on ganglionic transmission. In contrast, epibatidine enhanced ganglionic transmission, transiently at low doses (1 μg/kg) and in a more sustained manner at doses of up to 4 μg/kg (cumulative). In this dose range, the evoked responses reached a level comparable to that registered using maximally effective stimulation intensities before administration of the frog alkaloid. Ganglionic transmission started to fail after administration of a total cumulative dose of 8 μg/kg, and no evoked response could be generated after a total of 16 μg/kg of epibatidine. Identical results were obtained in the three other cases.

Discussion

The cardiorespiratory effects of epibatidine that were monitored in the present study were dose dependent and stereospecific, because the diastereoisomer endo-epibatidine was totally inactive in all experimental paradigms. Epibatidine exerted detectable effects on cardiorespiratory function in doses as low as 0.5 μg/kg, and maximum effects on ganglia were produced with a cumulative dose of 8 to 16 μg/kg. This dose range is similar to that at which activity has been detected in at least two behavioral analgesia tests (Spande *et al.*, 1992). Therefore, epibatidine is a very powerful drug *in vivo* with a potency that is comparable to that of such compounds as LSD, clonidine or fentanyl, which also require low doses (μg/kg) for half-maximal efficacy.

Epibatidine induced postganglionic neuronal activity in decentralized sympathetic ganglia. In addition, at low doses, the drug facilitated synaptic transmission between preganglionic and postganglionic neurons, whereas at higher doses, it blocked neurotransmission. These effects are typical of nicotinic ganglionic depolarizing drugs, and as expected, they were antagonized completely by the reversible ganglionic blocker trimethaphan (Taylor, 1990). Taken together, these results suggest strongly that epibatidine is a highly potent agonist of ganglionic nicotinic receptors that produces depolarization blockade at high doses. Clearly, more detailed neurophysiological investigations will be required to determine if the effects of epibatidine on synaptic transmission in sympathetic ganglia are due exclusively to its agonist effect on postsynaptic nicotinic receptors.

The pressor effect of epibatidine is partly due to ganglionic stimulation. However, a large pressor effect persists after gan-

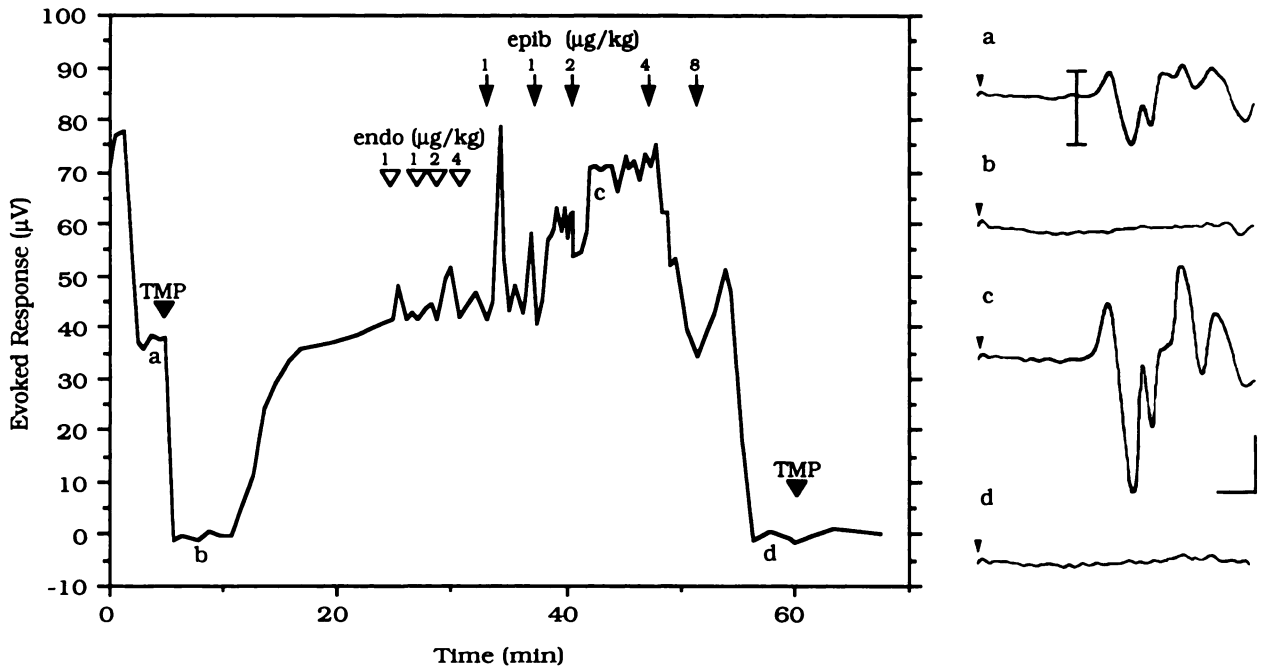

Fig. 6. Effect of epibatidine on ganglionic transmission *in vivo*. Sympathetic nerve activity, recorded in the cervical sympathetic chain distal to the superior cervical ganglion, was evoked by electrical stimulation proximal to the ganglion. Insets: a, half-maximal basal evoked response (vertical line in a indicates the waveform selected for measurement); b, blockade of the response by trimethaphan (TMP, 5.7 mg/kg i.v.); c, potentiated response following i.v. injection of 4 μg/kg epibatidine; d, blockade of ganglionic transmission after injection of 16 μg/kg epibatidine. Letters a through d also identify on the diagram on left the times at which the responses described in inset were observed. Calibration, 2 msec, 30 μV.

glionic blockade (fig. 4A). The magnitude of this remaining pressor effect was most likely magnified by the paradigm used for its study, *i.e.*, prior ganglionic blockade, which lowers MAP and reduces baroreflex control (see the difference between the pressor response to phenylephrine before and after ganglionic blockade in fig. 4A). Therefore, a comparison of the amplitude of the pressor response to epibatidine in rats with and without intact ganglionic function is not meaningful. Nevertheless, the origin of the pressor effect resisting ganglionic blockade could still be dissected pharmacologically. Because this remaining pressor effect was not affected by pretreatment with phentolamine, it is unlikely that epibatidine produces significant release of catecholamines from sympathetic postganglionic cells *via* a prejunctional mechanism. However, because the pressor effect of epibatidine resistant to ganglionic blockade was abolished by prior administration of a V_1-receptor-selective blocker, it is most likely that epibatidine, like nicotine, produces a powerful release of pituitary vasopressin. Finally, the complete blockade of the pressor effects of epibatidine by the combination of a ganglionic blocker, an *alpha*-1 adrenergic blocker and a V_1-antagonist suggests that the frog alkaloid has little or no direct effect on vascular smooth muscle.

In summary, epibatidine and nicotine both exert their effect on the cardiovascular system by stimulating ganglionic transmission and by releasing AVP. However, no evidence was found that epibatidine releases norepinephrine from sympathetic postganglionic cells *via* a prejunctional mechanism. Nicotine produces powerful respiratory stimulating effects attributed to a constellation of effects (stimulation of nociceptors, chemoreceptors and chemosensitive areas of the ventrolateral medulla; Taylor, 1990). Like nicotine, epibatidine produced powerful respiratory stimulation. These effects were not noticeably different after surgical elimination of peripheral chemoreceptors and vagal inputs. Their origin, central or peripheral, remains to be determined.

The effects of epibatidine on HR were complex, as are those of nicotine. The initial bradycardia produced by epibatidine could be due to direct stimulation of cardiac parasympathetic ganglia, and the following tachycardia could be due to cardiac *beta* adrenergic receptor stimulation *via* sympathetic and adrenal catecholamines.

The effect of epibatidine on neuromuscular transmission was not the focus of the present study. However, in the few animals in which pancuronium was deliberately not used, muscle fasciculations were not observed, and in the three cases in which an isotonic twitch response was tested, epibatidine produced no effect in doses of up to 32 μg/kg i.v., *i.e.*, doses considerably higher than those that produce complete ganglionic blockade. This apparent lack of effect on neuromuscular transmission and the previously mentioned lack of prejunctional catecholamine-releasing effect in sympathetic cells suggest that epibatidine has much higher affinity for ganglionic than neuromuscular nicotinic receptors and that epibatidine may interact with a narrower spectrum of nicotinic receptors than nicotine.

While the present article was being reviewed, others reported that the "analgesic" effect of epibatidine in rodents (increase in tail-flick latency) was (1) blocked by drugs with nicotinic antagonist properties, (2) probably of central origin and (3) mimicked by higher doses of nicotine (Quian *et al.*, 1993). This group also suggested that epibatidine has high affinity for presumptive central nervous system nicotinic receptors labeled with the radioligand [^{3}H]cytisine. Therefore, the results of Quian *et al.* (1993) appear to be fully congruent with ours.

The presence of a nicotinic agonist in the skin of *Dendrobatidae* poison frogs is particularly intriguing since the toxic alkaloid mixture of these animals also contains histrionicotoxin

(Daly *et al.*, 1992). This compound is a channel blocker that, in low doses, binds selectively to the agonist-liganded form of nicotinic channels (including the ganglionic nicotinic channels of chromaffin cells [e.g., Wada *et al.*, 1989]) and, in higher doses, blocks a variety of other channels. The presence of a nicotinic agonist in the frogs' venom would be expected to potentiate greatly the neurotoxicity of histrionicotoxin by facilitating its access to its site of action within nicotinic channels.

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