

Neurogenic Sympathetic Vasoconstriction of the Rabbit Basilar Artery

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SUMMARY When examined by fluorescence microscopy the rabbit basilar artery contains a rich adrenergic-like plexus at the adventitiomedial junction. The fluorescence disappears upon chronic reserpinization and bilateral superior cervical ganglionectomy. Transmural stimulation of intramural nerves results in a response which is predominantly constrictor but also contains a small, inconstant dilator component. The constrictor response is abolished by chronic reserpinization, bilateral superior cervical ganglionectomy, and cold storage of the preparation. The constriction is prevented by the adrenergic neuron blocking agents guanethidine and bretylium but not by such α -adrenergic receptor blocking agents as phenoxybenzamine (PBZ), phentolamine, and tolazoline. Our results

show that doses of the three latter agents sufficient to abolish contractions to norepinephrine (NE) in concentrations of up to 10^{-2} M only potentiate and prolong the contractile response to nerve stimulation. The β -adrenergic receptor blocking agent, propranolol, and inhibitors of NE neuronal uptake, such as desipramine (desmethyl-imipramine, DMI) and cocaine, do not influence the size of the neurogenic response. These results suggest that the vasoconstrictor component of the rabbit basilar artery response to transmural nerve stimulation (TNS) is mediated via sympathetic adrenergic-like neurons, but at the same time also raise the question whether the transmission process is typical of classic adrenergic neuroeffector mechanisms.

THE INNERVATION of cerebral blood vessels of many species has been intensively studied morphologically by light, ultraviolet fluorescence, and scanning and transmission electron microscopy techniques.¹⁻⁶ Unquestionably, the cerebral blood vessels of the cat, dog, mouse, monkey, rabbit, and rat are richly innervated. The presence of not only adrenergic but nonadrenergic nerves has been suggested on the basis of histochemistry and the appearance and size of the vesicles in the nerve terminals. However, reports on the vasomotor role of the innervation remain contradictory. Lack of response⁷⁻¹¹ or only a slight change in the caliber of cerebral vessels and of the cerebral blood flow^{12, 13} as a result of electrical stimulation of the cervical sympathetic system led these authors to believe that the innervation of these vessels is not functional. This concept was further supported by the observation that cerebral blood flow was not changed significantly following section of the sympathetic postganglionic neurons,^{14, 15} or blockade of the superior cervical ganglion with lidocaine.¹⁶ Recently many other authors have provided evidence that cerebral blood flow and the caliber of cerebral blood vessels are significantly altered by stimulation of the sympathetic nerve supply.¹⁶⁻²⁵

In an attempt to clarify these discrepancies an analysis of the response of isolated cerebral vessels to stimulation of their intramural nerves has been undertaken. The study was designed to permit the measurement of the size, and a description of the nature, of the contractile response. Through pharmacological analysis, characteristics of the involved transmitter were sought. It was anticipated that

results from this study should help elucidate the nature of the response of these vessels to neuronal activation and thus, perhaps, the possible role of the innervation in the regulation of cerebral vascular tone.

Methods

Adult white rabbits (2-3 kg) of either sex were stunned by a blow on the front of the head and exsanguinated. The entire brain with blood vessels attached and the most distal part of the saphenous artery were rapidly removed and placed in Krebs' bicarbonate solution equilibrated with 95% O₂ and 5% CO₂ at room temperature. The composition of the Krebs' solution was (mM): Na⁺, 144.2; K⁺, 4.9; Ca²⁺, 1.3; Mg²⁺, 1.2; Cl⁻, 126.7; HCO₃⁻, 25.0; SO₄²⁻, 1.19; glucose, 11.1; and calcium disodium ethylenediaminetetraacetate (EDTA), 0.023. Vessels were dissected and cleaned of surrounding tissue under a dissecting microscope.

Ring segments (4 mm long) of the arteries were cannulated with a stainless steel rod of hemispherical section and a short piece of platinum wire and mounted in an isolated tissue bath which contained 30 ml of Krebs' bicarbonate solution at room temperature. The platinum wire was bent into an inverted U shape and anchored to a plastic gate which could be moved up and down by a fine control micrometer. The steel rod was connected to a Statham strain gauge (GI0b, 0.15oz) for isometric recording of changes in force. This method has been described in detail.^{22, 26} After 5-10 minutes, the temperature of the Krebs' bicarbonate solution was gradually increased to and maintained at 37°C. Resting tension was then adjusted to 0.5 g and a period of 1 hour was allowed for equilibration. A pair of stimulating electrodes, one on either side of the vessel, was arranged for transmural nerve stimulation (TNS). Routinely trains of 200 biphasic square wave pulses of 0.3-msec duration were delivered at supramaximal voltage.

For studies on the response to exogenous drugs, both agonists and antagonists were added directly to the organ bath.

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FREQUENCY RESPONSE CURVES

Responses of a given basilar artery preparation to a set of TNS at 2, 4, 8, 16, 25, and 32 Hz in random sequence were elicited without changing the bath solution. Eight minutes was allowed following each stimulation, and 30 minutes later another set of TNS was applied. Drugs to be studied for their influence on the contractile response to TNS were added 15 minutes prior to the commencement of the second set. As the standard pulse duration (0.3 msec) used in this study sometimes caused a small direct (muscle) stimulation of the isolated vessel, a 90% or greater reduction in neurogenic contractile response was considered to be complete blockade. A shorter pulse duration, which would have been devoid of direct component, could not be employed because not all intramural nerves would have been stimulated.

FLUORESCENCE HISTOCHEMICAL TECHNIQUE

Freshly dissected, whole mount preparations of arteries were treated with glyoxylic acid to induce catecholamine fluorescence.²⁷

DENERVATION

Three rabbits were treated with reserpine (Serpasil), 3 mg/kg per day, intraperitoneally (ip) for 2 days and again intravenously (iv) for 1 day before they were killed. Six other rabbits were anesthetized with pentobarbital (40 mg/kg, ip). Both superior cervical ganglia were isolated and extirpated by cutting the sympathetic trunk at a point proximal to the ganglia and then removing them with short lengths of their other branches attached. Tissues were examined 1 and 3 weeks after ganglionectomy. The effectiveness of both chemical and surgical denervation was confirmed by fluorescence microscopy of arterial segments adjacent to those studied.

STATISTICAL METHODS

The data were statistically evaluated by Student's *t*-test for paired samples. The 0.05 level of probability was accepted as significant.

DRUGS USED

The drugs used were: *l*-norepinephrine bitartrate (Calbiochem), phenoxybenzamine (PBZ) hydrochloride (Smith, Kline and French), phentolamine methanesulfonate (Ciba), propranolol hydrochloride (Ayerst), desipramine (desmethylinipramine, DMI) hydrochloride (U.S. Vitamin Pharmaceutical Corp.), cocaine hydrochloride (Mallinckrodt), tetrodotoxin (TTX) (Sankyo-Tokyo), guanethidine sulfate (Ciba), bretylium tosylate (Burroughs Wellcome), and reserpine (Ciba).

Results

MORPHOLOGY OF INNERVATION OF RABBIT CEREBRAL ARTERIES

Whole mount preparations of normal rabbit cerebral arteries (basilar, middle cerebral, posterior cerebral, anterior cerebral, posterior communicating, and vertebral), after treatment with glyoxylic acid, exhibited a dense network of fluorescence which was typical of an adrenergic nerve plexus

(Fig. 1a) and which was quite clearly distinguishable from fluorescence due to serotonin. The fluorescence disappeared completely after reserpinization ($n = 3$) (Fig. 1b) and also 8 days after bilateral superior cervical ganglionectomy ($n = 6$) (Fig. 1c).

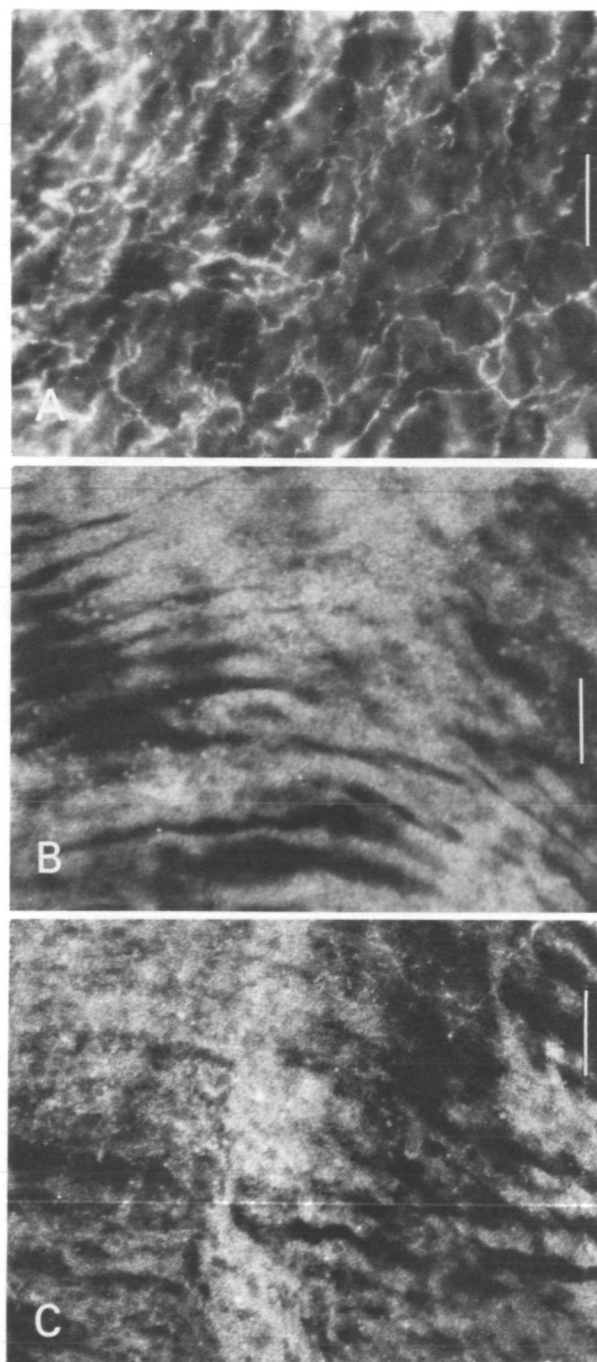


FIGURE 1 Whole mount glyoxylic acid-induced fluorescence photograph of comparable rostral sections of the basilar artery from rabbits. (a) untreated; the irregular, meshlike white lines represent fluorescence of catecholamine-containing nerves; (b) pretreated with reserpine; (c) superior cervical ganglionectomized. In neither b nor c was there any detectable catecholamine visible. Scale represents 100 μ m.

CHARACTERISTICS OF CONTRACTILE RESPONSES OF THE BASILAR ARTERY TO TRANSMURAL NERVE STIMULATION

Electrical TNS elicited a contraction in 103 of the 114 artery preparations tested. The contraction exhibited an initial rapidly rising phase followed by a fast decline to the resting tension level (Fig. 2a). On occasions (11 out of 114) the declining phase was slow. In six cases responses consisted of an initial rapid and an ensuing tonic phase (Fig. 2b), especially when higher stimulus frequencies were employed. Because all these contractions were blocked completely by TTX ($0.6 \mu\text{M}$) (Fig. 2a) and prevented by cold storage of the arteries for 6 days (Fig. 2c), and since the arteries still responded to *l*-norepinephrine (see below), it was assumed that the contractions must be due to activation of intramural nerves.

Contractions were frequency-dependent (Figs. 2a and 3). The average maximum tension developed by a 4-mm length of vessel at 32 Hz was about 200 mg. Because there were no significant qualitative or quantitative differences in the neurogenic contraction of segments taken from rostral and caudal parts of the basilar artery, in the subsequent analysis, data from all segments of the basilar artery were pooled.

PHARMACOLOGICAL CHARACTERIZATION OF THE NEUROGENIC CONTRACTILE RESPONSE

In view of published data (see introductory paragraphs) and the above observations, it was assumed as a working hypothesis that neurogenic contraction was the result of the liberation of norepinephrine (NE) from adrenergic nerves. This hypothesis was tested by classic pharmacological procedures.

EFFECT OF ADRENERGIC NEURONAL BLOCKING AGENTS

Guanethidine ($6 \mu\text{M}$), which by itself produced an increase in muscle tone of about 50 mg, and bretylium ($7 \mu\text{M}$) (Fig. 4) abolished the neurogenic response to TNS at all tested frequencies in 25 out of 30, and in 4 out of 6 basilar artery preparations, respectively. In the remaining cases a small residual response (less than 10% of the maximum) still persisted. This residual component was not abolished by TTX, indicating that it probably was due to direct stimulation of the muscle cells.

EFFECT OF α -ADRENERGIC RECEPTOR BLOCKING AGENTS

Basilar Artery. Neither PBZ ($10 \mu\text{M}$, $n = 14$), phentolamine ($10 \mu\text{M}$, $n = 3$), nor tolazoline ($10 \mu\text{M}$, $n = 3$) reduced the contractions of basilar artery segments to TNS at frequencies between 2 and 32 Hz. Unexpectedly, these concentrations of PBZ, phentolamine, and tolazoline which blocked the contractile responses to exogenous NE, *l*-epinephrine, and dopamine in concentrations up to 10^{-2} M , consistently increased the duration of neurogenic responses to TNS in 21 out of 21, in 3 out of 5, and in 4 out of 5 experiments, respectively (Fig. 5). Only at concentrations higher than $50 \mu\text{M}$, which abolished the response to direct muscle stimulation with long duration pulses, did these α -adrenergic blocking agents antagonize the neurogenic contraction.

As PBZ was more effective than either phentolamine or tolazoline in increasing the duration of neurogenic contraction, its effect on the magnitude of neurogenic response was further examined. As shown in Figure 6, responses of the

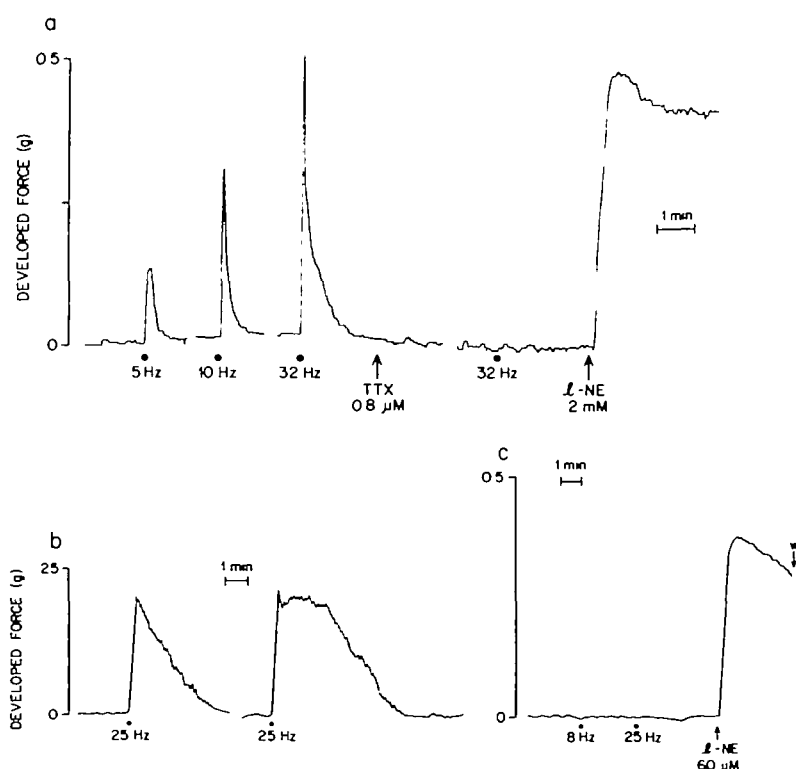


FIGURE 2 Responses of rabbit basilar artery ring to transmural nerve stimulation (TNS). (a) stimulation at different frequencies (Hz) and the effect of tetrodotoxin (TTX); (b) in some instances the rapid contractile responses were followed by a prolonged relaxation phase; (c) lack of response of cold-stored artery rings to TNS.

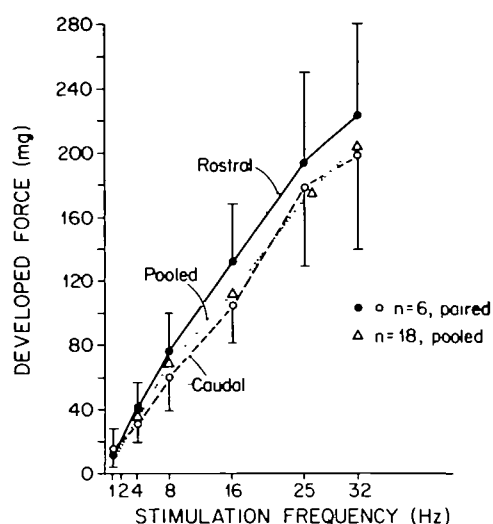


FIGURE 3 Frequency-response relationship of caudal and rostral portions of the rabbit basilar artery to transmural nerve stimulation. Vertical bars represent standard errors of the mean. There is no significant difference between the three mean values at any frequency.

basilar artery to TNS became greater upon repetition of stimulation even in the absence of any drug. However, the ratio of the second to the first response of the artery preparations ($n = 4$) given PBZ before the second response was greater than the corresponding ratio of the untreated artery preparations ($n = 7$) at all frequencies. At 4 and 8 Hz, in particular, the ratios of PBZ-treated preparations, 3.18 ± 0.75 and 2.79 ± 0.52 (mean \pm SE), respectively, were significantly greater than those of the untreated preparations, 1.77 ± 0.18 and 1.51 ± 0.13 ($P < 0.05$ at 4 Hz and P

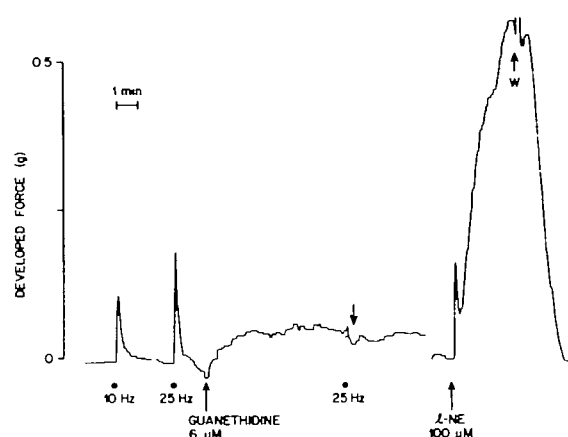


FIGURE 4 Effect of guanethidine ($6 \mu\text{M}$) on the contractile responses of basilar artery rings to transmural nerve stimulation (TNS). The contraction was abolished by guanethidine while a small component of relaxation of artery ring to TNS (arrow \downarrow) remained.

< 0.02 at 8 Hz by unpaired Student's t -test). Some basilar artery segments ($n = 4$) which initially did not respond to TNS became actively responsive after PBZ, $10 \mu\text{M}$ (Fig. 7a). The neurogenic contraction in the presence of PBZ was abolished by TTX.

After chronic sympathectomy TNS did not elicit a response either in the presence or absence of PBZ, $10 \mu\text{M}$ (Fig. 5). These results suggest that the potentiating action of PBZ depends on a presynaptic neuronal mechanism.

Saphenous Artery. In parallel studies, the neurogenic contraction of segments of the most distal part of the rabbit saphenous artery, a peripheral muscular artery which exhibits a rich catecholamine fluorescence, was invariably

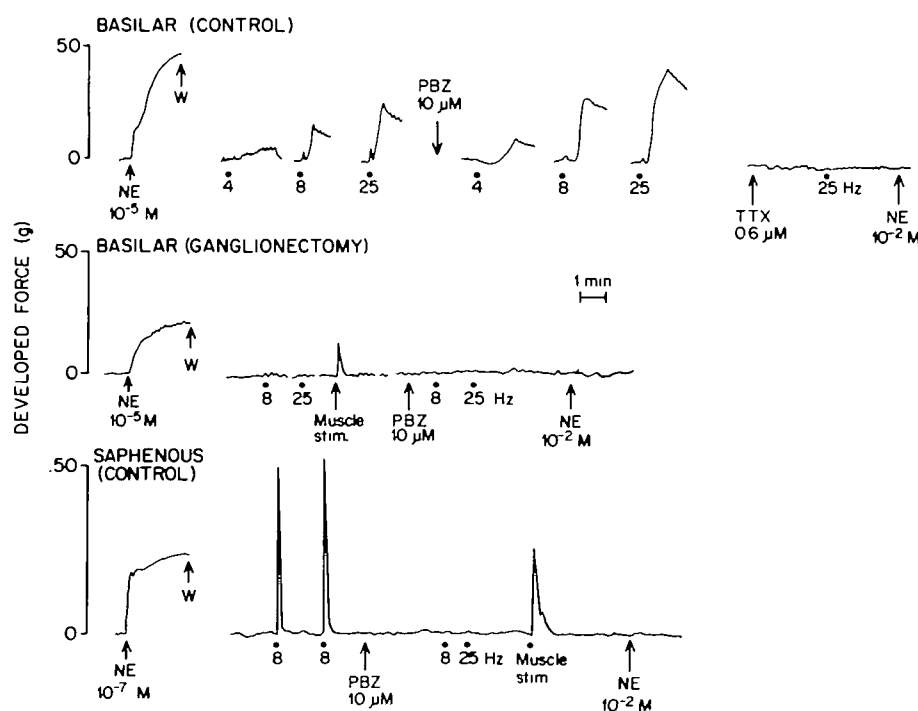


FIGURE 5 Effect of phenoxybenzamine (PBZ), $10 \mu\text{M}$, on the contractile responses of basilar artery and saphenous artery rings to transmural nerve stimulation (TNS). Basilar (control): PBZ increased both the duration and height of the contractile responses of the basilar artery to TNS at 4, 8, and 25 Hz, but blocked that to norepinephrine (NE) (10^{-5} M). The neurogenic response of the same vessel was abolished by tetrodotoxin (TTX). Basilar (ganglionectomy): basilar artery ring obtained from rabbit 8 days after bilateral superior ganglionectomy, which contracted in response to direct muscle stimulation at 3 msec, did not respond to TNS either before or after the addition of PBZ. Saphenous (control): the contractile responses of the saphenous artery ring to TNS and NE (10^{-2} M) were abolished by PBZ.

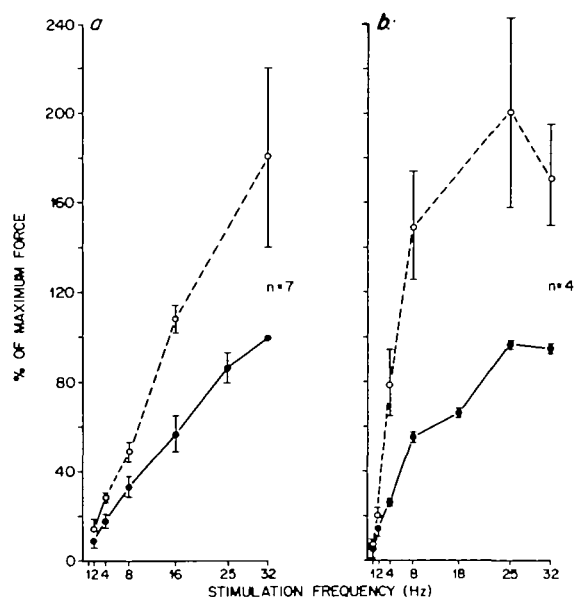


FIGURE 6 Responses to two consecutive sets of transmural nerve stimulation (TNS) applied at the given frequencies were determined for each artery preparation. Responses from the first set are shown in solid circles and the second in open circles. (a) control data are shown; (b) phenoxybenzamine (PBZ), $10 \mu\text{M}$, was added to the tissue prior to eliciting the second set (for details see text).

blocked by PBZ, $10 \mu\text{M}$ (Fig. 5) and also by phentolamine and tolazoline.

EFFECT OF β -ADRENERGIC BLOCKING AGENTS

The contractions of rabbit basilar artery segments to TNS were not influenced by propranolol ($1 \mu\text{M}$; 3 out of 3). Propranolol in this concentration will completely antagonize the relaxation caused by isoproterenol ($8 \mu\text{M}$) in other tissues. However, in the rabbit basilar artery, even when tone has been increased by serotonin ($0.25 \mu\text{M}$), isoproterenol exerts little relaxing effect.²⁸ Furthermore, the combination of PBZ ($10 \mu\text{M}$) and propranolol ($1 \mu\text{M}$) did not abolish neurogenic contractions.

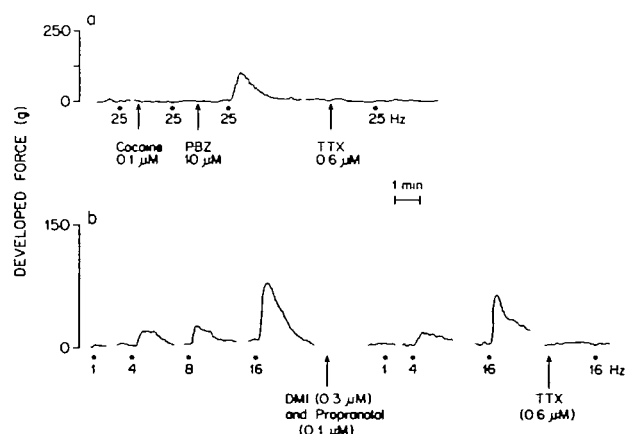


FIGURE 7 Effect of (a) cocaine ($0.1 \mu\text{M}$) and phenoxybenzamine (PBZ), $10 \mu\text{M}$ and (b) desmethylinipramine (DMI), $0.3 \mu\text{M}$, and propranolol, $0.1 \mu\text{M}$, on the contractile responses of basilar artery to transmural nerve stimulation (TNS). TTX = tetrodotoxin.

EFFECTS OF COCAINE AND DESIPRAMINE (DESMETHYLIMIPRAMINE, DMI)

Cocaine (10^{-7} to 10^{-6}M) and DMI (3×10^{-7} to 10^{-6}M), which block the uptake of catecholamines across the axonal membrane, did not affect the size of the neurogenic contractions of basilar artery segments to TNS at any frequency (3 out of 3) (Fig. 7a and b).

EFFECT OF CHRONIC RESERPINIZATION AND BILATERAL SUPERIOR CERVICAL GANGLIONECTOMY

Basilar artery segments from five chronically reserpinized rabbits, from which catecholamine fluorescence was absent, did not respond to TNS although the magnitude of responses to NE ($100 \mu\text{M}$) was of the same order of magnitude as in control tissues (Fig. 8). Eight to 24 days after bilateral superior cervical ganglionectomy, basilar artery segments from which catecholamine fluorescence was absent did not respond to TNS at any frequency (6 out of 8 rabbits), whereas the responses of the artery to NE (0.03 – 3mM) were of the same order of magnitude as in controls (Fig. 5). In two cases the denervated basilar artery showed small contractions to TNS, less than 10% of that expected from control tissues, which were not abolished by TTX ($0.6 \mu\text{M}$).

Discussion

The reason for the contradictory findings in vivo on the role of the innervation of cerebral blood vessels is not known. Species difference may be important. Regional differences in response of cerebral vessels to exogenously applied drugs²⁹ and to excitation of sympathetic nerves²⁰ may further complicate the interpretation of experimental results. Kobayashi et al.,¹⁹ using a method of comparatively low sensitivity for measuring vessel diameter, reported a dissociation between the change in caliber of regional blood vessels and changes in measured cerebral blood flow on electrical stimulation of the cervical sympathetic nerves. In addition, factors such as arterial blood pH, PCO_2 , and level of anesthesia and surgical trauma have been implicated.^{18, 21} Methodology in vitro provides a useful approach in that external conditions can be rigorously controlled. Neuro-muscular transmission processes can be initiated by electrical stimulation of intramuscular nerves and the resultant contraction can be subjected to analysis.²²

Our unequivocal finding of neurogenic vasoconstriction contrasts with that of Toda and Fujita,³⁰ who reported that

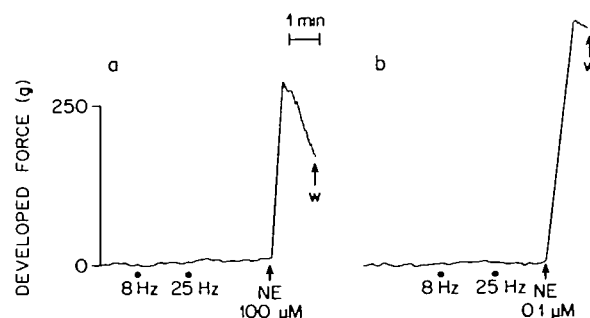


FIGURE 8 Effect of reserpine pretreatment (3mg/kg , ip and iv) on the contractile responses of basilar artery (a) and saphenous artery (b) to norepinephrine (NE) and transmural nerve stimulation.

the isolated dog basilar artery did not respond to TNS. Although a species difference cannot be excluded, these authors used helical strip preparations which might have suffered more trauma during dissection than the ring segments used in the present experiments.

Rabbit cerebral blood vessels have been shown by use of a fluorescence histochemical technique to receive a rich adrenergic-like innervation.^{31, 32} In the present study, the greenish-yellow catecholamine fluorescent network in the wall of the rabbit basilar artery demonstrated by the glyoxylic acid method²⁷ was typical of a peripheral adrenergic plexus. It disappeared after chronic reserpination and superior cervical ganglionectomy, indicating that this vessel is innervated by sympathetic nerves of an adrenergic type.

Since the frequency-dependent contractions of the rabbit basilar artery rings elicited by electrical stimulation were abolished by TTX (0.6 μ M), by cold storage, and also by chronic reserpination and bilateral superior cervical ganglionectomy, the contractions must have been the result of stimulation of adrenergic-type sympathetic neurons. Furthermore, neurogenic vasoconstriction was blocked by such adrenergic neuronal blocking agents as guanethidine and bretylium. These agents are considered specific to the adrenergic-sympathetic neuron. Their effects are not due to their local anesthetic activity in that the dilator component of nerve stimulation, when present, was unaffected. However, in rabbits (Lee, Su, and Bevan, unpublished results) and in other species,³³ dilation was invariably blocked by TTX (0.6 μ M).

The transmitter for the adrenergic nerve endings in cerebral blood vessels has been assumed by most investigators to be NE.^{25, 32, 34} However, the neurogenic response was not blocked but was potentiated by α -receptor adrenergic blocking agents in doses that abolished responses to doses of NE and also epinephrine and dopamine that matched the neurogenic response at 25 and 32 Hz. Furthermore, doses of these agents sufficient to abolish responses to NE, epinephrine, and dopamine in 100 times these concentrations did not diminish the neurogenic response. It might be pointed out that the median effective dose (ED_{50}) for NE in the rabbit basilar artery is high in comparison to that for other vessels;²⁶ it is more than 10^{-6} M even in the presence of neuronal uptake blocking agents (Duckles, Lee, and Bevan, unpublished data). This also appears to be true of the isolated cat cerebral artery.³⁴

The failure of α -adrenergic receptor blocking agents to block a sympathetic response in several nonvascular smooth muscle preparations has been reported,³⁵⁻³⁷ and led to the speculation that NE is probably not, or is only part of, the excitatory transmitter substance in the vas deferens.^{36, 37} Alternatively, the inability of PBZ, phentolamine, and tolazoline to block neurogenic responses, as opposed to responses to exogenous NE, might result from the narrowness of the neuromuscular cleft and the resulting inaccessibility of synaptic receptor sites to the blocking drugs.³⁸ This explanation is probably not applicable to the rabbit basilar artery, since the neuromuscular gap in this vessel, when examined under electron microscope, is considerably wider than that in the vas deferens (Lee, unpublished data). Besides, in parallel studies, the neurogenic vasoconstriction

of the rabbit saphenous artery was markedly blocked by PBZ (10 μ M) and in this vessel the neuromuscular gap is narrower than in the basilar artery (Lee, unpublished data).

The response of a small blood vessel to neural activation is complex; this is due in part to the varying intramural distribution of transmitter with time³⁹ and also to the bimodal pattern of response of the effector system.^{40, 41} It is possible that the first phasic component of response of the blood vessel to nerve activation at all frequencies is due to the transmitter reaction with α -receptors near the nerve terminals and the second phase, the result of transmitter diffusion into the muscle layers. However, neither component at any frequency of stimulation was diminished by PBZ or phentolamine. On the contrary, PBZ and phentolamine increased the duration and magnitude of neurogenic contractions to TNS. These drugs are known to increase NE release by inhibiting presynaptic α -receptors^{42, 43} and also by inhibiting the reuptake of neurally released catecholamines. As a result of these two effects the concentration and longevity of the transmitter at the postsynaptic α -receptors would increase. However, such effects would not explain the inability of these drugs to block neuromuscular transmission.

It is conceivable that another transmitter is involved in the transmission process. This is a departure from accepted ideas and there is no direct evidence for such a proposition. However, such a proposal has been made previously for other tissues (see above). It is well known that the "greenish-blue fluorescence" seen in sympathetic nerves after processing by the Falck or similar procedure can represent NE, epinephrine, or dopamine, or a mixture or combination of any of these. However, the effects of both epinephrine and dopamine are blocked by α -adrenergic receptor blocking agents at doses similar to those required for NE. Other studies²⁸ have shown that the α -adrenergic receptor in the rabbit basilar artery is not identical to that in other vessels. The NE dose-response curve is complex and can be resolved into two components. Relative potencies for a series of amines are not the same for both components and, like the phentolamine dissociation constant, differ from that in other arterial systems. Such a conclusion is supported by recent work in the cat cerebral vessels.¹⁴ The very high transient transmitter concentrations encountered in smaller vessels during nervous activity⁴⁴ associated with these unusual receptors may not be blocked by doses of blocking agents that antagonize equivalent steady state contractions. Higher doses of PBZ were not specific in that they also blocked other receptors. This, of course, is not to say that the action of these agents on the α -adrenergic receptor in these high doses is not responsible for the blockade of neurogenic contraction. Our results show quite clearly that neurogenic vasoconstriction depends on guanethidine- and bretylium-sensitive "adrenergic" neurons of sympathetic origin.

These experiments show that activation of intramural nerves can cause a measurable contractile response of several cerebral arteries. The functional consequences of this contraction with respect to cerebral blood flow cannot be determined from this *in vitro* data. It has been found previously that the neurogenic response of rabbit basilar arteries can be dramatically increased by as much as 3

orders of magnitude on exposure to histamine.⁴⁵ Since, under these circumstances, the response was comparable to that of a heavily innervated peripheral limb vessel, such observations show that neuronal activity is potentially capable of producing a significant contraction. Whether or not this potential is realized in vivo is not known.

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