Neurogenic Dilator and Constrictor Responses of Pial Arteries in Vitro

Differences between Dogs and Sheep

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SUMMARY Two distinct responses to transmural electrical stimulation of cerebrovascular smooth muscle isolated from dogs and sheep have been identified. Both these responses were blocked with tetrodotoxin and therefore were attributed to stimulation of intramural nerves. A constrictor response to transmural nerve stimulation (TNS) which was blocked with guanethidine was attributed to stimulation of sympathetic nerves. In cerebral arteries of the sheep this constrictor response was blocked by phentolamine, but in dog cerebral vessels it was not. Instead, phentolamine (10^{-6} M) potentiated the response to TNS. Postsynaptic $\alpha$-adrenergic receptors of dog cerebrovascular smooth muscle may be unusual and hence not susceptible to the action of phentolamine. The increased response to TNS may be due to blockade by phentolamine of presynaptic receptors, causing increased transmitter release. After blockade of the constrictor response to TNS and in the presence of smooth muscle tone, a dilator response, much more prominent in sheep, was unmasked. The dilator response was not blocked by guanethidine, propranolol, or phentolamine, in support of the hypothesis that cerebral vessels are innervated by nonsympathetic nerves. The possibility that these nerves are cholinergic was investigated, but the dilator response was neither blocked by atropine nor potentiated by physostigmine. The nature of the transmitter for this dilator response remains unclear. Some of the marked inconsistencies in studies of neurogenic control of the cerebral circulation may be attributed in part to both qualitative and quantitative differences in neuroeffector mechanisms among species.

RECENT EVIDENCE suggests that more than one type of nerve innervates the larger cerebral blood vessels. Histological studies support this concept. Thus at least two distinct types of nerve ending can be distinguished in the walls of pial vessels by the use of ultrastructural techniques (Iwayama et al., 1970; Edvinsson et al., 1972; Duckles et al., 1977). One type of nerve ending seems to correspond to the sympathetic nerves that originate in the superior cervical ganglion; the nature of the other type of nerve ending remains unclear. Corresponding to this ultrastructural evidence, functional studies of cerebral vessels in vitro have distinguished two types of response to transmural nerve stimulation. Stimulation of sympathetic nerves produces a contractile response in pial vessels of the rabbit (Lee et al., 1976), dog (Duckles et al., 1977; Muramatsu et al., 1977), sheep and monkey (Duckles et al., 1977). After sympathetic denervation or treatment with guanethidine, nerve stimulation produces a dilator response in pial vessels of the cat (Lee et al., 1978), sheep, and dog (Duckles et al., 1977).

There are several unusual features of nerve-induced responses of cerebral arteries that merit further study. A marked variation among species has been found in the relative importance to the neurogenic response of the constrictor and dilator components (Duckles et al., 1977). In addition, postsynaptic mechanisms for the sympathetic constrictor response are atypical. These atypical features include a relatively low sensitivity to the transmitter, norepinephrine, and, in the dog and the rabbit, an ineffectiveness of $\alpha$-adrenergic blocking agents on the nerve-induced contraction (Duckles et al., 1977; Lee et al., 1978; Duckles and Bevan, 1976). Furthermore, it has not been possible with pharmacological methods to identify the transmitter for the nonadrenergic dilator response.

Pial vessels of the dog and sheep were selected...
for further study. Responses to nerve stimulation in these two species show both constrictor and dilator components, but the dilator component is relatively small in the dog and much more prominent in the sheep. Thus the constrictor response can be studied in more detail in the dog, whereas sheep vessels make further study of the dilator response feasible after pretreatment with guanethidine.

Methods

Ring segments (4–5 mm long) of the basilar, middle cerebral, and anterior cerebral arteries were prepared from female sheep (50 kg) and mongrel dogs of either sex (30–40 kg). The animals were killed by an overdose of pentobarbital (30 mg/kg), and the entire brain with vasculature attached was removed quickly and placed in a Krebs' bicarbonate solution equilibrated with a 95% O₂-5% CO₂ gas mixture at room temperature. Composition of the Krebs' solution was (in mm): Na⁺, 144.2; K⁺, 4.9; Ca²⁺, 1.3; Mg²⁺, 1.2; Cl⁻, 126.7; HCO₃⁻, 25; SO₄²⁻, 1.19; glucose, 11.1; and calcium disodium ethylenediaminetetraacetate, 0.023. Vessels were removed carefully from the brain surface with the aid of a dissecting microscope.

Ring segments were cannulated with a stainless steel rod and a short piece of platinum wire, mounted in a tissue bath in Krebs' solution and maintained at 37°C. The platinum wire was bent into a U shape and anchored to a plastic holder. The steel rod was connected to a Statham strain gauge (G10B, 0.15 oz) for isometric recording of changes in force on a Sargent strip chart recorder or a Grass model 79 polygraph. After 1 hour in the bath at 37°C, resting tension was applied by moving the strain gauge with a fine micrometer. Optimal resting tension was defined as the tension at which maximal responses to a standard stimulation were obtained. This value was 1 g for all vessels studied except the anterior cerebral artery, for which 0.5 g was optimal. After the vessel was stretched to its resting tension, an additional hour of equilibration was allowed. Resting tension was maintained constant throughout the experiment. The viability of each vessel segment was confirmed at the end of each experiment by testing contraction to a high concentration of KCl, 150 mM.

Transmural electrical stimulation was provided by a Grass S-4 stimulator via platinum electrodes. These were parallel wires 6 mm in length positioned on either side of the vessel ring, 5–6 mm apart. Pulse duration was 0.3 msec, except when mentioned otherwise, and the voltage was supramaximal (15 V measured across the electrodes). All responses to electrical stimulation with these parameters could be blocked with tetrodotoxin (6 × 10⁻⁶ M), confirming their neurogenic origin. In such cases electrical stimulation is identified as transmural nerve stimulation (TNS). See Results for further details.

Drugs were added directly to the 50-ml bath in volumes of less than 50 μl. Concentrations are expressed as moles/liter final concentration in the bath. Drugs were dissolved in distilled water, with the exception of norepinephrine and phentolamine, which were dissolved in 0.001 N HCl. The following drugs were used: acetylcholine chloride (Calbiochem), atropine sulfate (Merck), guanethidine sulfate (Ciba), l-norepinephrine bitartrate (Sigma), phentolamine methanesulfonate (Ciba), physostigmine salicylate (Merck), propranolol HCl (Ayerst), prostaglandin F₂ (Upjohn, courtesy of Dr. John Pike), and tetrodotoxin (TTX) (Sankyo-Tokyo).

The data were evaluated statistically by Student's t-test for paired samples (Snedecor and Cochran, 1967). Standard errors are reported throughout.

Results

Responses to Transmural Nerve Stimulation

Both dog and sheep cerebral arteries respond to TNS with a contraction, but the characteristics of these responses are not identical (Fig. 1). The con-
contractile response of dog cerebral vessels increases with increasing frequency and is blocked with guanethidine. After smooth muscle tone is increased with serotonin, TNS produces only a small contractile response at 16 Hz. In the example shown there was no dilator response to TNS, but in some dog cerebral vessels a small dilator response to TNS was seen under similar conditions. In the sheep, on the other hand, the contractile response to TNS does not increase with stimulation frequency beyond 4 Hz. Furthermore, after TNS ceases, a further contractile response can be seen, and is particularly prominent at 8 and 16 Hz. Once again guanethidine blocks the contractile response to TNS, confirming its sympathetic nature. However, after smooth muscle tone is produced by treatment with serotonin, an additional, nonadrenergic dilator response to TNS can be seen. Thus the response to TNS of sheep cerebral arteries in the absence of drugs is composed of two simultaneous components: a contractile response superimposed on a dilator response. Evidently the transmitter for the dilator response is more short-lived, for after the cessation of TNS the constrictor response dominates.

Effect of TTX

Both constrictor and dilator responses to electrical stimulation at a pulse duration of 0.3 msec and voltage of 15 V are due to stimulation of intramural nerves. This was confirmed in each case with TTX, which blocks activation of nerves but will not block direct activation of smooth muscle itself (Gershon, 1967). Contractile responses to TNS in both dog and sheep cerebral arteries are blocked with TTX (Fig. 2). Figure 3 shows that the dilator response to TNS also is blocked by TTX (also see Fig. 13). In the experiment shown in Figure 3, responses to a longer pulse duration of 1 msec also were elicited. The dilator response to a pulse duration of 0.3 msec was blocked with TTX, but the contractile response to electrical stimulation at a pulse duration of 1 msec was not. This contractile response to electrical stimulation with a pulse duration of 1 msec is attributed to direct activation of smooth muscle.

**Figure 2** Effect of tetrotoxin (TTX, $6 \times 10^{-7}$ M) on contractile responses to TNS of dog (A) and sheep (B) artery segments. Duration of TNS is indicated by the length of the line under each response, and frequency of stimulation is indicated.

**Figure 3** Effect of tetrotoxin (TTX) on contractile responses of sheep basilar artery segment to electrical stimulation at a frequency of 4 Hz at two different pulse durations. The segment was pretreated with phentolamine ($5 \times 10^{-6}$ M) to block constrictor responses to nerve stimulation. Smooth muscle tone developed spontaneously.
Constrictor Response

Because dilator responses to TNS of dog cerebral arteries were very small or could not be measured, constrictor responses in this species could be examined in greater detail. A frequency-response curve for steady state responses to TNS is shown in Figure 4. The response to TNS reaches a maximum between 16 and 32 Hz, and half-maximal frequency is 5.7 Hz.

Since contractile responses to TNS in both dog and sheep cerebral arteries are blocked with guanethidine, they presumably are sympathetic in nature. It seems most probable that, as in other blood vessels, postsynaptic receptors for this response might be classified as α-adrenergic in type and would be blocked by phentolamine. Indeed, phentolamine blocked contractile responses to TNS of sheep cerebral arteries (Fig. 5B), but did not block dilator responses to TNS. However, as previously reported (Duckles et al., 1977; Muramatsu et al., 1977), contractile responses to TNS of dog cerebral arteries were not blocked by phentolamine, but actually were increased (Fig. 5A). As shown in Figure 6, phentolamine in a concentration of $10^{-7}$ M caused virtually no change in the frequency-response curve for the dog basilar artery. However, phentolamine ($10^{-6}$ M) caused a significantly increased response to TNS at 2 and 4 Hz and a change in the shape of the frequency response curve. A higher concentration of phentolamine ($10^{-5}$ M) depressed the response to TNS. This effect of phentolamine on contractile responses of dog basilar artery segments to norepinephrine. Developed force is plotted as a function of norepinephrine concentration. Means and standard errors (n = 7) are indicated.
Dilator Response

After blockade of the contractile response to TNS with guanethidine or phentolamine, sheep cerebral arteries relaxed in response to TNS, provided smooth muscle tone was present. In a few cases, vessel segments produced tone on their own, but in most cases it was necessary to add an agent such as serotonin, which would cause smooth muscle contraction. In the presence of tone, the frequency dependence of the dilator response could be seen (Figs. 8 and 9). With high frequencies of stimulation, the magnitude of relaxation produced by TNS was almost 90% of the total smooth muscle tone present.

To investigate the nature of the transmitter for the dilator response to TNS, the effects of several types of blocking agents were investigated. The effect of propranolol, a β-adrenergic blocking agent, on the dilator response to TNS is illustrated in...
Figure 10. Similar results were found in all three vessel segments studied: propranolol (10^{-6} \text{ M}) did not block the dilator response to TNS.

The possibility that acetylcholine is the transmitter for this dilator response also was considered. In all three vessels studied, atropine (10^{-5} \text{ M}) blocked the dilator response to exogenous acetylcholine (Fig. 11A), but the dilator response to TNS was not blocked with atropine (n = 4, Fig. 11B). As an alternative approach to the question of whether acetylcholine is the transmitter for this response, the effect of physostigmine, an inhibitor of acetylcholinesterase, was investigated. In four vessels studied, responses to acetylcholine were increased by physostigmine (10^{-6} \text{ M}) (Fig. 12B). However, dilator responses to TNS were not increased, even in the presence of a higher concentration of physostigmine, 10^{-5} \text{ M} (n = 4, Fig. 12A).

In the presence of guanethidine and smooth muscle tone, some dog cerebral vessels responded to TNS with a relaxation (Fig. 13). This response was not blocked by a high concentration of atropine, although it was blocked by TTX.

**Discussion**

The validity of this study depends on the assertion that transmural electrical stimulation elicits transmitter release from intramural nerves without...
stimulating smooth muscle directly. Several lines of evidence support this contention. In the present study, TTX was used to differentiate between excitation of nerves and direct activation of smooth muscle (Gershon, 1967; Bulbring and Tomita, 1967; Kao, 1966). Both constrictor and dilator responses to electrical stimulation with a pulse duration of 0.3 msec were abolished in the presence of TTX, confirming that these responses were due to activation of intramural nerves. In the presence of TTX, a longer duration pulse still could elicit contraction, and this effect was attributed to direct stimulation of smooth muscle. Furthermore, we have shown that constrictor responses to TNS were blocked with guanethidine, confirming that they are due to stimulation of sympathetic nerves. Other investigators, using similar electrode assemblies and stimulation parameters, have shown that constrictor responses to TNS of cerebral arteries of the rabbit disappear after removal of the superior cervical ganglion, reserpine pretreatment, cold storage, or addition of TTX (Lee et al., 1976). Dilator responses to TNS of cat cerebral arteries also were abolished by cold storage or treatment with TTX (Lee et al., 1978). Thus it seems reasonable to conclude that responses to electrical stimulation under the conditions of this study are due to excitation of intramural nerves.

In sheep cerebral vessels in the absence of blocking drugs, the response to nerve stimulation is a composite of both a constrictor and a dilator response. These two responses appear to have a somewhat different time course. After the cessation of stimulation, a large contractile response is seen (Figs. 1, 2, and 5), which suggests that the constrictor transmitter is more long-lived.

Dog cerebral vessels also showed both constrictor and dilator response to TNS, but the dilator response, when seen, was very weak. The absence of the dilator response in some vessels may have several explanations. Although these nerves may have been present in all vessel segments studied, the response may have been beyond the resolution of the recording system in some cases. Alternatively, since mongrel dogs were used, there may have been some variation in the effectiveness of this dilator system among individual dogs.

Constrictor Response

The hypothesis that contractile responses to TNS of both dog and sheep cerebral arteries are due to stimulation of sympathetic nerves seems justified. In both cases these responses were blocked with guanethidine, a drug that specifically blocks release of norepinephrine from sympathetic nerves (Boura and Green, 1965). Furthermore, it has been shown that $^3$H-norepinephrine is taken up by dog cerebral arteries and released during nerve stimulation (Muramatsu et al., 1977; Duckles and Oberhammer, unpublished observation). Some previous workers have failed to obtain responses to electrical stimulation from dog cerebral arteries (Toda and Fujita, 1973; Toda, 1977), but this may have been due to insufficient stimulus strength, since responses to electrical stimulation from dog cerebral arteries have been obtained by others (Muramatsu et al., 1977).

Frequency-response curves for the contractile response to sympathetic nerve stimulation could be obtained from dog cerebral arteries, since interference by a simultaneous dilator response was very small or entirely absent. The shape of the frequency response curve is similar to that which has been found for sympathetic nerve stimulation in arteries from other vascular beds, as is the frequency for half-maximal nerve stimulation (Bevan and Bevan, 1977; Bevan, 1977). However, dog cerebral arteries appear to be more responsive to sympathetic nerve stimulation than those of the rabbit. The half-maximal frequency is smaller and the maximal response larger than comparable values for the rabbit basilar artery (Bevan and Bevan, 1977).

Although the bulk of evidence suggests that constrictor responses to TNS are due to stimulation of sympathetic nerves, there appear to be important differences in the postsynaptic receptor mechanisms for this response. As one would predict, phentolamine blocks the contractile response to TNS of sheep cerebral arteries, but this is not true for cerebral arteries of the dog. In this species, contractile responses to TNS are increased by phentolamine (Fig. 5A), even though responses to exogenous norepinephrine are blocked competitively by the same phentolamine concentration (Fig. 7). A similar observation has been made in cerebral arteries of the rabbit (Lee et al., 1976). It is true that a higher concentration of phentolamine ($10^{-5} \text{ M}$) effectively blocked contractile responses to TNS (Fig. 6). However, the specificity of this high concentration is doubtful, since we have observed that phentolamine ($10^{-6} \text{ M}$) also will block contractile responses to serotonin (Duckles, unpublished observation). Phentolamine did not increase the size of the maximal response to TNS; rather, the frequency for half-maximal contraction appears to be shifted (Fig. 6).

It is possible that phentolamine at a concentration of $10^{-6} \text{ M}$ is more effective at presynaptic $\alpha$-adrenergic receptors than at postsynaptic receptors of dog cerebral vessels (Duckles et al., 1977; Muramatsu et al., 1977). Indeed, phentolamine increases the stimulation-evoked release of tritiated norepinephrine from dog cerebral arteries (Muramatsu, 1977; Duckles et al., unpublished observation), suggesting the presence of active presynaptic $\alpha$-adrenergic receptors in this tissue. One would have to conclude that phentolamine at a concentration of $10^{-5} \text{ M}$ does not effectively block the high concentration of norepinephrine in the region of the smooth muscle receptors, whereas presynaptic receptors located on adrenergic nerves are blocked. This implies significant differences in characteris-
The large magnitude of the constrictor response to TNS of dog cerebral arteries (Fig. 4) suggests that sympathetic nerves should have a profound effect on cerebral blood flow. Indeed, D'Alecy and Feigl (1972) have demonstrated a large decrease in cerebral blood flow with sympathetic nerve stimulation in dogs. However, in contrast to our findings, this effect of nerve stimulation was blocked by α-adrenergic blocking agents (D'Alecy, 1973). It is difficult to compare drug concentrations in vitro with in vivo doses, but it seems likely that the dose range used in D'Alecy's work (1–2 mg/kg, iv) may have been comparable to the in vivo concentration range of 10⁻⁷ m. Perhaps the specific range of α blockade may have been exceeded. An additional problem is that the validity of the methods used by D'Alecy and co-workers has been questioned (Traystman and Rapela, 1975). Furthermore, some workers, using other methods, have been unable to demonstrate such a profound influence of sympathetic nerves on cerebral blood flow in dogs (Heistad and Marcus, 1977; Heistad et al., 1977; Mueller et al., 1977). Measurements in vivo of the resistance of large arteries of the circle of Willis suggested that they did not constrict in response to sympathetic nerve stimulation (Heistad et al., 1977). This is not to say that an effect of sympathetic nerves on cerebral blood vessels could not be demonstrated; a significant change in cerebral blood flow could be demonstrated during hypertension, and sympathetic stimulation during hypertension had a protective effect on the blood-brain barrier. Although we have shown clearly that smooth muscle of pial arteries of both the dog and sheep can be constricted profoundly by sympathetic nerve stimulation, the physiological significance of this effect remains unclear.

**Dilator Response**

Both dog and sheep cerebral arteries showed evidence of a dilator response to TNS, but this response was much greater in the sheep vessels. The magnitude of this response in the sheep, in which contractile responses to TNS were almost neutralized by the dilator response, suggests that these nerves might well play an important role in the control of cerebral blood flow in some species.

Several lines of evidence can be marshalled to determine a likely candidate for the transmitter of this dilator response. Electron microscopy has been used to identify a type of nerve ending in the wall of cerebral blood vessels that is distinct from sympathethic nerve endings. Unlike adrenergic nerve terminals, which contain small granular vesicles, nerve endings that contain exclusively small agranular vesicles and do not accumulate 6-hydroxydopamine have been identified (Iwayama et al., 1970; Duckles et al., 1977). Although it has been assumed that such nerve endings are cholinergic, the validity of this identification is by no means certain (Daniel et al., 1977).

Histofluorescence methods have been used to show that acetylcholinesterase is present in the adventitia of cerebral blood vessels (Edvinsson et al., 1972). However, much more convincing evidence comes from recent measurements of the activity of choline acetylase (Florence and Bevan, unpublished observation). This enzyme is present in cerebral vessels from a number of species, with particularly high activity in vessels from the sheep. Most peripheral vessels exhibit little enzyme activity. However, it is worth noting that high levels of this enzyme have been found in a non-innervated tissue (Welsh, 1977). In addition to this evidence for a cholinergic innervation of cerebral vessels, immunohistochemical techniques have been used to demonstrate a number of putative polypeptide transmitters in the walls of cerebral blood vessels (Larsen et al., 1976; Chan-Palay, 1977).

The in vitro studies of the dilator response suggest that acetylcholine is not the transmitter. Thus atropine does not block the dilator response to TNS (Fig. 11), and physostigmine does not potentiate it (Fig. 12). Pharmacological studies of a similar dilator response in cat cerebral arteries suggested that the transmitter was not acetylcholine, histamine, ATP, prostaglandins, gamma aminobutyric acid, dopamine, or serotonin (Lee et al., 1978). However, it is possible that acetylcholine, or another of the substances tested, is the transmitter, but postsynaptic mechanisms are unusual.

There is some support from in vivo studies for the concept of a dilator innervation to cerebral vessels (Forbes and Wolff, 1928; Cobb and Finesinger, 1932). Most recently, an increase in cerebral blood flow during stimulation of the left major petrosal nerve in the dog has been demonstrated (D'Alecy and Rose, 1977). In contrast to our results, this response was blocked partially with atropine. It is possible that the effectiveness of atropine reflects contamination from extracranial sources in the method used by these workers (Traystman and Rapela, 1975).

**Species Variation**

The advantage of studies of vascular smooth muscle in vitro is that standard conditions facilitate comparison of vessels from different locations as well as different species. Thus, differences in fundamental mechanisms of nerve-smooth muscle interactions can be elucidated.

Our studies have shown important quantitative and qualitative differences among species in the constrictor and dilator responses to nerve stimulation of cerebrovascular smooth muscle (Duckles et al., 1977). In some species (dog, monkey, rabbit) a constrictor response dominates, and dilator responses to nerve stimulation are very small. In
contrast, a dilator response dominates in cerebral vessels of the cat. Both these responses are important in cerebral vessels of the sheep. Not only are there differences in the relative importance of the two types of innervation, there are also qualitative differences in receptor mechanisms. Thus, phenolamine effectively blocks the constractor response to TNS of sheep cerebral arteries, but does not in the dog and the rabbit (Lee et al., 1976; Muramatsu et al., 1977).

These differences among species mean that any examination of the influence of nerves on cerebral blood flow will be dependent on the species used. It seems possible that some of the conflicting evidence about the role of cerebrovascular innervation may be resolved on this basis. However, the crucial question of the importance of these mechanisms for the control of cerebral blood flow in man remains to be answered.

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