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FUNCTIONAL ACTIVITY OF THE NORADRENERGIC INNERVATION OF LARGE CEREBRAL ARTERIES

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1 The role of the sympathetic innervation of cerebral arteries remains controversial. Therefore, the functional activity of the adrenergic innervation of the rabbit basilar artery was characterized and compared to that of a peripheral artery, the ear artery.

2 Both the ear artery and basilar artery have similar endogenous noradrenaline (NA) contents but accumulation of [^{3}H]-NA was considerably greater in the basilar artery.

3 Studies of tritium efflux after loading with [^{3}H]-NA demonstrated a considerable non-neuronal component since neither guanethidine nor tetrodotoxin completely blocked tritium efflux during nerve stimulation. Pretreatment with blockers of uptake$_2$ did not eliminate this problem.

4 Comparison of methods for estimating the functional activity of adrenergic nerves showed that, for the vessels studied, NA content and [^{3}H]-NA accumulation gave markedly different answers. Fractional release of [^{3}H]-NA did not correspond to fractional release of endogenous NA.

5 Adrenergic nerves innervating cerebral arteries are shown to have a high activity relative to a peripheral artery. While cerebrovascular sympathetic innervation may not play an important role in normal circumstances, its influence may be seen in pathological conditions.

Introduction

Under normal conditions, sympathetic nerves innervating cerebral arteries have little effect on cerebral blood flow (Alm & Bill, 1973; Traystman & Rapela, 1975; Heistad, Marcus, Sandberg & Abboud, 1977; Heistad, Marcus & Gross, 1978). However, during severe hypertension, sympathetic nerve stimulation decreases cerebral blood flow and reduces damage to the blood-brain barrier (Bill & Linder, 1976; Heistad *et al.*, 1978). Furthermore, sinoaortic deafferentation which produces hypertension and also activates sympathetic pathways has similar effects on the cerebral circulation (Gross, Heistad, Stuart, Marcus & Brody, 1979). This recent evidence that sympathetic innervation of cerebral blood vessels may play an important role in preventing hypertensive damage to the blood-brain barrier underscores the importance of a systematic characterization of the sympathetic neuroeffector mechanism of cerebral arteries.

Previous studies have shown that cerebral blood vessels *in vitro* contract when intramural sympathetic nerves are stimulated (Lee, Su & Bevan, 1976; Muramatsu, Ikushima & Fujiwara, 1977; Duckles, 1979a). Postsynaptic adrenoceptors have been characterized (Edvinsson & Owman, 1974; Duckles & Bevan, 1976), but little work has been done to analyze the functional activity of adrenergic nerves. Release of tritium after labelling with [^{3}H]-noradrenaline ([^{3}H]-NA) has been used to identify presynaptic adrenoceptors (Muramatsu *et al.*, 1977; Duckles, 1979b) and cholinoceptors (Edvinsson, Falck & Owman, 1977) in cerebral arteries. However, in the light of recent reports that field stimulation-evoked release of [^{3}H]-NA is in part of extraneuronal origin (Schrold & Nedergaard, 1977) it is important to determine whether [^{3}H]-NA efflux is a valid representation of endogenous NA release. Recent development of techniques for measuring NA has made it possible to measure release of endogenous NA from cerebral arteries (Duckles & Rapoport, 1979). Therefore, in the present paper, results using [^{3}H]-NA labelling to measure transmitter release are compared with measurements of endogenous NA release obtained under similar conditions in the same laboratory.

[^{3}H]-NA accumulation was measured as a further index of functional nerve capacity and all these measurements compared with previous measurements of NA content. For comparison with peripheral arteries, parallel studies were done on the rabbit ear artery, a well innervated vessel (Bevan, Bevan & Duckles, 1980). Thus results of four independent approaches to characterizing the noradrenergic inner-

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vation of blood vessels are synthesized; these include measurements of endogenous NA content and release as well as [^{3}H]-NA accumulation and efflux.

Methods

Adult white rabbits (2 to 3 kg) of either sex were stunned by a blow on the anterior portion of the head and exsanguinated. The basilar and central ear arteries and thoracic aorta were carefully dissected out and placed in Krebs bicarbonate solution equilibrated with 95% O_2 and 5% CO_2. The composition of Krebs solution was (mM): Na^+ 148, K^+ 5.1, Ca^{2+} 1.6, Mg^{2+} 1.2, Cl^- 127, HCO_3^- 26, SO_4^- 1.2, glucose 11, disodium ethylenediamine tetraacetate 0.027 and ascorbic acid 0.11.

[^{14}C]-*sucrose accumulation*

Segments of the basilar and ear arteries and the thoracic aorta were incubated in Krebs solution at 37°C for 60 min. This was followed by incubation for 60 min in Krebs solution containing [^{14}C]-sucrose (Amersham/Searle, 610 mCi/mmol) in a concentration of 1.3 μM. Tissues were then briefly rinsed in fresh Krebs solution, blotted and weighed on a Cahn electrobalance. Tissues were solubilized in 0.5 ml soluene-100 (Packard), and radioactivity of solubilized tissues and a bath aliquot were determined using liquid scintillation counting. Accumulation was calculated as ml of bath fluid cleared per wet wt. of tissue.

[^3H]-*noradrenaline accumulation*

Accumulation of [^{3}H]-NA was measured in a manner similar to that described above for sucrose accumulation with the following differences: Incubation in [^{3}H]-NA was preceded by a 60 min equilibration period. To measure non-neuronal accumulation, cocaine (10^{-4} M) was present during the last 30 min of the equilibration period and during incubation with [^{3}H]-NA (Su, Duckles & Florence, 1977). Tissues were incubated with (−)-[7,8 ^{3}H]-noradrenaline (New England Nuclear, 27.7 Ci/mmol) in a concentration of 10^{-8} M. Tissues were exposed to [^{3}H]-NA for periods varying from 5 to 180 min. Neuronal accumulation was calculated as (total accumulation − accumulation in the presence of cocaine).

[^3H]-*noradrenaline efflux*

The entire basilar artery or a 1 cm length of the ear artery were each cut into a spiral strip and suspended by threads between two parallel platinum electrodes (60 mm length, 1 mm apart) as previously described (Su & Bevan, 1970). The upper end of the strip was attached to a Statham strain gauge (G10b, 0.15 oz) for isometric recording of force on a potentiometric strip chart recorder. Arterial strips were stretched to a resting tension of 1 g, and an initial equilibration period of 60 min was allowed. The strips were then incubated in Krebs solution containing (−)-[7,8-^{3}H]-NA (New England Nuclear, 27.7 Ci/mmol) in a concentration of 10^{-7} M for 90 min. Strips were then superfused with pre-warmed and oxygenated Krebs solution at 3 ml/min for a further 60 min. At the end of this washout period, transmural electrical stimulation was delivered in three periods for 60 s each. These three stimulation periods were at 60, 90 and 120 min after the start of washout. Superfusate samples were collected at 1 min intervals, 4 before each stimulation, 1 during, and 5 after.

Transmural electrical stimulation was delivered across the electrodes via a stimulator (Grass S-88) and coupling device to provide a low source impedance (Duckles & Silverman, 1980). Stimulation parameters (0.3 ms, 6 V across the electrodes) were selected to achieve supramaximal nerve stimulation.

The efflux of tritium was estimated by measuring tritium content of a 1 ml aliquot of each sample by liquid scintillation spectrometry. Counting efficiency was estimated by the channels ratio method. Stimulation-evoked tritium efflux was calculated as the total amount of tritium released minus the resting (pre-stimulation) tritium efflux. Stimulation-induced efflux of tritium during the second (S_2) and third (S_3) periods of stimulation is expressed as a percentage of efflux during the initial stimulation (S_1).

At the end of the experiment, tissues were solubilized in 0.5 ml soluene-100 (Packard) and tritium remaining in the tissue was measured. The fractional release of tritium per stimulation pulse was calculated using the following formula:

$$\frac{\text{stimulation evoked efflux (d/min)}}{[\text{tissue d/min} + \text{total efflux (d/min) after stimulation}] \times \text{number of pulses}}$$

Drugs used

The following drugs were used: guanethidine sulphate (Ciba), tetrodotoxin (Calbiochem), (±)-normetanephrine HCl (Sigma), desoxycorticosterone (Aldrich

Table 1 Summary of parameters related to noradrenergic innervation of rabbit blood vessels

		[^3H]-NA		Endogenous NA	
	[^{14}C]-sucrose uptake (ml cleared/g)	Neuronal‡ accumulation (ml cleared 5 min^{-1} g^{-1})	Stimulation-evoked efflux (Fractional release/ pulse ($\times 10^{-5}$))	NA content* ($\mu g/g$)	Stimulation-evoked† release (Fractional release/ pulse ($\times 10^{-5}$))
Basilar artery	0.3 ± 0.04 (3)	17.4 ± 2.2 (4)	8.53 ± 1.06 (9)	2.5 ± 0.5	9.7
Ear artery	0.32 ± 0.05 (3)	3.26 ± 0.25 (8)	15 ± 0.3 (3)	2.1 ± 0.2	1.9
Thoracic aorta	0.36 ± 0.05 (3)	1.17 ± 0.50 (8)	—	0.78 ± 0.08	—

* R. Rapoport, personal communication; † Duckles & Rapoport, (1979); ‡ [^{3}H]-NA = 10^{-8} M
Figures in parentheses indicate the number of measurements.

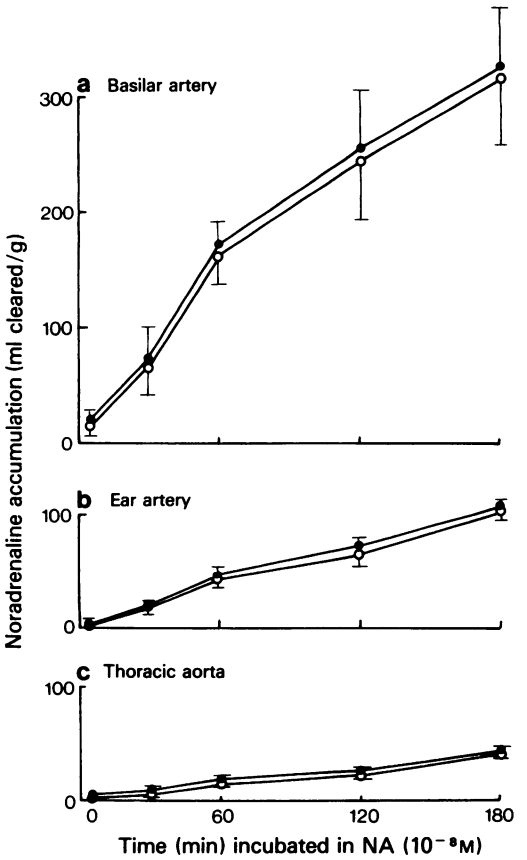

Figure 1 Accumulation of [^{3}H]-noradrenaline [^{3}H-NA] (ml bath cleared/g tissue wet wt) as a function of time of incubation in [^{3}H]-NA (10^{-8} M). Total (●) and neuronal (○) accumulation are shown for three vessels as indicated. Means (n = at least 5) and standard errors are shown.

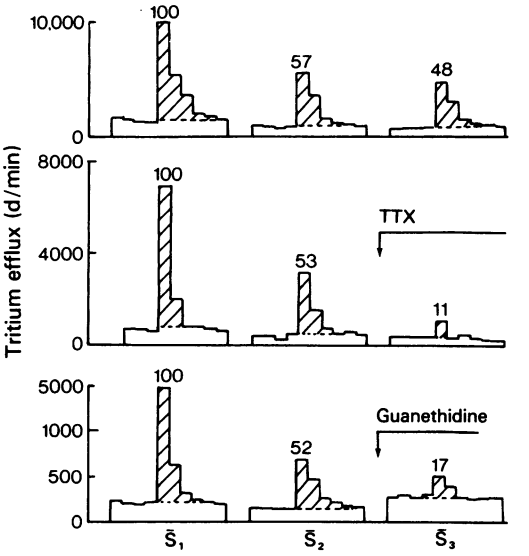

Figure 2 Efflux of tritium from the rabbit basilar artery after loading with [^{3}H]-noradrenaline ([^{3}H]-NA). Efflux for three stimulation periods (8 Hz) is shown S_1, S_2 and S_3. Hatched portions indicate the stimulation-evoked efflux (efflux above spontaneous levels). Results from three basilar arteries are illustrated; drugs were administered during S_3 in the following concentrations: tetrodotoxin (TTX, 10^{-6} M) and guanethidine (5×10^{-6} M). Numbers above each set of columns indicate the stimulation-evoked release as % of release during S_1.

Chemical), (−)-noradrenaline bitartrate (Sigma) and cocaine (UCLA pharmacy).

Results

Differences in [^{14}C]-sucrose accumulation among the three tissues studied were not statistically significant ($P > 0.1$, Table 1). However, significant differences were found in both total and neuronal [^{3}H]-NA accumulation (Figure 1). With a [^{3}H]-NA concentration of 10^{-8} M, in all three tissues non-neuronal accumulation was only a small percentage of total accumulation (shown by difference between solid and open circles, Figure 1). With a NA concentration of 10^{-8} M, NA accumulation was nearly linear up to 180 min (Figure 1).

Stimulation-evoked tritium efflux from the basilar artery after loading with [^{3}H]-NA is shown in Figure 2. As previously found (McCulloch, Bevan & Su, 1975; Schrold & Nedergaard, 1977), stimulation-evoked efflux of tritium is high during the first stimulation period and decreases in subsequent periods (Figures 2 and 3). When tritium efflux is calculated as the fraction of total tritium in the tissue released with each pulse, there is a marked decrease between the first and second stimulation periods (Figure 3). However, fractional release/pulse remains constant for the third stimulation.

Although treatment with guanethidine or tetrodotoxin completely eliminates contractile responses to transmural nerve stimulation (Lee *et al.*, 1976; Duckles, 1979a), the increase in tritium efflux above baseline levels was not totally abolished by these agents in 6 vessels studied (Figure 2). A similar observation was also made in the rabbit ear artery.

As shown in Figure 4, the fraction of total tritium released per pulse increased when stimulation fre-

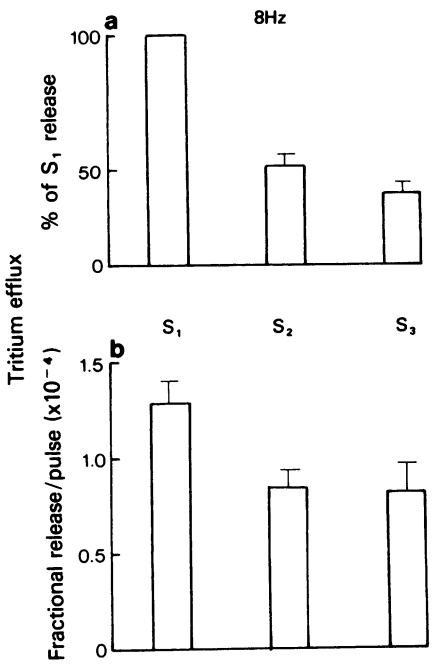

Figure 3 Stimulation-evoked tritium efflux from the rabbit basilar artery after loading with [^{3}H]-noradrenaline ([^{3}H]-NA). Efflux for three successive stimulation periods (8 Hz) is shown. Data are expressed in two ways: as % of release during the first stimulation period (a) and as the fraction of total tissue content released per pulse (b). Values shown are the means and standard errors of three experiments.

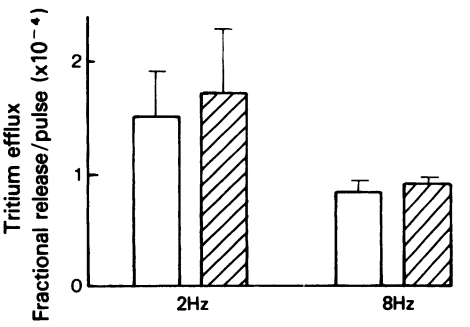

Figure 4 Stimulation-evoked tritium efflux from the rabbit basilar artery after loading with [^{3}H]-noradrenaline ([^{3}H]-NA). Efflux for the second stimulation period is expressed as the fraction of total tritium released per stimulation pulse. Results for stimulation frequencies of 2 and 8 Hz are shown in the absence of drugs (open columns) and in the presence of cocaine (10^{-5} M) and desoxycorticosterone (4×10^{-5} M) (hatched columns) which were added after the first stimulation period. Values shown are means and standard errors of 3 experiments.

quency was lowered from 8 Hz to 2 Hz. However, addition of cocaine and desoxycorticosterone to block neuronal and extraneuronal uptake had no effect on total tritium efflux at either frequency (Figure 4). It has been suggested by Schrold & Nedergaard (1977) that electrical stimulation releases tritium from extraneuronal storage sites, possibly corresponding to the uptake$_2$ sites described by Iversen (1967). Therefore, the basilar artery was pretreated during the [^{3}H]-NA loading period and throughout the rest of the experiment with desoxycorticosterone or normetanephrine to block uptake$_2$ sites. However, as shown in Figure 5, blockade of these extraneuronal uptake sites did not prevent the rise in stimulation-evoked efflux in the presence of tetrodotoxin or guanethidine, nor did it prevent the greatly increased efflux during the first stimulation period.

Table 1 compares results for [^{3}H]-NA accumulation and efflux with measurements of endogenous NA content and stimulation-evoked release of endogenous NA. As can be seen, although values for stimulation-evoked transmitter release using the two different methods are similar for the basilar artery, they are not the same for the ear artery. The ratio of NA content in the basilar and ear arteries is close to 1, while the ratio of neuronal accumulation in these two vessels is 5.3. The ratio of stimulation-evoked NA release (basilar/ear) is close to 5.

Discussion

A number of arguments can be marshalled to suggest that accumulation of [^{3}H]-NA is not a good index of the density of adrenergic innervation. It has been suggested that NA metabolism may significantly alter the amount of NA accumulated, and the influence of this could vary from tissue to tissue. It has been shown that, of the total amount of NA processed by the rabbit aorta during a 60 min incubation in 0.2 μM NA, 25% remained in the tissue (Takimoto, Cho & Schaeffer, 1977). While this may suggest that neuronal NA accumulation would be significantly affected by metabolism, in these experiments treatment with cocaine to block neuronal uptake did not significantly decrease the amount of NA metabolites released into the bathing solution. Thus, most of the NA metabolized by the tissue interacts with extraneuronal uptake sites. At any rate, incubation with low concentrations of NA and for short time periods should minimize the contribution that metabolism makes to neuronal NA accumulation (Graefe, 1976). In the present work, at the shortest incubation time used (5 min) and a low concentration of NA, there was a large difference in

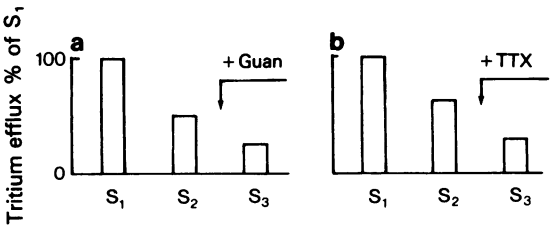

Figure 5 Effect of pretreatment during [^{3}H]-noradrenaline ([^{3}H]-NA) loading with blockers of extraneuronal uptake on stimulation-evoked tritium efflux. Two representative experiments are shown: (a) pretreatment with desoxycorticosterone (4×10^{-5} M) and (b) pretreatment with normetanephrine (10^{-5} M). Guanethidine (Guan, 5×10^{-6} M) was added to tissue (a) before the third stimulation period (S_3) and tetrodotoxin (TTX, 10^{-6} M) was added to tissue (b) before S_3.

NA accumulation between the three arteries studied; the ratio of NA accumulation was 5.34:1:0.36 for the basilar artery, ear artery and thoracic aorta, respectively. The validity of this measurement as a reflection of the density of neuronal uptake sites is further supported by the observation that accumulation of [^{3}H]-metaraminol shows similar ratios in these three vessels (unpublished observations). It has been suggested that accumulation of metaraminol, which is not metabolized, would provide a more appropriate measure of nerve density than NA (Herman & Graefe, 1977).

Release of NA during nerve stimulation might possibly be a more reliable parameter for estimating the functional activity of perivascular adrenergic innervation. The most common approach to measuring NA release has been to label transmitter stores by incubation with [^{3}H]-NA. However, it has recently been suggested by Schrold & Nedergaard (1977) that stimulation-evoked release of tritium in such preparations does not entirely originate from neuronal stores, as has previously been assumed. Results from the present experiments support this concept. Neither tetrodotoxin nor guanethidine completely blocked the stimulation-evoked increase in tritium release in spite of the fact that these drugs completely blocked contractile responses to transmural electrical stimulation (Lee *et al.*, 1976; Duckles & Silverman, 1980). Furthermore, unlike release of endogenous NA (Duckles & Rapoport, 1979), tritium release falls off sharply after the first stimulation period. Contrary to suggestions made by Schrold & Nedergaard (1977), pretreatment during the labelling period with blockers of uptake$_2$ did not alter this situation (Figure 5). This suggests that tritium released during electrical stimulation comes at least partly from extraneuronal sites distinct from uptake$_2$ sites.

An even more important issue, though, is whether these difficulties with the [^{3}H]-NA labelling method of measuring NA release have a substantial effect on the validity of the results obtained. That this is an important problem can be seen when results obtained from tritium efflux are compared with measurements of endogenous NA release obtained under very similar conditions (Table 1). When calculated as fractional release/stimulation pulse, values for NA release with these two methods are fairly similar for the basilar artery whereas values for the ear artery are not at all the same. This suggests that the relationship of pools labelled with [^{3}H]-NA, the pool of NA subject to release, and endogenous NA stores may be markedly variable in different blood vessels. Thus, results obtained when tissues are labelled with [^{3}H]-NA must be carefully considered in the light of this problem.

It seems reasonable to accept stimulation-evoked release of endogenous NA as inherently the most reliable of the methods used to estimate functional activity of adrenergic nerves. In that case, it is interesting that accumulation of [^{3}H]-NA is a better predictor of functional nerve activity than is NA content. Of course, there is more than one explanation for the differences seen between the ear and basilar arteries. Higher fractional release of NA in the basilar artery could mean that each adrenergic varicosity has the same NA content but releases more transmitter with each pulse than varicosities in the ear artery. On the other hand, it could mean that each varicosity in the basilar artery has a lower NA content but releases the same amount of NA per pulse as in the ear artery. More work needs to be done to sort out the relationships between NA content, NA released, and NA accumulation.

No matter what the explanation is for the high fractional release of NA in the basilar artery, it is clear that sympathetic nerves innervating cerebral arteries are functionally active. These nerves have a high NA content, and transmitter is accumulated and released by nerve stimulation in large quantities. As suggested previously (Duckles & Bevan, 1976; Duckles, 1979a), the relatively small responsiveness of cerebrovascular smooth muscle to sympathetic nerve stimulation can probably be accounted for by the unusual postsynaptic mechanisms seen in this tissue. Presynaptically this tissue has a dense and active adrenergic innervation. While this innervation may play little role in normal circumstances, its ability to function may come into play in pathological situations, such as extreme hypertension.

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