Movement of Norepinephrine through the Media of Rabbit Aorta

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ABSTRACT

The entry and movement of norepinephrine through the isolated rabbit thoracic aorta has been investigated using an isotopic frozen section technique. After 1-minute exposure of the intimal surface of a helical strip to Krebs bicarbonate solution containing tritiated norepinephrine (3H-NE), the 3H material was distributed throughout the thickness of the wall. The highest concentration occurred at the innermost layer of the media and gradually decreased toward the adventitial side. The shape of the tritium distribution curves was not significantly changed by previous treatment of aortic strips with either cocaine, phenoxybenzamine or the MAO and COMT inhibitors, pargyline and tropolone, respectively. Temperature reduction to 27°C had no significant effect on the tritium distribution curves. The apparent diffusion coefficient for 3H-NE calculated for movement into the extracellular (inulin) space of the media was 7.29 x 10^{-9} cm²/sec. This relatively slow rate of diffusion is probably due to physical barriers such as elastic lamellae within the tunica media.

ADDITIONAL KEY WORDS

catecholamine smooth muscle
cocaine isotopic frozen section technique MAO and COMT inhibitors
phenoxybenzamine
reduced temperature

A fraction of the norepinephrine released from the terminal effector plexus in the blood vessel as a result of neurogenic activity enters the tunica media to cause contraction (1). In vessels in which propagated activity is minimal or absent, the spatial and temporal distribution of transmitter in the tunica media is an important determinant of response (2). Since the amounts of transmitter released from the terminal effector plexus of elastic vessels, 52.8 pg • pulse⁻¹ • g⁻¹ wet weight (3), are too small to permit a direct study of transmitter entry and movement within the media during nervous activity, other approaches must be employed.

The entry and movement of NE through the media has been investigated by studying the passage of 3H-NE through the rabbit aorta in vitro from the intimal surface using a frozen section technique. For technical reasons, it was necessary to study movement in this direction rather than in the direction, adventitia to media. There is no reason to suspect that 3H-NE movement in the media in the direction intima to adventitia is different from that in the opposite direction.

Methods

All studies were performed on helical strips of rabbit thoracic aorta prepared and treated as previously described. The adventitial surface of each strip was coated with Vaseline so that 3H-NE entered only from its intimal surface. After exposing the aorta strips to 3H-NE contained in Krebs bicarbonate solution for a specific time, either 1 or 2 minutes, the strips were rapidly dipped once in Krebs solution containing an equivalent concentration of unlabeled NE, frozen and sectioned at 20μ parallel to the intimal surface in a cryostat. The approximate position of
The adventitia-media junction was established visually from the change in translucency and texture of the sections.

The appearance of each section was recorded. If part of the frozen artery specimen chipped away during cutting or other irregularities such as a fold appeared, the preparation was discarded. Since the last few frozen sections were rarely complete and contained both ice and arterial tissue, visual estimates of completeness of these were made. Mounts in which more than three successive sections contained less than 90% of the complete surface area of the artery were discarded. Each slide was transferred to a separate vial and its radioactivity determined by liquid scintillation spectrometry (4).

Accumulation of radioactivity in each section was expressed as a percent of the value expected if the extracellular space (inulin space) was saturated with \(^{3}H\)-NE. The inulin space for the tunica media was taken as 42% (Torbøk, Nedegaard and Bevan, unpublished observation). Counts from incomplete sections were expressed as the value expected if the section had been complete.

Chromatographically pure dl-7-\(^{3}H\)-norepinephrine from commercial sources, specific activity 6.8 and 6.88 c/mmole, was used at a concentration of 0.128 \( \mu \)g/ml (7.5 \( \times \) 10\(^{-4}\)m). In terms of pharmacological potency, this initiated an equilibrium contraction approximately 70 to 75% of maximum.

In general, preparations were equilibrated in Krebs bicarbonate solution maintained at 37°C for 60 to 90 minutes and then transferred to a bath containing Krebs solution with \(^{3}H\)-NE. The strip was previously treated with the following drugs in certain experiments before transference to tritium-containing solution: tropolone (10\(^{-4}\)M), 30-minute soak; pargyline (Eutonyl) (10\(^{-5}\)M), 30-minute soak followed by washing in Krebs solution for an additional 30 minutes; cocaine, concentration (10\(^{-3}\)M), 30-minute soak; phenoxybenzamine hydrochloride (3.3 \( \times \) 10\(^{-5}\)M), 30-minute soak followed by washing in Krebs solution for an additional 30 minutes.

Using the standard Student's t-test, a statistical inference of significance was made when \( P < 0.05 \).

**Results**

Aortic strips previously treated with cocaine (10\(^{-4}\)M) for 30 minutes were exposed to \(^{3}H\)-NE, 7.5 \( \times \) 10\(^{-4}\) (5 \( \mu \)g/ml) in Krebs bicarbonate solution for 60 and 120 seconds. They were then washed, frozen, sectioned, and the radioactivity of each section determined. The mean values of tritiated activity expressed as percent saturation of the inulin space from a total of 16 successful serial section studies are presented in Figure 1. Section designated

![Figure 1](http://circres.ahajournals.org/fig-1.png)
number 1 was that closest to the intima which was more than 90% complete.

As expected, the greatest accumulation of $^3$H-activity was found towards the intimal side of media. The concentration fell smoothly towards the adventitia. Since the mean count in those sections most distant from the intima is significantly higher than background, tritiated material must penetrate the arterial wall and emerge from the other side of this vessel within 1 minute.

The mean saturation of the media at 60 and 120 seconds was 32.7% and 51.4%, respectively. Thus, the half time for saturation was approximately 2 minutes.

**CALCULATED DIFFUSION COEFFICIENT**

The apparent diffusion coefficient for the penetration of the artery wall through the intimal surface by $^3$H-NE can be calculated by a method similar to that employed by Hill (5) and Creese (6). From the Fick equation for one dimensional drift in a concentration gradient of a single substance into plane sheet of tissue, the amount of $^3$H-NE entering the media of thickness $d$, at time $t$, expressed as a fraction $f$ (percent) of the equilibrium value, is given by

$$f = 1 - \frac{8}{\pi^2} \left[ e^{-\frac{4}{d^2}} + \frac{1}{3} e^{-\frac{4}{3d^2}} + \frac{1}{25} e^{-\frac{4}{25d^2}} + \cdots \right],$$

where $k = \frac{\pi^2}{4d^2}$ and $D'$ is the apparent diffusion coefficient.

The value for medial saturation after 1 or 2 minutes was used to calculate the apparent diffusion coefficient in the interspaces. The values for longer times were not determined experimentally, since it seemed likely a priori that the contribution of drug-insensitive or "nonspecific" binding might become proportionately more important with time.

Assuming a medial thickness of 240 $\mu$, the apparent diffusion coefficient in the interspaces (inulin space) for the tritiated material $7.29 \times 10^{-3}$ cm$^2$/sec (calculated from fractional saturation at 1 minute) and $9.97 \times 10^{-7}$ cm$^3$/sec (calculated from fractional saturation at 2 minutes). This coefficient was determined assuming a straight line diffusion pathway.
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(which is clearly not the case) and ignoring any other influence, as binding, charge interaction, etc., which might modify the rate of entry of the molecule.

**EFFECT OF MAO AND COMT INHIBITORS**

There is evidence that NE is metabolized in both the media and the nerve-containing adventitia (1, 7-10). To estimate the magnitude of potential errors resulting from breakdown of \(^3H\)-NE by degrading enzymes, vessels were previously treated with MAO and COMT inhibitors, pargyline \((10^{-8}M)\) and tropolone \((10^{-8}M)\), respectively, and then exposed to \(^3H\)-NE for 60 seconds (Fig. 2). These strips were not previously treated with cocaine. When comparisons were made between the mean radioactivity in corresponding sections from inhibitor-treated and control tissues, no significant differences were found, at the P < 0.05 level, even in the region of adventitio-medial junction.

**EFFECT OF COCAINE AND PHENOXYBENZAMINE**

The apparent slow rate of penetration may be the consequence of specific and nonspecific uptake or binding processes to cellular elements in the artery wall. Cocaine in high concentrations has been shown to depress NE binding in the media (11) and phenoxymethylamine not only blocks specific uptake of NE into neural and muscular tissue (11, 12) but inactivates many types of agonist receptors (13). In Figure 3, distribution curves for \(^3H\)-NE at 60 seconds after previous treatment with cocaine \((10^{-8}M)\) and phenoxymethylamine \((3.3 \times 10^{-5}M)\) are shown. The rate of entry of the tritiated material is unaffected by previous treatment with either of these agents. It will be noted that the distribution of radioactive material in tissues previously treated with cocaine in concentrations that depress specific NE transport mechanisms (Figs. 1 and 3) represents material in the extracellular space plus that bound nonspecifically to tissue constituents.

The radioactive material which accumulates in the tunica media after 1 or 2 minutes can be almost completely washed out. In preparations not previously treated with cocaine, a small peak of radioactivity, approximately 5 to 10% of that in the incubation medium, persisted in the region of adventitio-medial junction after washing for 60 minutes. As this peak did not occur in cocaine-treated material, it is possible that \(^3H\)-accumulation probably represents specific uptake into nerve terminals.

**EFFECT OF TEMPERATURE ON \(^3H\)-NE ENTRY**

The influence of temperature on NE movement was determined in four arterial strips by
measuring \(^3\)H-NE distribution after 60-second exposure at 27°C to the standard concentration of \(^3\)H-NE and also to \(^3\)H-NE 0.64 \(\mu\)g/ml (25 \(\mu\)c/ml), the pharmacologically equivalent concentration at this temperature (Török and Bevan, unpublished data). A reduction in temperature from 37°C to 27°C produced no significant change in the radioactivity concentration curves. The apparent diffusion coefficient in the interspaces at 27°C was \(10.14 \times 10^{-7}\) cm\(^2\)/sec (Fig. 4).

**Discussion**

The penetration of NE into the media has been studied using tritiated norepinephrine and a frozen section technique. The concentration distribution of NE within the vessel wall was determined after varying times of exposure of the intimal surface to a large reservoir of tritiated material. The general form of these distribution curves, their shape, and level of origin suggest that the factors determining \(^3\)H-NE distribution in the media in terms of 24-\(\mu\) increments are evenly distributed. These factors are not evenly distributed through the adventitia. It was for this reason, and because the uneven adventitial surface made the tissue more difficult to mount, on the cryostat, that the movement was studied in the direction intima to adventitia. Furthermore, the general form of the distribution curves (Fig. 1) and in particular their common origin from a level of concentration equal to that in the tissue both suggest that \(^3\)H-NE movement through the intima, the endothelium, and basement membrane is not slow in comparison to that through the media. It will be remembered that the tissue slice containing the intima was discarded from the data represented in Figure 1.

The apparent diffusion coefficient for \(^3\)H-NE in the interspaces is low in comparison with the free diffusion coefficient of similar sized molecules. The diffusion coefficient for glucose in water at 37°C is \(9.1 \times 10^{-6}\) cm\(^2\)/sec (14). Since the molecular weights of glucose (180) and \(^3\)H-NE (169) are similar, the apparent diffusion coefficient for \(^3\)H-NE in the interspaces of the media is in the order of 10 times lower than that expected in free aqueous solution. Creese (15) found that the apparent diffusion coefficient for sodium (radius 0.95 Å) in the interspaces of the rat diaphragm (2.1 \(\times\) \(10^{-6}\) cm\(^2\)/sec) was 8 that in saline solution. Krojević and Mitchell (16), studying acetylcholine, found a factor of seven in the same muscle.
The apparent slow rate of penetration cannot be accounted for by specific binding or uptake mechanisms, nor by binding to acceptor or receptor sites, at least those sensitive to phenoxybenzamine or cocaine. The role of nonspecific binding cannot be assessed at present, but it is probably negligible because of the short time of exposure of strips to 3H-NE. The consequences of the lengthened path of diffusion caused by the tissue elements per se (the extracellular space in this tissue is extremely complex in shape) cannot be allowed for quantitatively. Since drug diffusion through agar gel is similar to that in free solution (16), the presence of colloidal material in the extracellular space probably does not account for the low diffusion coefficient. The media contains many elastic lamellae which appear as practically continuous sheets with only occasional gaps (17) and are distributed throughout the wall thickness fairly uniformly (18). This provides a morphological basis for the slow rate of penetration of NE through the tunica media. Indeed, it would be surprising if these did not significantly affect penetration.

Calculations of the apparent diffusion coefficient for 3H-NE were based on a number of assumptions: first, that the tissues are sectioned parallel to the intima. The criteria of adequacy of tissue preparation (see above) were based upon the completeness of the last few sections of medial and subintimal tissue. This ensures that only tissues in which obliquity was less than approximately 1° to the plane of the cutting blade were accepted. Because of the thickness of the intima, we could never be certain of obtaining a complete section that contained only intimal tissue. Consequently, the concentration of tritiated material in tissue beyond the internal elastic lamina could not be determined. Because the calculated value of the diffusion coefficient is very sensitive to the value assigned to the thickness of the preparations (15), we restricted the data used for calculation of the diffusion coefficient to preparations providing 10 ± 1 medial sections. During exposure of the vessel to NE in vivo there is an increase in medial thickness (19). In our studies, the change of thickness was minimal, because of the short time of exposure to 3H-NE (maximal contraction of aortas is reached between 5 and 10 minutes) and because the strip once mounted on the tissue holder contracted under isometric conditions.

Second, the accuracy of our calculation depends upon the assumption that 3H-NE breakdown in the media is minimal under our experimental conditions. The distribution of tritiated material in the wall is uninfluenced by MAO and COMT inhibition, and by phenoxybenzamine which has been shown to reduce metabolite production (1). Since 3H-NE must break down into tritiated substances, significant breakdown would be expected to alter the shape of the tritium distribution curve. Degradative enzymes are present in the blood vessels (7, 9, 30, 21), and there is evidence of their activity (1, 7, 10, 22). For example, transmitter material overflowing from the pulmonary artery during nervous activity may contain up to 50% of metabolites (1). However, the conditions of experimentation in these two studies are not comparable and could presumably account for these inconsistencies. In the experiments described in this paper, the early phase of NE entry into the media in high concentration from a large reservoir is studied. It would seem feasible that under these circumstances either mechanisms of inactivation are swamped or else only an unimportant fraction of the total catecholamines contained in the wall have reached sites of metabolism.

Finally, the homogeneity of the extracellular space within the media is assumed. Experimental evidence suggests that the inulin space is evenly distributed through the media (Torok, Nedergaard and Bevan, unpublished data), at least when this tissue is measured in 24μ increments. A lamella is 16μ thick (18).

References


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