Evidence for Uneven Alpha-Receptor Distribution in the Rat Portal Vein

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ABSTRACT

The smooth muscle of the rat portal vein which supports propagation becomes supersensitive to exogenous l-norepinephrine after elimination of the vessel's intramural neuronal uptake mechanism. Such supersensitivity is generally associated with intimate neuromuscular connections. Since the adrenergic vasmotor fibers in the portal vein do not enter the muscle tissue, it has been proposed that muscle cells close to the nerve terminals must determine the response to norepinephrine. With the aim of elucidating the mechanism behind the prejunctional supersensitivity, the total uptake of labeled norepinephrine in the portal vein was analyzed and its distribution within the vessel wall was evaluated directly using isotopically labeled frozen sections. The total uptake of the portal vein, related to its endogenous norepinephrine content, was comparable with that in other vascular and nonvascular tissues. The $^3$H-uptake profile of the vessel wall showed that the neurogenic uptake was confined to the narrow adrenergic plexus between the longitudinal and the circular muscle layers. The neuronal uptake mechanism therefore could only influence the concentration of exogenous norepinephrine at muscle cells close to the nerve terminals. These results support the hypothesis that the prejunctional supersensitivity in the rat portal vein indicates that the alpha receptors are located on muscle cells in the vicinity of the adrenergic nerve terminals.

KEY WORDS cocaine prejunctional supersensitivity denervation phenoxybenzamine $^3$H-l-norepinephrine uptake propranolol

Inhibition of neuronal l-norepinephrine uptake in a tissue can be achieved by chronic denervation or by pharmacological blockade of the amine-uptake mechanism. Such inhibition can lead to increased local tissue concentrations during administration of exogenous norepinephrine, which in several adrenergically innervated organs has been shown to result in "prejunctional supersensitivity" (1).

In studies of isolated large blood vessels little or no prejunctional supersensitivity to norepinephrine has been found (2). This observation seems compatible with the characteristics of their innervation: the adrenergic ground plexus is confined to the adventitio-medial junction and the large neuro-muscular gap.

It has recently been shown that the longitudinal propagation-supporting vascular muscle of the rat portal vein develops a considerable prejunctional supersensitivity to norepinephrine after chronic denervation or cocaine treatment (3). In the same study, the pattern of innervation of the portal vein was analyzed by the fluorescence histochemical method. The longitudinal media layer is lined with an inner terminal ground plexus and an outer, rather sparse, network of adrenergic nerve elements. No adrenergic nerve terminals are found within the muscle layer. The outer nerve layer appears to be dense enough to prevent exogenous norepinephrine from entering the media via the adventitial side. Therefore, the high degree of prejunctional supersensitivity cannot be attributed to a reduction in the average concentration of norepinephrine in the greater part of the vascular muscle as a result of neurogenic norepinephrine uptake. Instead, it has been suggested that the alpha receptors which are functionally important for the response to exogenous norepinephrine are limited to muscle cells in the vicinity of the nerve terminals. Only at this site would an intact norepinephrine uptake mechanism be expected to reduce the exogenous amine concentration in the muscle layer.
The arguments for the proposed uneven alpha-receptor distribution in the portal vein are indirect and rest on a relative difference between the uptake capacity of the outer adrenergic plexus and the inner adrenergic plexus. The previously cited morphological analysis of the adrenergic innervation (3) does not provide quantitative information about the magnitudes of the neuronal amine uptake in these two plexuses. In the present study, the uptake of labeled norepinephrine in the isolated rat portal vein preparation was analyzed, and the distribution of the ^3^H-norepinephrine uptake within the vascular wall was evaluated directly using isotopically labeled frozen sections (4). The results provided further information about the neural mechanisms underlying prejunctional supersensitivity in the portal vein and supported the concept that the alpha receptors are predominantly located on muscle cells adjacent to the vasmotor nerve terminals in this propagating vascular smooth muscle.

**Methods**

Portal vein preparations from male rats of the Sprague-Dawley strain were used in this study. The rats were killed by a blow on the neck, and the portal vein was freed from surrounding tissue and cut open longitudinally before it was removed and used in the experiments.

*Isometric Recording of Mechanical Activity.—* Isolated preparations from 250-g rats were mounted longitudinally in an organ bath. Recordings of isometric contractile force were made using a Statham transducer and rest on a relative difference between the uptake capacity of the outer adrenergic plexus and the inner adrenergic plexus. The previously cited morphological analysis of the adrenergic innervation (3) does not provide quantitative information about the magnitudes of the neuronal amine uptake in these two plexuses. In the present study, the uptake of labeled norepinephrine in the isolated rat portal vein preparation was analyzed, and the distribution of the ^3^H-norepinephrine uptake within the vascular wall was evaluated directly using isotopically labeled frozen sections (4). The results provided further information about the neural mechanisms underlying prejunctional supersensitivity in the portal vein and supported the concept that the alpha receptors are predominantly located on muscle cells adjacent to the vasmotor nerve terminals in this propagating vascular smooth muscle.

*Denervation.—* Chronic denervation of the portal vein was performed as previously described (3). In this earlier study the completeness of the denervation was confirmed by transmural electrical stimulation and fluorescence histochemistry. The celiac-mesenteric ganglia and the postganglionic fibers on both sides were removed under sodium pentobarbital anesthesia (30 mg/kg, ip). The portal vein was studied in an acute experiment 5–7 days later.

*^3^H-norepinephrine Uptake and Distribution.—* Preparations from rats weighing approximately 500 g were used. Five vessels were studied in each uptake experiment. Each of these vessels was divided longitudinally into two equal preparations which were preincubated in Krebs solution at 37°C for 60 minutes. The solution contained calcium disodium ethylenediaminetetraacetate (EDTA) (2.4 X 10^-4 m) to stabilize the norepinephrine (5). During the last 30 minutes of this period and for the subsequent incubation period, one of the two paired tissues was exposed to phenoxybenzamine (10^-5 m). The preparations were then incubated in ^3^H-norepinephrine (6 X 10^-5 m, specific activity 5.8 c/mmole) for 1, 3, 10, 30, or 60 minutes. After a rapid rinse in l-norepinephrine (6 X 10^-3 m), the tissues were blotted between filter paper, weighed on a Cahn electrobalance, and digested in 0.5 ml of Soluene (Packard). The radioactivity in the samples and in aliquots of the incubation medium was measured by scintillation spectrometry. Total tissue uptake was expressed as milliliters of bath fluid cleared per gram wet weight of tissue (ml/g). For further details see reference 5.

The frozen section technique used for studying the distribution of labeled substances in the vascular wall (4) was employed in the modified form described in detail by Török and Bevan (6). The portal vein was opened longitudinally, and under a dissecting microscope the vessel wall was mounted in situ with its intimal side up on a flat plastic holder. The holder permitted unimpeded access of bath solution to both surfaces of the vessel. The two ends were stretched to the same transverse width while the tissue was tied to the holder. Then the mounted preparation was removed from the rat. Thus, all tissues were studied at approximately their in vivo length and under comparable transverse stress.

The preparations were preincubated for 60 minutes, then exposed to ^3^H-norepinephrine (6 X 10^-5 m, specific activity 6.6 c/mmole) for 10 minutes, rapidly rinsed in l-norepinephrine (6 X 10^-5 m), and frozen in isopentane at the temperature of liquid nitrogen with the intimal side against a flat steel surface.

In some experiments cocaine (10^-5 m) was added to the preincubation medium for the last 30 minutes and to the incubation medium.

The frozen tissue was fixed, intimal side down, to the tissue turret in a cryostat at -16°C and serially sectioned at 8μm. Each section was solubilized in 0.5 ml of Soluene, and its radioactivity was counted. Tissue weight was calculated from the area and the thickness of the section, assuming that the specific gravity was 1.05. During the sectioning it was difficult to distinguish between the frozen Krebs solution used for fixation and the first adventitial tissue slices. Normally it was possible to visually identify the last slice, which contained the intima. In denervated and cocaine-treated preparations, the uptake values of the last 20 tissue slices were utilized in the ^3^H-distribution profiles. In control preparations, the tissue section with the peak value, 15 slices on its adventitial side, and 4 slices on its intimal side were included (Fig. 3). In some control experiments, this procedure meant that the uptake values of the last few sections on the intimal side were discarded.

*Nature of the Tritiated Material in Portal Vein.—* Portal veins weighing 2.5-4.0 mg were isolated from six rats. After 60 minutes of conditioning in Krebs solution, ^3^H-norepinephrine was added to a final concentration of 8 X 10^-5 m. The veins were removed from the bath 10 minutes later, rapidly rinsed in Krebs solution containing l-norepinephrine (6 X 10^-5 m), blotted, weighed, and homogenized in 5% trichloroacetic acid.
After centrifugation, the supernatant fluid was analyzed for $^3\text{H}$-norepinephrine and its metabolites by adsorption on alumina and extraction with ethylacetate as described previously (7). The radioactive material unadsorbed by alumina was further extracted with ethylacetate after acidification to assay the deaminated O-methylated metabolites. Although no attempt was made to identify the metabolites, the ethylacetate extract of the acid eluate from alumina might have contained both 3, 4-dihydroxymandelic acid and 3, 4-dihydroxyphenylglycol and the extract from material unadsorbed by alumina might have contained 3-methoxy-4-hydroxymandelic acid and 3-methoxy-4-hydroxyphenylglycol, since all these metabolites along with normetanephrine have been found in rabbit aortas treated with $^3\text{H}$-dl-norepinephrine (8).

Differences between mean values were calculated using Student's t-test and were considered significant when $P < 0.05$.

**Drugs.**—The following drugs were used: L-norepinephrine bitartrate (Levophed, Winthrop Laboratories), $^3\text{H}$-L-norepinephrine hydrochloride (specific activity 5.8 and 6.6 c/mmole) (New England Nuclear Corporation), cocaine hydrochloride, phenoxybenzamine hydrochloride (Dibenzyline, Smith, Kline and French), and propranolol hydrochloride. All drugs were dissolved or diluted in Krebs solution prior to use.

The Krebs solution (5) was bubbled with 95% O$_2$-5% CO$_2$, and the temperature was kept at 37°C.

**Results**

The potentiation of the contractile responses to exogenous norepinephrine by cocaine was studied in one series of 12 experiments. Each preparation was exposed to norepinephrine ($6 \times 10^{-7}$M) during an initial 3-minute period. Norepinephrine was again applied 30 minutes later in the same concentration to a control group and to tissues which had been pretreated with cocaine ($10^{-6}$M) during the previous 30-minute period. The effect of norepinephrine was recorded for 30 minutes in both of these groups of preparations and in another group to which norepinephrine ($8 \times 10^{-7}$M) had been administered. Representative recordings from three of these experiments are illustrated in Figure 1.

Characteristic spontaneous activity was seen during the control periods. Administration of norepinephrine ($6 \times 10^{-7}$M) (Fig. 1, top recording) resulted in an initial tetanuslike contraction. Within a few minutes, a new pattern of activity followed; this pattern was characterized by intermittent contractions of high amplitude and long duration. The middle recording shows the initial 3-minute response to norepinephrine ($6 \times 10^{-7}$M) and the response to the same norepinephrine concentration in the presence of cocaine ($10^{-6}$M). The effect of norepinephrine was clearly potentiated: the amplitude and the pattern of maintained contraction closely resembled the response to norepinephrine ($6 \times 10^{-6}$M) shown in the bottom recording. The responses were quantified by integration and expressed as a percent of the initial 3-minute control response to norepinephrine ($6 \times 10^{-7}$M).

The total uptake of $^3\text{H}$-L-norepinephrine ($6 \times 10^{-7}$M) was studied as a function of time (Fig. 2). In normal Krebs solution, $^3\text{H}$-L-norepinephrine
Accumulation of $^3$H in the rat portal vein during incubation in $^3$H-$l$-norepinephrine ($6 \times 10^{-7} M$) as a function of time in the presence (open circles) and the absence (solid circles) of phenoxybenzamine ($10^{-6} M$). The mean uptake is expressed as a tissue-medium ratio. Vertical bars represent ±SE accumulated in the tissue at a progressively decreasing rate. In the presence of phenoxybenzamine ($10^{-6} M$), the rate of uptake was much lower and tended to decrease, particularly after 3 minutes of incubation. This initial phase of uptake presumably represented the occupation by $^3$H-$l$-norepinephrine of the extracellular space. The uptake at 10 minutes was 4.7 ml/g, and at the end of 1 hour it was 13 ml/g.

As a result of these studies, a tissue incubation time with $^3$H-$l$-norepinephrine ($6 \times 10^{-7} M$) of 10 minutes was chosen to elucidate the distribution of exogenous norepinephrine uptake in the vessel wall. After 10 minutes, the tracer had had sufficient time to evenly distribute itself within the extracellular space of the muscle. This time requirement was consistent with the rapid rate of $^3$C-sucrose equilibration in the rat portal vein (9). Furthermore, the cocaine supersensitivity (Fig. 1) was maintained for a period that well exceeded 10 minutes. The distribution of norepinephrine was studied under three experimental conditions, i.e., control (nine intact portal vein preparations), after cocaine ($10^{-6} M$) pretreatment for 30 minutes (six veins), and 5-7 days after surgical denervation (six veins).

In Figure 3A the $^3$H uptake in each of the $8 \mu$ thick sections of the vascular wall has been plotted against wall thickness to illustrate the $^3$H-distribution profile in two representative control preparations. In one vessel (squares), low uptake values were seen throughout the tissue with the exception of two peak values, approximately eight and five times higher, which presumably corresponded to the adrenergic plexus. The plexus is located between the wide longitudinal layer and the narrow circular smooth muscle layer. In the other preparation (crosses), the uptake values which prevailed throughout the tissue were higher than those in the first tissue, but the peak value was only two and a half times the average tissue uptake value. The profiles obtained in three of the control experiments were characterized by high peak values which were at least six times greater than the uptake in the remainder of the vascular wall. In the other six control experiments, the peak values were merely two to four times greater (Fig. 2). In no instance was an increased section uptake found which corresponded to the site of the outer adrenergic plexus in the rat portal vein (3). The superimposed $^3$H-$l$-norepinephrine distribution profiles for these nine control preparations are shown in Figure 3B. The mean section uptake value for the pooled data was 4.15 ml/g, which was in good agreement with the 10-minute value for total uptake (4.7 ml/g, Fig. 2).

The $^3$H activity of the portal vein extracts was 93.8 ± 1.7% $^3$H-$l$-norepinephrine, 4.1 ± 1.8% deaminated catechols (3, 4-dihydroxyphenylglycol and 3, 4-dihydroxymandelic acid), 2.4 ± 0.3% normetanephrine, and 0.7 ± 0.6% deaminated O-methylated metabolites (3-methoxy-4-hydroxyphenylglycol and 3-methoxy-4-hydroxymandelic acid) ($n = 6$ veins). These results are in contrast to those from the rabbit pulmonary artery in which the O-methylated metabolites predominated (7) and the rabbit aorta in which the degree of metabolism was very extensive after 60 minutes of incubation (8).

Cocaine treatment ($10^{-4} M$) and chronic denervation of the portal vein eliminated the peak uptake and reduced the uptake values throughout the vessel wall by approximately the same extent (Fig. 3). The uptakes under these circumstances approached those in the longitudinal muscle layer in tissues with high peak uptake values (Fig. 3).
Discussion

The degree of supersensitivity to exogenous norepinephrine seen after elimination of the neuronal uptake mechanisms has been shown to vary with the density of the adrenergic innervation and with the width of the junctional separation in different tissues (10). It is understandable that the proximity of the site of norepinephrine uptake to the individual muscle cells influences the consequence of that uptake on the local muscle norepinephrine concentration and thus on the contractile response. The crucial factor must be the distance between the sites of uptake and the receptors on the muscle cells which are functionally important for the production of a response. In the following discussion we shall point out that such receptors cannot always be assumed to be evenly distributed through the thickness of the vessel wall.

In most blood vessels the adrenergic nerve terminals are arranged in a two-dimensional plexus outside the media; consequently, most of the muscle cells are comparatively distant from the varicosities. In the rabbit aorta, Bevan and Verity (2) found no supersensitivity after mechanical removal of the nerve plexus. Only a small potentiation attributable to prejunctional mechanisms has been observed after cocaine treatment (2, 11). This observation is consistent with the paucity and the pattern of the innervation, the width of the neuromuscular separation in elastic arteries (12), and the finding that the muscle tissue in the aorta seems to exhibit the same responsiveness throughout the media (Bevan, unpublished observation). The perfused rabbit ear artery is more sensitive to exogenous norepinephrine when it is applied from the lumen than it is when the drug is administered via the surrounding bath fluid. This finding has been attributed to the efficient neuronal uptake of norepinephrine at the adventitio-medial junction, which reduces the norepinephrine concentration in the media when norepinephrine enters from the outside but not when it enters from the intimal side (13). These findings imply that the medial tissue in this muscular artery has a uniform sensitivity to exogenous norepinephrine throughout its thickness.

The longitudinal muscle of the rat portal vein develops a considerable prejunctional supersensitivity to exogenous norepinephrine after denervation or cocaine treatment (3). In organ bath experiments in which norepinephrine has access to the media from both sides of the vascular wall, the median effective dose (ED50) of norepinephrine decreases by a factor of 13 after chronic denervation (3-21 days). Cocaine (10^-6M) produces a slight further shift of the dose-response curve to the left, which might indicate an additional postjunctional effect (2, 3, 14, 15). It is apparent, however, that in the portal vein this drug in the concentration of 10^-4M exerts its action predominantly at the prejunctional level. In the present experiments, a comparable degree of cocaine supersensitivity was shown. The drug potentiated the response to norepinephrine (6 x 10^-5M) so that it equaled, both with regard to contractile pattern and amplitude, the response of control preparations to a 13 times higher norepinephrine concentration (8 x 10^-4M) (Fig. 1). The rat portal vein responds to norepinephrine in low and moderate concentrations with rhythmic contractions of increased amplitude and frequency (16, 17). At higher concentrations of norepinephrine the contractions tend to fuse, and a tetanus ensues. The contractile activity is closely associated with action potentials in the portal vein (16-18) so that the pattern of the mechanical response can be assumed to indicate the degree of membrane excitation. The present finding of a consistently tetanic response for 30 minutes to norepinephrine (6 x 10^-5M) in the presence of cocaine and to norepinephrine (8 x 10^-4M) in the control preparations suggests that the state of the membrane and thus the cocaine supersensitivity was maintained for the 30-minute period studied. The observed slow decrease in force development might then be ascribed to a "fatigue" phenomenon.
rather than to a reduced effective norepinephrine concentration.

The morphological features of the rat portal vein and its adrenergic innervation have been described by Johansson et al. (3). The media consists of an outer longitudinal muscle layer approximately 60μ thick and an inner circular muscle layer 15μ thick. The longitudinal muscle coat is interposed between an outer sparse plexus composed of predominantly nonterminal fibers and an inner terminal plexus with characteristics similar to the plexus found at the adventitio-medial junction of most vessels.

The fact that the muscle tissue under study, the longitudinal layer, is surrounded by two nerve layers might suggest that exogenous norepinephrine would be lost on entry into the muscle by neuronal uptake on both sides. If these uptakes are abolished by cocaine or denervation, the tissue would appear to become more sensitive. Such a mechanism would be analogous to that which pertains to the rabbit ear artery when norepinephrine is applied from the outside only and to the apparent supersensitivity to norepinephrine applied in this manner after cocaine administration. Such an explanation is rendered unlikely by our distribution profiles for 3H uptake in the vascular wall obtained using the frozen section method (Fig. 3). A very distinct peak of 3H accumulation was found at the site of the adrenergic plexus on the inside of the longitudinal layer. No increased uptake was found at the site of the outer adrenergic plexus. Since this peak uptake was absent after chronic denervation and completely blocked by cocaine treatment and since the 3H activity in the portal vein was represented almost entirely by 3H-l-norepinephrine, the profile peak must portray neuronal 3H-l-norepinephrine accumulation. Thus, the functionally important nerve terminals are confined to the ground plexus between the two muscle layers. This finding is compatible with the morphological pattern seen with fluorescence histochemistry (3).

The mean 3H-l-norepinephrine uptake from all the preparations in those sections corresponding to the longitudinal muscle layer was significantly reduced by denervation and by cocaine treatment. This fact suggests that no slices were completely free from neural elements. In the fluorescent study cited above (3), no fluorescent nerve structures were found in the longitudinal muscle layer. In control experiments, frozen portal vein preparations were sectioned transversely and examined histologically. It was found that the longitudinal muscle layer tended to wrinkle under the experimental conditions, presumably as a result of the transverse force exerted by the circular muscle layer. A similar tendency was apparent in transverse sections of the partially distended rat portal vein frozen in situ, (3, Figs. 2 and 3); moreover, the adrenergic ground plexus followed the wrinkled inner surface of the longitudinal muscle layer. Such a phenomenon would explain the presence of some neurogenic 3H-l-norepinephrine uptake throughout the vascular wall (Fig. 3). It would be expected that the flatter a preparation, the lower its medial uptake values and the higher the peak value (Fig. 3A).

The frozen section technique thus gave a less clear image of the 3H-l-norepinephrine distribution in the portal vein than that previously obtained in other blood vessels (19, 20), but the results strongly indicated that the functionally important adrenergic nerve terminals of the rat portal vein are restricted to a narrow network on one side of the muscle tissue. In this way, the vein is analogous to most other blood vessels. The inner plexus is a continuation of the innervation at the adventitio-medial junction of the superior mesenteric vein, which has no longitudinal muscle layer (3). The outer nerve fibers probably represent postganglionic axons which follow the portal vein but supply other sections of the splanchnic vasculature.

The capacity of the neuronal uptake mechanism in the portal vein can be compared with that reported for other tissues, if the uptake values (Fig. 1) are related to the endogenous norepinephrine content, which was 2.2 μg/g (21). Due to variations in experimental methods, such a comparison can only be approximate. The 60-minute clearance value (5.9 ml/μg norepinephrine) was of the same order of magnitude as that in the rabbit aortic strip (22). The phenoxybenzamine-sensitive uptake after 3 minutes of incubation (total uptake minus uptake in the presence of phenoxybenzamine, 3.4 ng 3H-l-norepinephrine/μg norepinephrine) corresponded to the uptake values reported by Trendelenburg et al. (23, Fig. 1) for the isolated cat nictitating membrane. The rate of neuronal uptake in the portal vein thus appeared to be similar to that in the aorta, which does not develop denervation supersensitivity (2), and to that in the nictitating membrane, in which a high level of sensitivity occurs (24).

It seems that the general localization and the capacity of the neuronal uptake mechanism are comparable in the rat portal vein and the rabbit aorta. Therefore, the fact that one vessel develops denervation supersensitivity but that the other does
not cannot be explained by a difference in any prejuncional mechanism.

The minimum neuromuscular separation in the rat portal vein is on the order of 0.1 μ (25), whereas it is 4 μ in elastic arteries (12). In two recent reports (26, 27), it has been pointed out that prejuncional supersensitivity has only been detected in tissues in which the neuromuscular separation is less than 0.1 μ. The muscle cell diameter in the portal vein is approximately 4 μ (28). Within its longitudinal muscle layer only those muscle cells next to the terminal ground plexus are closer to the site of the neuronal uptake mechanism than are the muscle cells in the media of the aorta. The very considerable prejuncional supersensitivity exhibited by the rat portal vein consequently implies that the response to exogenous norepinephrine must be determined predominantly by the local amine concentration which prevails in the immediate vicinity of the nerve terminals. Thus, the functionally important alpha receptors must be confined to "innervated" muscle cells and probably to that part of the muscle cell immediately subjacent to the varicosities.

Johansson et al. (3) have proposed that neurogenic transmitter and exogenously administered norepinephrine act on the same alpha-receptor population and that this population is located on a limited number of muscle cells, namely those immediately adjacent to the neural plexus. From this layer, excitation is myogenically propagated to the remainder of the media (29, 30). A receptor kinetic model of neuroeffector function in the rat portal vein based on the principle of a single receptor-agonist reaction for responses to both neurogenic and exogenous norepinephrine (31) favors the validity of this concept.

In a study of the adrenergic control of the peripheral vascular resistance in vivo, Click et al. (32) concluded that the physiological sites of alpha and beta receptors differ in the resistance vessels. These authors considered the possibility that at least a portion of the vasoconstrictor effect of injected norepinephrine was exerted via alpha receptors located in the vicinity of the neuroeffector junction. The present discussion suggests that, in any blood vessel in which the innervation is confined to the adventitio-medial junction and the media consists of several layers of muscle cells, prejuncional supersensitivity to intravascularly administered norepinephrine indicates that the functionally important alpha receptors are concentrated in muscle cells close to the adrenergic terminals.

References

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