Trophic Effect of Norepinephrine on the Rat Portal Vein in Organ Culture

PETER W. ABEL, ANGELO TRAPANI, OCTAVIO APRIGLIANO, AND KENT HERMSMeyer

SUMMARY Rat portal veins were maintained in organ culture to study the development of characteristic denervation changes and a possible trophic effect of the neurotransmitter norepinephrine (NE). Vessels maintained in organ culture for 2 days showed supersensitivity to NE and Ba\(^{2+}\), a more rapid rate of relaxation from a Ba\(^{2+}\) contracture, and partial depolarization of the myovascular cells. All of these changes except the quicker relaxation from Ba\(^{2+}\) contracture could be prevented by incubating the preparations in a NE-containing medium. This evidence suggests that functional changes in vascular muscle cells are caused by the removal of a trophic influence of NE, but can be prevented by NE replacement. However, the failure of NE in the culture media to prevent the increased rate of relaxation from Ba\(^{2+}\) contracture found after 2 days in organ culture suggests that NE is not the only trophic influence acting on the portal vein. In addition, incubation of veins in a NE-containing medium produced a marked subsensitivity to the contractile effects of NE, but not Ba\(^{2+}\), and thus possible desensitization of noradrenergic receptors. The data thus support a trophic role for NE in the rat portal vein.

Methods

Organ Culture

Portal-mesenteric veins from male albino rats (175–200 g) anesthetized with sodium pentobarbital (30 mg/kg, ip) were used in this study. The vessels were dissected aseptically, freed of surrounding tissue, removed from the animals, and pinned in an organ bath for in vitro denervation using 6-hydroxydopamine (6-OHDA) (Sigma) as previously described (Aprigliano and Hermsmeyer, 1976) with prevention of contracture due to released NE by 1 μM phentolamine (Ciba). Following the denervation procedure, the veins were placed in cardiovascular culture medium based on Eagle’s MEM (CVIE), containing 50% minimum essential medium (MEM, Kansas City Biological), 49% salts to complement Eagle’s MEM (CVIE-S) (composition in mm: NaCl, 130; NaHCO\(_3\), 28; NaH\(_2\)PO\(_4\), 0.5; KCl, 4; CaCl\(_2\), 2.3; MgCl\(_2\), 0.4; MgSO\(_4\).7H\(_2\)O, 0.4; HEPES, 13; glucose, 5.6), 1% fetal calf serum (Kansas City Biological), 4.0 mM 1-glutamine (Microbiological Associates), and 40 μg/ml gentamicin (Schering Corporation). The final composition was 330 mOsM with Ca\(^{2+}\) = 1.8 mM and K\(^+\) = 4.7 mM. All subsequent washing and incubation steps were performed using this medium at a pH of 7.4.

The veins were washed 3 times in separate sterile Falcon plastic culture dishes, transferred to sterile glass Petri dishes, stretched as close as possible to their in situ length, and fixed in place by means of sterile glass pins in paraffin. The flask containing the vessels were then placed in a tissue culture incubator at 37°C and maintained in an atmosphere of 5% CO\(_2\), 95% air. Control veins were removed for measurements after 1–2 hours in culture while cultured veins were removed for measurements after 2 days. Control veins would reveal any immediate effect of CVIE medium on the veins, but would not have time to show long-term effects. Approximately every 8 hours, beginning 5 hours after removal of the veins from the animals, three-fourths of the
incubation medium was removed from all culture dishes and replaced with fresh medium.

For those cultures to be exposed to L-norepinephrine, the bitartrate (Sigma) was prepared by diluting sterile drug solution containing 20 μM glutathione (Sigma) as antioxidant in sterile incubation medium to give a final NE concentration of 1 μM. Approximately every 8 hours, three-fourths of the incubation medium was removed from the culture dishes and replaced with the NE-containing medium. Although oxidation of NE takes place in the culture medium, experiments with the medium removed after 5 hours showed that it had an effect on freshly dissected portal veins equivalent to that of 10 nM NE. Cultured NE-treated veins were used in paired experiments with cultured veins not exposed to NE to determine possible trophic effects of NE.

**Pharmacological and Electrophysiological Measurements**

After 1 hour or 2 days of incubation, portal veins were mounted as 15-mm long strips in a muscle chamber. The preparations were continuously superfused without recirculation with ionic solution for mammals (ISM) (composition in mM: NaCl, 130; NaHCO3, 16; NaH2PO4, 0.5; KCl, 4.7; CaCl2, 1.8; MgCl2, 0.4; MgSO4, 0.4; HEPES, 13; glucose, 5.5). The ISM was maintained at 37°C, gassed with 95% O2, 5% CO2, and had a pH of 7.4. Recordings of isometric tension were made with Grass FT.03 transducers connected to a Grass polygraph. The initial tension applied to the portal vein preparations was 750 dynes/4 X 10^-3 cm^2. Methods of contractile data collection and analysis, including 2.5-minute drug exposures, computer analysis of tension-time integral, and concentration-response curves have been described elsewhere (Aprigliano and Hermsmeyer, 1977). Glass microelectrodes (40-80 MΩ) filled with 3 M KCl and suspended from 25-μm Ag wire were used to record membrane potential (Em). The maximum potential value measured during quiescent periods between spike bursts was taken as resting E m. Impalements were considered acceptable only if the reference potential and electrode resistance were stable and had the same value (± 2 mV or ± 2 MΩ) before and after an impalement, cell impalement was signaled by a sharp voltage change, and cell input resistance was 4-40 MΩ. For a more complete discussion of E m measurements, see Aprigliano and Hermsmeyer (1977) and Hermansmeyer (1976). All recordings were made after a period of equilibration of at least 1 hour in the muscle chamber. All EC50 values were calculated by geometrical mean distribution theory (Fleming et al., 1972). Statistical significance was tested by analysis of variance followed by the Bonferroni t-test for comparison of more than two groups (Morrison, 1976). All cases, the 0.008 confidence level (which is equivalent to a P < 0.05 t-test comparison of two groups) was accepted as significant.

**Results**

**Contractility of Denervated and Organ-Cultured Veins**

Portal veins kept in organ culture for 2 days, as well as 1-hour controls, exhibited spontaneous contractile activity consisting of intermittent contractions of varying amplitude, shape, and duration (Fig. 1). These spontaneous contractions, however, were of smaller amplitude than those observed in veins after only 1 hour in culture. Transmitter depletion by the in vitro 6-OHDA treatment was verified by lack of contractile responses to short duration, field stimulation pulses (0.3 msec at 10 Hz). In all denervated cultured veins, field stimulation elicited no response (Fig. 1).

**Denervation Supersensitivity**

The changes in NE sensitivity with time in organ culture and complete concentration-response curves are shown in Figure 2. After 2 days in organ culture, the EC50 for NE fell from the 1-hour control of 87 ng/ml to 47 ng/ml, which represents a 1.8-fold increase in sensitivity. Concentration-response curves obtained from veins incubated in NE-containing medium demonstrated decreased sensitivity.
by a significant increase in the mean EC\textsubscript{50} value to 166 ng/ml. The maximum contractions were obtained with NE concentrations between 3,000 and 10,000 ng/ml. The magnitudes of the tension-time integrals for maximum responses of control and cultured veins were not significantly different, showing that full contractility was maintained in culture. Concentration-response curves of the 2-day NE-free veins showed a significant shift to the left, whereas veins incubated in NE-containing medium showed a significant shift to the right.

The Ba\textsuperscript{2+} mean EC\textsubscript{50} and concentration-response curves from cultured portal veins are shown in Figure 3. Without NE, the Ba\textsuperscript{2+} sensitivity was increased by approximately 1.5-fold after 2 days. With NE-containing medium, the EC\textsubscript{50} values remained at control levels. The Figure 3 concentration-response curves for 1-hour control and 2-day NE curves overlap at all concentrations. Concentrations of Ba\textsuperscript{2+} between 10 and 30 mM induced maximal contractions similar to those obtained with maximal concentrations of NE, i.e., an immediate increase in tension interspersed with some spontaneous fluctuations, eventually fusing in a sustained contraction. A few minutes after Ba\textsuperscript{2+} was washed out of the preparations, this contraction subsided. Low concentrations of Ba\textsuperscript{2+} always induced an increase in spontaneous contractions of the veins.

Relaxation and Membrane Potential Alterations

The procedure employed for determining quickness of relaxation consisted of the three steps shown in Figure 4. (1) NE or Ba\textsuperscript{2+} (to give 80% of maximum contraction) was allowed to act on the preparations for 2.5 minutes. The time integral of the tension developed over this period of time was evaluated and taken as 100% contraction. (2) After the 2.5-minute exposure, the contractile agent was washed out of the preparations by continuous suffusion for 30 seconds. (3) Finally, the tension remaining again was measured by integration for a 2.5-minute period. Table 1 shows that the residual contractile activity after washing out NE was not significantly different from 1-hour controls (52%) in either the 2-day veins (66%) or veins maintained in NE-containing medium (44%). However, the NE-containing group relaxed to a significantly greater extent than the NE-free group. In contrast, the NE incubation did not alter the time course of relaxation from
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Table 1  Membrane Potential and Residual Contraction

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>2 day</th>
<th>2 day + NE</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Relaxation from E&lt;sub&gt;C50&lt;/sub&gt; NE ± se</td>
<td>52 ± 4</td>
<td>66 ± 4†</td>
<td>44 ± 3§</td>
</tr>
<tr>
<td>(n)*</td>
<td>(7)</td>
<td>(10)</td>
<td>(9)</td>
</tr>
<tr>
<td>% Relaxation from E&lt;sub&gt;C50&lt;/sub&gt; Ba&lt;sup&gt;2+&lt;/sup&gt; ± se</td>
<td>70 ± 42§</td>
<td>31 ± 6</td>
<td></td>
</tr>
<tr>
<td>(n)**</td>
<td>(8)</td>
<td>(9)</td>
<td>(10)</td>
</tr>
<tr>
<td>E&lt;sub&gt;m&lt;/sub&gt; ± se</td>
<td>-47 ± 1.2§</td>
<td>-43 ± 1.0‡</td>
<td>-47 ± 0.9§</td>
</tr>
<tr>
<td>(n)†</td>
<td>(6, 50)</td>
<td>(7, 60)</td>
<td>(7, 56)</td>
</tr>
</tbody>
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* Number of portal veins.
† Number of portal veins, number of impalements.
§ Significantly different from 2 day + NE.
|| Significantly different from 2 day.
|| Significantly different from control.

Ba<sup>2+</sup> contractures. The residual contractile activity after washing out Ba<sup>2+</sup> was significantly decreased in both 2-day veins (31%) and 2-day veins maintained in NE medium (24%) compared to 1-hour controls (70%).

Mean values of E<sub>m</sub> from controls and cultured portal veins are also shown in Table 1. Veins maintained in organ culture for 2 days developed a partial depolarization of approximately 4 mV. Vessels maintained in the NE-containing medium had E<sub>m</sub> values that were not significantly different from control. In addition, the NE-containing cultures were significantly different from the NE-free 2-day cultures. In all the incubated veins, spontaneous spikes underlying the observed contractile activity were recorded. The spontaneous spikes tended to be more closely spaced in the 2-day cultured veins, but bursts were shorter.

Discussion

The evidence presented shows that full contractile function of rat portal-mesenteric veins can be successfully maintained in organ culture, and supports a trophic role of the neurotransmitter NE in blood vessels. The cultured veins showed pharmacological and electrophysiological changes typical of denervation in the vascular muscle cells in vivo (Aprigliano and Hermmsmeyer, 1977). Evidence for a trophic action of NE was the prevention of some, but not all, of the postjunctional alterations by NE-containing medium.

We previously have described (Aprigliano and Hermmsmeyer, 1977) the pharmacological and electrophysiological changes that occur following in vivo denervation of the rat portal vein by 6-OHDA, which were: (1) a time-dependent increase in sensitivity to both NE and Ba<sup>2+</sup>, (2) a partial depolarization of the myovascular cells in supersensitive veins, and (3) prolongation of relaxation times following a NE contracture. To draw correlations between the present results and those previously obtained in our laboratory, it is necessary to demonstrate that incubation of the veins in culture medium produced no deleterious effects in the muscle cells. Evidence supporting this assumption is that the magnitude of the maximum tension integrals of cultured veins was not significantly different from those of 1-hour control preparations or non-cultured veins, whether animals had been vehicle treated or denervated in vivo with 6-OHDA (cf. Aprigliano and Hermmsmeyer, 1977). The values for 1-hour and 2-day denervated preparations from the present experiments are in good agreement with previous in vitro (Aprigliano and Hermmsmeyer, 1976) and in vivo (Aprigliano and Hermmsmeyer, 1977) values. The present NE E<sub>C50</sub> of 47 ng/ml vs. 40 ng/ml, the present Ba<sup>2+</sup> E<sub>C50</sub> of 1.3 mM vs. 1.38 mM, and the 4 mV vs. 4 mV depolarization all show the same 2-day denervation values whether the portal veins are in vivo (Aprigliano and Hermmsmeyer, 1977) or in organ culture.

The supersensitivity observed after 2 days in organ culture is likely to be due to changes in the muscle cells. The increased sensitivity to Ba<sup>2+</sup> shows agonist nonspecificity, which also suggests postjunctional supersensitivity (Fleming et al., 1973). The mechanism for the postjunctional supersensitivity in organ culture probably involves the 4-mV depolarization observed after adrenergic denervation here in organ culture or previously in vivo (Aprigliano and Hermmsmeyer, 1977). In addition, a similar depolarization of supersensitive tissues has been reported for heart (Taylor et al., 1976), vas deferens (Fleming and Westfall, 1975), and saphenous artery (Abel et al., 1976). The displacement of the E<sub>m</sub> toward a less negative value would be a facilitatory mechanism for NE, Ba<sup>2+</sup>, and other excitatory agents, because partial depolarization might be expected to poise the cells near threshold, if the threshold did not increase enough to compensate.

The elimination of nerve endings before organ culture allowed us to separate effects due to development of supersensitivity from the influences of remaining nerve terminals. In previous experiments, we placed portal veins in organ culture with-
out denervation, allowing the nerve endings to continue NE uptake and release. In those experiments, NE failed to prevent depolarization of the muscle cells (Hermsmeyer and Aprigliano, 1980). In the present experiments, we used the 6-OHDA method (Aprigliano and Hermsmeyer, 1976) to destroy rapidly the adrenergic nerve endings, thus preventing all of their influences on the muscle cells in culture. Since constant suffusion with phentolamine prevented α-adrenergic receptor stimulation by released NE during the 6-OHDA in vitro denervation (Aprigliano and Hermsmeyer, 1976), the portal veins in the NE-free-cultures were abruptly freed of NE stimulation before culturing began. The quick transition to an NE-free environment without exposure to a high NE concentration allowed the muscle cell changes to be observed with an optimum time course. In the present experiments, we noted that NE in the cultures prevented the depolarization as well as preventing the NE supersensitivity. Thus, the lack of prevention of depolarization by NE shown previously (Hermsmeyer and Aprigliano, 1980) probably was due to the influence of deteriorating nerve endings during the 2-day culture period. Elimination of the adrenergic nerve endings before culture began may also have been important in allowing the measurement of subsensitivity to NE in the NE-incubated cultures. The denervation and organ culture approach would appear to be practically ideal for the demonstration of muscle changes due to the abrupt termination of NE influence.

The data presented provide support for a trophic role of the neurotransmitter. Maintenance of the veins in a NE-containing medium prevented the appearance of the increase in sensitivity to NE and Ba2+. The partial depolarization was also reversed by the NE-containing medium. These data provide direct evidence that these changes may be directly related to the removal of NE from the cell’s environment by denervation. However, the mechanisms by which NE promotes this effect are not clear. The constant presence of neurotransmitter in the cell’s milieu could exert multiple actions involving membrane alterations. For example, Hermsmeyer and Robinson (1977) and Marvin et al. (1979) showed that cultured myocardial cells, free of any NE influence, have the highest NE sensitivity so far demonstrated, and rapidly desensitize after NE exposure. Cultured vascular muscle cells show similar very high sensitivity (Hermsmeyer and Aprigliano, 1980). In the present study, the subsensitivity to NE of portal veins incubated in NE-containing medium may also be due to desensitization following NE exposure. This desensitization would be caused by the maintenance of NE in the culture medium and would probably represent a mechanism constantly present during in vivo conditions.

On the other hand, NE incubation did not prevent the quicker relaxation following Ba2+ contrac-

ture found in 2-day cultured veins, which suggests that the lack of some other trophic factor in addition to the neurotransmitter also is important. Veins cultured with or without NE relaxed more quickly from Ba2+ contraction. In contrast, 2-day veins cultured without NE produced slower relaxation from NE contraction than veins cultured with NE, suggesting a different mechanism for relaxation from NE vs. Ba2+ contraction. NE incubation altered the relaxation mechanism from NE but not Ba2+ contracture. Support for the contribution of another trophic substance can be inferred from this and other available evidence. For example, other trophic factors could be conveyed to the muscle cells by a mechanism of orthograde axonal transport (Hofmann and Thesleff, 1972; Thoenen and Stockel, 1975).

In conclusion, these experiments have shown that maintenance of portal veins in organ culture produces supersensitivity of the preparations associated with pharmacological and electrophysiological changes in the muscle cells. The evidence suggests that the supersensitivity to NE and Ba2+ and membrane depolarization can be attributed to the removal of a trophic influence of the neurotransmitter.

References


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SUMMARY In an attempt to understand the way automatic cells in the sinus node (SN) control the cardiac rhythm, we studied extracellular electrograms recorded from the SN region in conscious dogs. A SN electrode, containing 48 silver terminals arranged 1.5 mm apart, was implanted over the node, and an indifferent electrode was implanted on the superior vena cava. Through terminals of the SN electrode paired with the vena caval electrode, "unipolar" electrograms were recorded at 100 μV/cm and with a time constant of 0.1 second. Low amplitude and low frequency deflections (dV/dt ≤ 20 mV/sec) which resulted from electrical activity of the node could be differentiated from the more rapid deflections due to atrial electrical activity. Electrical activity due to the inherent automaticity of what appeared to be groups of automatic cells was recognized as a slow negative-going diastolic slope followed by a slow negative-going, or negative and then positive-going, SN potential. Impulse propagation toward the SN electrode terminal in groups of automatic cells appeared as a slow positive-going deflection interrupting the diastolic slope. Adjacent groups of automatic cells located near the sites of earliest atrial activation discharged asynchronously before the earliest atrial activity; this suggests that multiple groups of automatic cells might initiate atrial activation. In addition to changes in rate and in location of the pacemaking groups of automatic cells, significant beat-to-beat variation in the sinoatrial interval contributed to the changes in atrial rate in "sinus arrhythmia." These studies provide a better understanding of SN function in conscious animals.
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Circ Res. 1980;47:770-775
doi: 10.1161/01.RES.47.5.770

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