Prejunctional Inhibition of Norepinephrine Release Caused by Acetylcholine in the Human Saphenous Vein

Duane K. Rorie, Nancy J. Rusch, John T. Shepherd, Paul M. Vanhoutte, and Gertrude M. Tyce

SUMMARY We performed experiments to determine whether or not acetylcholine exerts a prejunc-
tional inhibitory effect on adrenergic neurotransmission in the human blood vessel wall. Rings of
human greater saphenous veins were prepared 2 to 15 hours after death and mounted for isometric
tension recording in organ chambers filled with Krebs-Ringer solution. Acetylcholine depressed con-
tractile responses to electric activation of the sympathetic nerve endings significantly more than those
to exogenous norepinephrine; the relaxations caused by the cholinergic
transmitter
were antagonized
by atropine. Helical strips were incubated with [1H]norepinephrine and mounted for superfusion.
Electric stimulation augmented the fractional release of labeled norepinephrine. Acetylcholine caused
a depression of the evoked [H] release which was antagonized by atropine but not by hexamethonium.
These experiments demonstrate that, as in animal cutaneous veins, there are prejunctional inhibitory
muscarnic receptors on the adrenergic nerve endings in the human saphenous vein. By contrast, the

IT has been shown in a variety of isolated blood vessels of different species and in several perfused
vascular beds of the dog that, in the presence of sympathetic nerve activation, acetylcholine causes
relaxation of vascular smooth muscle cells in part because it exerts an inhibitory prejunctional effect
on the evoked release of norepinephrine (Vanhoutte, 1977; Shepherd et al., 1978; Vanhoutte and
Levy, 1980). The present experiments were de-
signed to determine whether or not acetylcholine
inhibits adrenergic neurotransmission in the human
blood vessel wall.

Methods

Source of Tissues

Segments 6–8 cm in length of the greater saphen-
ous vein were removed from the area of the medial
malleolus in cadavers donated to Mayo Foundation
for the purpose of medical science. The age at death
was 17–63 years; most deaths were sudden and
related to trauma. If resuscitation had been at-
ttempted, or if adrenergic blocking drugs or antihy-
pertensive agents had been administered shortly
before death, the veins were not studied. The veins
were removed between 2 and 15 hours after death,
cleaned of perivascular tissue, placed for 2 hours in
Krebs-Ringer bicarbonate solution (millimolar
composition: NaCl, 118.3; KCl, 4.7; CaCl₂, 2.5;
MgSO₄, 1.2; KH₂PO₄, 1.2; NaHCO₃, 25.0; calcium
EDTA, 0.026; glucose, 11.1), aerated with 95% O₂
and 5% CO₂, and kept at 37°C.

Isometric Tension Recording

We suspended rings of vein (4 mm wide) in
chambers by placing two hooks in the lumen of the vessel. The lower hook was anchored in the bottom
of the chamber; the upper was connected by a
thread to a force-displacement transducer (Grass
FT 03C) for isometric tension recording. The cham-
bber was filled with Krebs-Ringer solution main-
tained at 37°C and aerated continuously with a 95%
O₂-5% CO₂ mixture. For electrical stimulation, two
platinum electrodes (4 mm by 4 mm; 0.5 mm thick)
were positioned parallel to the veins; the electric
impulses consisted of rectangular pulses (9 V, 2
msec, 0.5-16 Hz) from a direct current power supply
with a switching transistor (RCA 1-2N-3034) trig-
gered by a stimulator (Grass SD5). After an equili-
bration period of 60 minutes, the preparations were
placed at the optimal point of their length-tension
curves, using a 10-second, 10-Hz electrical stimula-
tion. For the 14 segments studied, the optimal ten-
sion averaged 3.51 ± 0.53 g; at the optimal tension,
the response to the 10-Hz stimulation averaged 9.23
± 1.19 g.

Superfusion Experiments

Helical strips (3–4 mm wide, 70–100 mm long)
were incubated for 2 hours in Krebs-Ringer solution
containing L-[7-3H]norepinephrine (5.5 × 10⁻⁷ M;
specific activity, 4.5 Ci/mmol; New England Nuclear 377). After incubation, the preparations were rinsed in fresh Krebs-Ringer solution and mounted for superfusion (Vanhoutte et al., 1973; Muldoon et al., 1978). Superfusion was with Krebs-Ringer solution, aerated continuously with 95% O₂ and 5% CO₂, flowing at 2 ml/min; a 90-minute wash-out interval was allowed prior to experimentation to remove [³H]norepinephrine from extraneuronal and extravesicular sites. Each strip was connected to a force-displacement transducer for isometric tension recording. The initial tension was set at 2 g; after this initial stretch, tension decreased and stabilized within 30 minutes. For electrical stimulation of adrenergic nerves, two platinum wires (0.5 mm in diameter) were placed parallel to, and in contact with, the tissue preparation; both the vessel and the electrodes were superfused continuously. Electrical stimulation was applied (10 V, 2 msec, 8 Hz) for 2 minutes every 16 minutes. Acetylcholine was added to the superfusate immediately after the second period of electrical stimulation; in addition atropine or hexamethonium was added after the fourth period. Control experiments were done without addition of acetylcholine. The superfusate was collected at 2-minute intervals by means of a fraction collector. The start of each stimulation period coincided with the onset of a new collection period. To determine the amount of norepinephrine released by each period of electrical stimulation, we pooled the three samples (after the start of that stimulation) for analysis by column chromatography of [³H]norepinephrine and its radiolabeled metabolites.

At the end of the experiments, veins were blotted dry and weighed, and [³H]norepinephrine and its metabolites were extracted with 1 N acetic acid containing 0.03 mm EDTA and ascorbic acid, 0.2 mg/ml (Rorie et al., 1980).

Column Chromatography

[³H]Norepinephrine was separated from its metabolites in the superfusates, and in the extracts of vein prepared after termination of superfusion, by the method of Graefe et al. (1973).

Radioactivity Measurements

Aliquots (1-ml) of superfusate and of the effluents from the columns were added to 10 ml of Insta-Gel (Packard Instrument Company, Inc.), and the radioactivity was measured in a liquid scintillation counter. Corrections for quenching were made with an external standard. The counting efficiency was 30 to 35%. The samples were counted for 10 minutes or until 10,000 counts had been reached.

Drugs

L-Norepinephrine bitartrate (Levophed, Winthrop), acetylcholine chloride (Sigma), hexamethonium bromide (K and K Laboratories), and atropine sulfate (Lilly) were dissolved in Krebs-Ringer solution. The concentrations are expressed as final concentrations (m) in either the bath or the superfusion solution.

Calculations

The release of total radioactivity evoked by electrical stimulation was expressed as fractional release per 2-minute interval; i.e., the radioactivity released per time interval was divided by the radioactivity present in the tissue just prior to that interval. The amount of radioactivity in the tissue at any given time was calculated by adding to the radioactivity in the vein at the end of the study all the tritium collected in samples of superfusate from that time to the end of sample collection.

For the organ bath studies, more than one ring from each vein was studied. The responses of rings from each vein were averaged. These averages were used for calculating statistical probability. The paired t-test was used to test for significance of differences; P values < 0.05 were considered significant.

Results

Organ Bath Experiments

Nine rings prepared from saphenous veins of four humans were studied. They contracted when stimulated electrically or with the addition of exogenous norepinephrine (Table 1). Sustained contractions were obtained with electrical stimulation at 1 Hz. When the response had stabilized, increasing concentrations of acetylcholine (10⁻⁹ to 5 x 10⁻⁷ m) were added in a noncumulative manner. From 10⁻⁶ to 5 x 10⁻⁷ m acetylcholine caused marked relaxations. Examples of these studies are shown in Figure 1. The relaxations caused by acetylcholine were attenuated or abolished by incubation of four rings from four humans for 10 minutes in solutions con-

<table>
<thead>
<tr>
<th>Norepinephrine (m)</th>
<th>Increases in tension above basal (g)</th>
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</thead>
<tbody>
<tr>
<td>5 x 10⁻⁹</td>
<td>0.16 ± 0.08</td>
</tr>
<tr>
<td>10⁻⁹</td>
<td>0.41 ± 0.21</td>
</tr>
<tr>
<td>5 x 10⁻⁸</td>
<td>3.50 ± 1.38</td>
</tr>
<tr>
<td>10⁻⁷</td>
<td>5.53 ± 1.65</td>
</tr>
<tr>
<td>5 x 10⁻⁷</td>
<td>12.80 ± 2.40</td>
</tr>
<tr>
<td>10⁻⁶</td>
<td>14.40 ± 2.50</td>
</tr>
</tbody>
</table>

* Data shown as means ± SE for vein rings from four subjects.
taining $10^{-8}$ M to $10^{-7}$ M atropine. Examples of these studies are shown in Figure 2.

When contractions were obtained with norepinephrine ($10^{-7}$ to $5 \times 10^{-7}$ M) comparable in amplitude to those with electrical stimulation (1 Hz), the addition of acetylcholine ($5 \times 10^{-8}$ to $5 \times 10^{-7}$ M) also caused a decrease in tension (Fig. 1). However, the relaxations induced by acetylcholine during contractions evoked by exogenous norepinephrine were significantly smaller than the relaxations induced by the same concentration of acetylcholine obtained during electrical stimulation (Fig. 3). In three rings from three subjects in which tests were made, the effects of acetylcholine were antagonized by atropine ($10^{-7}$ M).

Superfusion Studies

Unstimulated Preparations

In veins from 10 humans, the basal fractional overflow of total radioactivity after 90 minutes of washout was $0.18 \pm 0.03\%$ (mean $\pm$ se) per 2 minutes. Acetylcholine ($3 \times 10^{-6}$ M), or a combination of acetylcholine and atropine ($10^{-8}$ M) or acetylcholine and hexamethonium ($3 \times 10^{-4}$ M) did not alter significantly this release or the basal tension.

**Figure 1** Effect of acetylcholine on contractions of a human saphenous vein ring induced by electrical stimulation and norepinephrine. Ach = acetylcholine. Time scale applies throughout the experiments.

**Figure 2** Isolated human saphenous vein contracted by electrical stimulation; the effect of atropine on the relaxation induced by Ach. Two veins from the same human. Ring one serves as the control. Time scale applies throughout the experiments.

**Figure 3** Comparison of the effects of Ach ($10^{-7}$ M) on the response of the same human saphenous vein rings to electrical stimulation (1 Hz) and norepinephrine ($5 \times 10^{-8}$ to $10^{-7}$ M). In each ring, the norepinephrine concentration was selected to give contractions comparable to those given by electrical stimulation at a frequency of 1 Hz. Data from four humans are expressed as percent of the response in the absence of Ach (means $\pm$ se). * The depression caused by Ach is significantly less than that during electrical stimulation.

**Figure 4** Effects of Ach ($3 \times 10^{-6}$ M), atropine ($10^{-8}$ M; $n = 7$), and hexamethonium ($3 \times 10^{-4}$ M; $n = 3$) on fractional release evoked by electrical stimulation (ES, 8 Hz) from the saphenous veins from 10 humans. Means $\pm$ se. * Evoked release is depressed significantly by Ach but was returned toward control values by atropine** (average absolute increase in fractional release was $0.165 \pm 0.04$, $P < 0.02$), but not by hexamethonium.

**Figure 3** Comparison of the effects of Ach ($10^{-7}$ M) on the response of the same human saphenous vein rings to electrical stimulation (1 Hz) and norepinephrine ($5 \times 10^{-8}$ to $10^{-7}$ M). In each ring, the norepinephrine concentration was selected to give contractions comparable to those given by electrical stimulation at a frequency of 1 Hz. Data from four humans are expressed as percent of the response in the absence of Ach (means $\pm$ se). * The depression caused by Ach is significantly less than that during electrical stimulation.
FIGURE 5: Effect of Ach (3 x 10^{-6} M) and atropine (10^{-8} M) on fractional release of \(^{3}\)H from human saphenous veins prelabeled with \(^{3}\)Hnorepinephrine. The veins were stimulated electrically at 8 Hz during the 2-minute intervals indicated by the rectangles. Shown are the ratios of each increase compared to that of the second stimulation as described by Schrold and Nedergaard (1977). Upper: fractional release of radioactivity in control veins; atropine did not affect pattern of release. Lower: Ach caused a depression of fractional release that was reversed by atropine.

Stimulated Preparations

A 2-minute period of electrical stimulation increased the fractional release of \(^{3}\)H to a mean value of 1.66\% (Fig. 4). In most preparations, more \(^{3}\)H was released in the first period of electrical stimulation than in the second (Fig. 5). The addition of acetylcholine (3 x 10^{-6} M) to the superfusion fluid decreased significantly the release of radioactivity (Fig. 4). Hexamethonium bromide (3 x 10^{-4} M) did not significantly affect the reduction of \(^{3}\)H release evoked by acetylcholine, but atropine (10^{-8} M) reversed the inhibitory effect of acetylcholine on total radioactivity release (Fig. 4).

Table 2: \(^{3}\)H]NE and Its Metabolites in Superfusate and in Human Saphenous Vein

<table>
<thead>
<tr>
<th>Sample</th>
<th>NE</th>
<th>DOPEG</th>
<th>NMN</th>
<th>OMDA</th>
<th>DOMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superfusate collected during ES at 8 Hz*</td>
<td>35.95 ±13.53</td>
<td>10.26 ±3.11</td>
<td>2.70 ±1.33</td>
<td>10.40 ±2.39</td>
<td>4.46 ±1.18</td>
</tr>
<tr>
<td>Superfusate collected during ES at 8 Hz with acetylcholine (3 x 10^{-6} M) present</td>
<td>18.34 ±6.02</td>
<td>10.29 ±2.02</td>
<td>2.52 ±0.80</td>
<td>10.15 ±3.10</td>
<td>3.56 ±1.62</td>
</tr>
<tr>
<td>Δ with acetylcholine</td>
<td>-17.61 ±4.46</td>
<td>0.02 ±0.01</td>
<td>-0.18 ±0.21</td>
<td>-0.25 ±0.44</td>
<td>0.89 ±0.07</td>
</tr>
<tr>
<td>Tissue after ES</td>
<td>1086.94 ±245.30</td>
<td>11.54 ±2.73</td>
<td>21.16 ±5.27</td>
<td>144.73 ±22.45</td>
<td>22.45 ±16.90</td>
</tr>
</tbody>
</table>

Abbreviations: NE = norepinephrine, DOPEG = 3,4-dihydroxyphenylglycol; NMN = normetanephrine; OMDA = O-methyldihydroxyphenylglycol; DOMA = 3,4-dihydroxyphenylglycol; ES = electrical stimulation.

Discussion

The present experiments demonstrate that isolated human saphenous veins taken a few hours after death exhibit a contractile response to electrical stimulation and to exogenous norepinephrine similar to that reported in the isolated saphenous veins of dogs and rabbits (Vanhoutte and Shepherd, 1973; De Mey and Vanhoutte, 1980). The adrenergic nerve endings in the human vein wall can take up \(^{3}\)Hnorepinephrine, metabolize the labeled transmitter, and release it upon electrical stimulation in a manner similar to that reported for isolated dog and rabbit cutaneous veins (Vanhoutte et al., 1973; Muldoon et al., 1976; De Mey and Vanhoutte, 1979; Rorie et al., 1980).

In dog and rabbit saphenous vein, acetylcholine causes an atropine-sensitive reduction of the contractile response to electrical stimulation; the relaxation caused by the cholinergic transmitter is paralleled by a decreased release of \(^{3}\)Hnorepinephrine (in preparations incubated previously with the labeled transmitter) or by a reduced evoked overflow of endogenous norepinephrine (Vanhoutte and Shepherd, 1973; Vanhoutte et al., 1973; Vanhoutte, 1974; Vanhoutte et al., 1979; De Mey and Vanhoutte, 1980). The present study shows that the same is true in the human saphenous vein. Thus, as in all animal blood vessels tested so far, the adrenergic nerve endings of the human saphenous veins contain cholinergic receptors capable of causing inhibition of the exocytotic release of norepinephrine. The observation that atropine, but not hexamethonium, can curtail the inhibitory effect of acetylcholine on adrenergic neurotransmission indicates that a muscarinic rather than a nicotinic effect is involved (Vanhoutte and Shepherd, 1973; Vanhoutte, 1974; Edvinson, 1975).

Column chromatographic analyses show that the changes in total radioactivity overflowing from the veins caused by acetylcholine was due primarily to changes in \(^{3}\)Hnorepinephrine (Table 2).
In contrast with earlier observations on dog and rabbit saphenous veins (Vanhoutte and Shepherd, 1973), acetylcholine reduced the contractile responses of the human veins to exogenous norepinephrine. Relaxations of isolated arteries caused by acetylcholine during norepinephrine-induced contractions of different animal species have been reported (Vanhoutte, 1977). Thus the human saphenous vein differs from animal veins in that the cholinergic transmitter exerts a postjunctional inhibitory effect in the former, similar to that noted in animal arteries. Thus the postjunctional effect of the cholinergic transmitter explains only part of the inhibition of the response to activation of the sympathetic nerves, since relaxations induced by acetylcholine were significantly more marked during contractions evoked by electric impulses than during the addition of exogenous norepinephrine.

References

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