A COMPARATIVE STUDY OF CHANGES IN INNERVATION AND DEVELOPMENT OF SUPERSENSITIVITY IN THE RAT VAS DEFERENS AFTER VARIOUS PROCEDURES

Akira SAITO, Yutaka KASUYA and Katsutoshi GOTO*

Department of Chemical Pharmacology, Faculty of Pharmaceutical Sciences, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan and *Department of Pharmacology, Institute of Basic Medical Sciences, The University of Tsukuba, Niihari-gun, Ibaraki 305, Japan

Accepted October 29, 1981

Abstract—The relationship between morphological and functional changes in adrenergic nerves and the development of supersensitivity in the rat vas deferens was comparatively investigated after surgical denervation, chemical sympathectomy by 6-hydroxydopamine, daily treatment of animals with reserpine, or local application of colchicine to the hypogastric plexus. The order of ability to produce supersensitivity, as judged by the extent of the increase in the pD₂ value of norepinephrine and the maximum response to norepinephrine, was as follows: denervation=colchicine>6-hydroxydopamine>reserpine. These procedures produced alterations in morphological characteristics of the nerve ending with severeness of degeneration in the following order: denervation>6-hydroxydopamine>colchicine>reserpine. Twitch contractions induced by transmural nerve stimulation were slightly reduced after colchicine or 6-hydroxydopamine treatment, markedly reduced by reserpine, and abolished by denervation. Therefore, the development of supersensitivity in the rat vas deferens is not necessarily in proportion to the morphological or functional changes in adrenergic nerves. The results suggest that some neurofactor, e.g. trophic factor, is involved in the control of the drug sensitivity of smooth muscle in addition to the neurotransmitter itself.

A number of studies concerning denervation supersensitivity in skeletal muscle have indicated that some trophic effect of the motoneuron is involved in regulation of the drug sensitivity of skeletal muscle (1-4). However, there has been little understanding of the trophic influence of autonomic nerves on smooth muscle.

Chronic postganglionic denervation of adrenergically innervated smooth muscle is known to result in the development of both pre- and postjunctional supersensitivity. Prejunctional supersensitivity characterized by a large increase in sensitivity which is specific for norepinephrine and closely related amines is attributed to a loss of the neuronal uptake mechanism into adrenergic nerves. On the other hand, postjunctional supersensitivity is a phenomenon in which the sensitivity of the smooth muscle cells is elevated to a variety of unrelated agonists as a consequence of the cessation of physiological activities of the smooth muscle due to a loss of neurotransmitter (5-7).

In the rat vas deferens, supersensitivity is induced by surgical denervation (8, 9), pretreatment with reserpine (9-11) or pretreatment with 6-hydroxydopamine (11,
Recently Goto et al. (13) showed that local application of colchicine to the hypogastric plexus produced denervation-like supersensitivity in the rat vas deferens without causing over-all degeneration of the adrenergic nerves. Postjunctional supersensitivity in the rat vas deferens induced by denervation or colchicine is accompanied by a large increase in the maximum responses to agonists (8, 9, 13) as well as the leftward shift of the dose-response curves. However, a causal factor for this increase in the maximum response is still obscure.

In the present study, to elucidate the role of alteration in the nervous environment of smooth muscle in the development of supersensitivity, the changes in innervation of the smooth muscle layer were comparatively investigated from morphological and functional viewpoints after various procedures which produce supersensitivity.

MATERIALS AND METHODS

Various procedures for producing supersensitivity

Male Wister-strain rats weighing from 220 to 340 g were subjected to the following procedures which produce supersensitivity:

1. Surgical denervation: Postganglionic denervation of the vas deferens was performed according to the method of Kasuya et al. (8). The left vas deferens was denervated and the other tissue was left intact to serve as the control. The animals were killed 4 days after the operation and both right and left vasa deferentia were removed for in vitro studies.

2. Reserpine treatment: The animals were treated with reserpine (1.0 mg/kg/day, i.p.) for 5 to 6 days before sacrifice as described elsewhere (9).

3. Chemical sympathectomy with 6-hydroxydopamine: The animals received two intravenous injections of 6-hydroxydopamine hydrobromide. The first dose was 100 mg/kg and the second dose administered on the next day was 250 mg/kg (12). The animals were killed 4 days after the second injection.

4. Colchicine treatment: The animals were anesthetized with ether. Colchicine (20 μg) was applied locally to the left hypogastric plexus as described previously (13). The right vas deferens was left intact to serve as the control. The animals were killed 4 days after this treatment.

Organ bath studies

On the day when the supersensitivity was fully developed following each treatment, the animals were stunned and bled. Both right and left vasa deferentia were subsequently removed, desheathed and set up in an organ bath of 45 ml capacity. The bathing medium was a modified Krebs' solution of the following composition (millimolar concentration): NaCl, 120; KCl, 6.0; CaCl2, 2.2; MgCl2, 1.2; NaHCO3, 25.0; and D-glucose, 14.0. The solution was maintained at 37°C and gassed with 95% O2 and 5% CO2. A resting tension of 0.5 g was applied. One hour was allowed for equilibration before the tissue was examined. Contractions of the tissues were recorded isometrically with a force-displacement transducer (Nihon Kohden, SB-1T) and an ink-writing oscillograph.

Full dose-response curves to norepinephrine were obtained by a stepwise increase in the concentration of norepinephrine. Norepinephrine was applied for 1 min and then washed out thoroughly. There were 10 to 15 min between successive doses of norepinephrine. The peak tension developed after application of each dose of norepinephrine was used as the response for constructing the dose-response curve. In each dose-response curve the highest tension development (g) in response to norepinephrine was taken as the maximum response of each tissue.

Transmural nerve stimulation was performed on the prostatic portion of the vas
deferens using platinum electrodes. In expectation of an efficient stimulation, a relatively short preparation was used in this particular experiment. A short length of platinum wire which just entered one end of the lumen served as one electrode and a coaxial circle of the wire at the outside of the other end of the tissue served as the second electrode. A square pulse (1 msec in duration, various voltages) was generated through a Nihon-Kohden SEN 3101 stimulator. A twitch contraction of the tissue elicited by one pulse at each voltage, which was sensitive to tetrodotoxin (3×10⁻⁷ g/ml) and guanethidine sulfate (10⁻⁵ M), was used for the construction of the voltage-response curves.

Electron microscopic studies

Rats received 5-hydroxydopamine chloride, 30 mg/kg i.v., to enhance the granulation of the vesicles in adrenergic nerve endings (14) 30 min before the perfusion of the abdominal aorta by glutaraldehyde (2%) in Millonig’s buffer (pH=7.4). A prostatic portion of the vas deferens was dissected out and cut into less than 1 mm³ cubes and fixed for 1 hr in the same fixative at 4°C. The specimens were treated with 3% tannin for 30 min, postfixed in 1% OsO₄ for 1 hr in Millonig’s buffer, stained with uranylacetate in veronal buffer (pH=6.8), dehydrate using polyethylene glycol 200 (15), and embedded in epoxy resin (Poly Bed 812, Poly Sci. Inc.) (16). These sections of longitudinal muscle layer were placed on 300 mesh copper grids, stained with uranylacetate and lead citrate, and examined with a JEM 100-C or a 100-S electron microscope. Nerve endings (varicosities) and muscle cells per grid square were counted for quantitative study, and the density of innervation was expressed by the following index:

\[
\text{varicosities/grid square} \times 100 \div \text{muscle cells/grid square}
\]

**Drugs:** Apoplon (Daiichi Seiyaku), colchicine cryst (Wako Pure Chemical Industries) 6-hydroxydopamine hydrobromide (Sigma Chemical Comp.) and L-noradrenaline bitartrate (Wako Pure Chemical Industries).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>pD2&lt;sup&gt;a&lt;/sup&gt;</th>
<th>pD2&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Maximum response (g)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>JMax. (g)&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.23±0.12</td>
<td>1.85</td>
<td>1.88±0.21</td>
<td>1.36</td>
</tr>
<tr>
<td>Denervated&lt;sup&gt;4&lt;/sup&gt;</td>
<td>6.88±0.20</td>
<td></td>
<td>3.24±0.06</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5.41±0.24</td>
<td>1.75</td>
<td>1.92±0.21</td>
<td>1.23</td>
</tr>
<tr>
<td>Colchicine&lt;sup&gt;5&lt;/sup&gt;</td>
<td>7.16±0.27</td>
<td></td>
<td>3.15±0.21</td>
<td></td>
</tr>
<tr>
<td>Control&lt;sup&gt;6&lt;/sup&gt;</td>
<td>5.23±0.09</td>
<td>1.07</td>
<td>1.58±0.17</td>
<td>0.88</td>
</tr>
<tr>
<td>6-OHDA&lt;sup&gt;7&lt;/sup&gt;</td>
<td>6.30±0.04</td>
<td></td>
<td>2.26±0.12</td>
<td></td>
</tr>
<tr>
<td>Control&lt;sup&gt;8&lt;/sup&gt;</td>
<td>5.26±0.10</td>
<td>0.53</td>
<td>1.24±0.09</td>
<td>0.15</td>
</tr>
<tr>
<td>RSP&lt;sup&gt;9&lt;/sup&gt;</td>
<td>5.79±0.19</td>
<td></td>
<td>1.39±0.08</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Values represent the mean±S.E.

<sup>b</sup> \( \Delta pD2 = pD2 \) (treated) – \( pD2 \) (control)

<sup>c</sup> JMax. = Maximum response (treated) – Maximum response (control)

<sup>d</sup> Denervation was performed 4 days before sacrifice.

<sup>5</sup> Colchicine (20 μg) was applied to the hypogastric plexus 4 days before sacrifice.

<sup>6</sup> The animals received two injections of 6-hydroxydopamine hydrobromide. The first dose was 100 mg/kg and the second dose, injected on the next day, was 250 mg/kg. The animals were killed 4 days after the second injection.

<sup>7</sup> The animals were treated with reserpine 1 mg/kg/day i.p. for 5 to 6 days before sacrifice.

<sup>9</sup> Vasa deferentia from vehicle-treated rats were used as the control tissues.
RESULTS

Organ bath studies: Table 1 summarizes the effects of various procedures on changes in pD₂ (−log ED 50) value for norepinephrine and the maximum contractile response to this agent. Denervation or colchicine treatment produced the greatest changes in both the sensitivity and the maximum response. The increases in pD₂ value and the maximum response induced by 6-hydroxydopamine were less than those induced by denervation or colchicine, indicating that 6-hydroxydopamine did not produce the maximal supersensitivity in the present study. A slight increase in pD₂ value was observed without appreciable change in the maximum response in the vas deferens from reserpine-treated rats.

Since the endogenous norepinephrine content was reported to decrease following these procedures (8, 9, 12, 13, 17), changes in neuromuscular function were examined by measuring contractile responses to nerve stimulation. Shown in Fig. 1 are voltage-response relationships in the rat vas deferens which were subjected to the various procedures. Each point represents the mean of 5 experiments. Vertical bar indicates the S.E. RSP: The animals were treated with reserpine 1 mg/kg/day i.p. for 5 to 6 days before sacrifice. Colchicine: Colchicine (20 µg) was applied locally to the hypogastric plexus 4 days before sacrifice. 6-OHDA: The animals received two intravenous injections of 6-hydroxydopamine hydrobromide. The first dose was 100 mg/kg and the second dose, injected on the next day, was 250 mg/kg. The animals were killed 4 days after the second injection. Denervation: Denervation was performed 4 days before sacrifice.

![Fig. 1. Voltage-response relations of transmural nerve-stimulated rat vas deferens which developed supersensitivity. A single pulse stimulation (duration =1 msec) was applied at various voltages. Each point represents the mean of 5 experiments. Vertical bar indicates the S.E. RSP: The animals were treated with reserpine 1 mg/kg/day i.p. for 5 to 6 days before sacrifice. Colchicine: Colchicine (20 µg) was applied locally to the hypogastric plexus 4 days before sacrifice. 6-OHDA: The animals received two intravenous injections of 6-hydroxydopamine hydrobromide. The first dose was 100 mg/kg and the second dose, injected on the next day, was 250 mg/kg. The animals were killed 4 days after the second injection. Denervation: Denervation was performed 4 days before sacrifice.](image-url)
procedures. Nerve stimulation-induced twitch contractions were completely abolished by denervation and markedly reduced by reserpine treatment. On the other hand, in the tissues treated with colchicine or 6-hydroxydopamine, the twitch contractions induced by nerve stimulation were only slightly smaller than those in the control tissues.

Electron microscopic studies: In order to quantitate the changes in nerve profiles after various procedures, the nerve endings were classified into several types according to the severity of morphological changes, from normal to degenerative types.

In the control tissue, nerve endings could easily be distinguished in the smooth muscle layer by the presence of numerous small vesicles (ca 400–600 Å in diameter) and a few mitochondria. The varicosities containing small granular vesicles (Fig 2-A) were labelled type I and varicosities containing small agranular vesicles but not small granular vesicles (Fig 2-B) were labelled type II. Nerve endings in the control tissue were composed of approximately 90% type I and

---

**Fig 2.** Electron micrographs of nerve endings observed in the control vas deferens of the rat. Bar equals 0.5 μm. A. Varicosity with small granular vesicles. This varicosity was labelled type I. ×30,000. B. Varicosity without small granular vesicles. This varicosity was labelled type II. ×30,000.

**Fig 3.** Electron micrographs of degenerating nerve endings observed in the denervated vas deferens. Bar equals 0.5 μm. A. Varicosity with deteriorated and scattered vesicles and relatively electron-lucent cytoplasm 1 day after denervation. ×30,000. This varicosity was labelled type III. B. Varicosity with electron-dense cytoplasm 1 day after denervation. ×30,000. This varicosity was labelled type IV.
10% type II, which is in agreement with values reported elsewhere (18).

On the 1st and 2nd days after denervation, the number of type I and type II varicosities decreased, and various types of nerve endings of abnormal appearance were found (Fig. 3). Some nerve endings appeared swollen and had electron-lucent cytoplasms containing scattered or deteriorated synaptic vesicles (Fig. 3-A) and were labelled type III. In some other varicosities, the axoplasm was more electron-dense and occasionally surrounded by a Schwann cell as shown in Fig. 3-B, and the varicosities of these profiles were labelled type IV. It may be considered that type III and type IV varicosities reflect some degenerative changes in adrenergic nerve endings, with a more severe change in type IV than type III (19–21).

Figure 4 represents the time course for the alterations in the number of varicosities of type I to IV per 100 muscle cells after denervation. The number of varicosities of type I and type II decreased on the 1st and 2nd days and disappeared on the 4th day after denervation. In contrast, the number of varicosities of type III and type IV showed a temporal increase, especially on the 1st and 2nd days. These indicate that degeneration of adrenergic nerves is almost complete within 2 days following denervation in the rat vas deferens as was demonstrated by the depletion of endogenous norepinephrine (8).

Figure 5 shows the results of measuring the number of various types of varicosities in the vas deferens which exhibited maximal supersensitivity after each treatment. In the tissues of reserpine-treated rats, the

![Fig. 4. Time course of changes in the number of varicosities in the smooth muscle layer of the vas deferens after denervation. Top panel shows the change in number of type I (●) and type II (○), and bottom panel shows that of type III (□) and type IV (■). N: number of animals. n: number of observations. (N, n)=(6, 23) C: control; (5, 19) 1 day; (4, 16) 2 days; (3, 11) 4 days; (4, 16) 7 days. Each point and vertical bar represent the mean and the S.E. Type I and type II rapidly decreased in number, but type III and type IV increased after denervation.](image)

![Fig. 5. Changes in the number of varicosities in the supersensitive smooth muscle layer of the rat vas deferens. N: number of animals. n: number of observations. RSP: Daily treatment of the rat with reserpine (1 mg/kg/day i.p.) for 5 to 6 days. (N, n)=(4, 15). 6-OHDA: The animals received two intravenous injections of 6-hydroxydopamine hydrobromide. The first dose was 100 mg/kg and the second dose, injected on the next day, was 250 mg/kg. The animals were killed 4 days after the second injection. (N, n)=(3, 12). COLCHICINE: Colchicine (20 μg) was applied locally to the hypogastric plexus 4 days before sacrifice. (N, n)=(5, 13). Each column and bar represent the mean and the S.E.](image)
varicosities of type I were only about 10% of all varicosities and the others were all type II varicosities. Even in the remaining varicosities of type I, the majority of small vesicles were of an agranular type (Fig. 6). Reserpine produced no alteration in the number of varicosities of type III and type IV.

6-Hydroxydopamine produced severe morphological changes in adrenergic nerve endings. Numerous varicosities of type III and type IV already appeared on the 1st day after the injection of 100 mg/kg of 6-hydroxydopamine (Fig. 7). The time course of morphological changes in adrenergic nerve endings was quite similar to that in the case of denervation, though the extent was slightly less (data not shown). On the 4th day after the 2nd injection of 6-hydroxydopamine, the number of type I varicosities were about 25% of that in the normal tissue (Fig. 5).

Colchicine produced a slight alteration in morphological features of the adrenergic nerve endings. On the 4th day after colchicine treatment, the number of type I varicosities was approximately 60% of those in the control tissue. The type I varicosity in the smooth muscle layer of colchicine-treated vas deferens showed almost a normal appearance; and, in contrast to reserpine treatment, a reduction in the number of small granular vesicles in respective varicosities was not conspicuous (Fig. 8). However, the number of granular or agranular vesicles in each varicosity was not analyzed quantitatively in the present study.

Quantitative analysis by means of electron

Fig. 6. An electron micrograph of the varicosity of type I in the smooth muscle layer of the vas deferens from a reserpine-treated rat (1 mg/kg/day i.p. for 5 days). A small number of granular vesicles are seen in the nerve ending (cf. Fig. 2-A). ×30,000. Bar equals 0.5 μm.

Fig. 7. An electron micrograph of a varicosity of type IV in the vas deferens from a rat, after the i.v. injection of 100 mg/kg of 6-hydroxydopamine hydrobromide. This profile appears similar to that observed after denervation (cf. Fig. 3-B). ×30,000. Bar equals 0.5 μm.

Fig. 8. An electron micrograph of a varicosity of type I in the smooth muscle layer of the vas deferens, 4 days after the colchicine (20 μg) treatment. This varicosity is almost of normal appearance and there seems to be no decrease in the number of granular vesicles (cf. Fig. 2-A and Fig. 6). ×30,000. Bar equals 0.5 μm.
microscopy was also performed on the other epididymal portion of the vas deferens. In comparison with the prostatic portion, the density of adrenergic nerve endings was generally less in this epididymal portion. Nevertheless, the effects of the various procedures were detected to be quantitatively and qualitatively similar to those in the prostatic portion.

**DISCUSSION**

The results of the present study demonstrate that there was no unequivocal relationship between the extent of morphological and functional changes in adrenergic nerves and the development of pre- and postjunctional supersensitivity. This is in agreement with the previous finding that the manifestation of denervation-like supersensitivity in the rat vas deferens induced by colchicine is not exclusively attributed to the degeneration of adrenergic nerves (13, 22, 23).

Pretreatment of rats with reserpine produced a leftward shift of the dose-response curve to norepinephrine with an approximate 4-fold decrease in the ED50 value, which is comparable to that reported by Lee et al. (9) and Westfall (10, 11). Following reserpine treatment, the nerve varicosities underwent specific changes from type I to type II, but not degenerative changes. This may bring about a reduction in physiological activities of the vas deferens due to a diminution of neurotransmitter (9), and this consequently produces a nonspecific increase in the sensitivity of the smooth muscle to various agonists, i.e. postjunctional supersensitivity (6, 7).

Denervation, colchicine and 6-hydroxydopamine produced further displacement of the dose-response curve to norepinephrine to the left, suggesting that prejunctional supersensitivity is also involved. The extent of supersensitivity due to the loss of neuronal uptake would simply be expected to be proportional to the reduction in the number of normal nerve endings. In the present study, 6-hydroxydopamine and colchicine produced decreases in adrenergic nerve endings (type I) by approximately 75% and 40%, respectively. Nevertheless, colchicine produced a leftward shift of the dose-response curve to norepinephrine, to the same extent as that due to denervation, but greater than that by 6-hydroxydopamine. This may indicate that only a small number of normal varicosities remained after 6-hydroxydopamine treatment and these still participated in the norepinephrine inactivation through an uptake process or that postjunctional supersensitivity is not maximally produced as yet because of incomplete degeneration of adrenergic nerves. In contrast, because the preferential action of colchicine on adrenergic nerves rather than smooth muscle cells was shown (13), colchicine is assumed to impair the pumping mechanism for norepinephrine uptake at the neuronal membrane, thereby resulting in the maximal development of prejunctional supersensitivity.

Nerve stimulation-induced contractions were abolished by denervation, reduced markedly by reserpine, but only slightly affected by colchicine and 6-hydroxydopamine. If the development of postjunctional supersensitivity is exclusively due to a cessation of physiological stimuli on the effector cells (7), the extent of postjunctional supersensitivity induced by colchicine or 6-hydroxydopamine would be predicted to be less than that induced by denervation or reserpine. However, the supersensitivity induced by colchicine was quantitatively and qualitatively identical to that induced by denervation. It seems to be most reasonable that because the direct action of colchicine on the smooth muscle is inconceivable (13), colchicine might interrupt the subtle activity of the physiological neurotransmission and
thus produce postjunctional supersensitivity maximally.

Postjunctional supersensitivity in the rat vas deferens induced by denervation or colchicine was accompanied by an increase in the maximum responses to various drugs (8, 9, 13). The increase in the maximum response was demonstrated to be due to an improved electrical coupling between cells (24, 25) resulting from an enhanced spread of current through the tissue following denervation or colchicine treatment (22, 26). Because reserpine did not produce an increase in the maximum response, this particular phenomenon was formerly considered to be closely related to degeneration of adrenergic nerves (8, 9, 12). In the present study, however, even if colchicine produced less degeneration of adrenergic nerves than 6-hydroxydopamine, the increase in the maximum response was greater than that induced by 6-hydroxydopamine. Thus, the effect of colchicine can be considered to be independent of the degenerative changes of adrenergic nerves. Colchicine has been known to suppress axoplasmic transport in adrenergic nerve fibers (27-30); and, therefore, a likely mechanism to consider is that the action of colchicine may be exerted through interfering with this axoplasmic transport.

The results of the present study appear to be most consistently accounted for by assuming that some neurotrophic factor in addition to neurotransmitter is involved in the regulation of smooth muscle responsiveness (13, 22, 23). Reserpine might produce a cessation of physiological neurotransmission resulting from norepinephrine depletion and bring about postjunctional supersensitivity. Degeneration of adrenergic nerves induced by either surgical denervation or 6-hydroxydopamine treatment can be anticipated to inevitably lead to the disappearance of both neurotransmitter and some neurotrophic factor, thereby resulting in the development of postjunctional supersensitivity accompanied by an increase in the maximum responses, with simultaneous loss of neuronal uptake, i.e. prejunctional supersensitivity. In the present study, however, the effect of 6-hydroxydopamine was incomplete, and the extent of supersensitivity was less than that induced by denervation. High doses of colchicine were shown to produce greater depletion of norepinephrine (13) presumably due to degenerative changes in many adrenergic nerves resulting from more severe suppression of axoplasmic transport (31). However, it is of particular importance in the present study that denervation-like supersensitivity could be produced by colchicine at a dose which did not bring about degenerative features in the majority of adrenergic nerves. It appears to be most likely, therefore, that the action of colchicine was exerted through interrupting the influences of some neurotrophic factor as well as a neurotransmitter on the smooth muscle cells via interference with the axoplasmic transport in adrenergic nerve fibers.

Acknowledgements: We wish to thank Dr. H. Shinozaki, Dr. T. Masaki and Mr. T. Tanaka for generously allowing us the use of the electron microscope. We also thank Mr. O. Inagaki for his technical assistance.

REFERENCES
5) Trendelenburg, U.: Supersensitivity and subsensitivity to sympathomimetic amines. Phar-
macol. Rev. 15, 225–276 (1963)


29) Hökfelt, T. and Dahlström, A.: Effects of two mitosis inhibitors (colchicine and vinblastine) on the distribution and axonal transport of...
noradrenaline storage particles, studied by fluorescence and electron microscopy. Z. Zellforsch. 119, 460–482 (1971)
