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Acetylcholinesterase-positive afferent axons in mucosa of urinary bladder of adult cats: retrograde tracing and degeneration studies

Y. Wakabayashi[1], Y. Kojima[2], Y. Makiura[1], T. Tomoyoshi[1] and T. Maeda[2]
Departments of [1]Urology and [2]Anatomy, Shiga University of Medical Science, Otsu, Shiga, Japan

Summary. Acetylcholinesterase (AchE)-positive afferent axons in the mucosa of the cat urinary bladder were examined in the present experiments. Small-sized dorsal root ganglion cells containing AchE enzyme activity were labelled by injection of retrograde tracer (wheat germ agglutinin conjugated to enzymatically inactive horseradish peroxidase gold complex) into the bladder mucosa of adult cats. Results show that 48.9% (90/184) of the labelled ganglion cells possessed AchE enzyme activity. Following unilateral dorsal root ganglionectomy (L_{2-5}, S_{1-3}), a total of 6619 unmyelinated axon terminals were examined in the bladder mucosa, including 691 degenerating axon terminals. Percentages (8.6-16.1%) of degenerating axon terminals in the ganglionectomized animals (1, 2, 3, 10 and 21 days post-operated) were significantly higher than those of controls (3.1%) and the 60-day post-operated animals (3.2%). Approximately one-half (47.9%) of the degenerating axon terminals observed in the 1-21 day post-operated animals were AchE-positive. Further examination also disclosed that the population of the intact (not affected by ganglionectomy) AchE-positive axon terminals at 60 days (59.3%) was significantly greater than that of controls (45.6%). The AchE-positive terminals containing few synaptic vesicles were significantly increased in number in the 60 day post-operated cats. In conclusion the present study demonstrates that one half of afferent axons in the mucosa were AchE-positive. The increase in AchE-positive afferent axon terminals containing few synaptic vesicles may be derived from contralateral dorsal root ganglia resulting from sprouting following dorsal root ganglionectomy.

Key words: Acetylcholinesterase, Cat, Urinary bladder, Afferent axon, Sprouting

Offprint requests to: Dr. Yoshihiko Wakabayashi, Department of Physiology, Faculty of Medicine, The University of British Columbia, 2146 Health Sciences Mall, Vancouver, B.C., Canada V6T 1Z3

Introduction

Although the presence of acetylcholinesterase (AchE) enzyme activity has been demonstrated in dorsal root ganglia (Cauna and Naik, 1963; Novikoff et al., 1966; Ambrose and McNeill, 1978), the biological significance of AchE in primary sensory neurons of the dorsal root ganglia is not clear. The presence of AchE-positive nerve plexus in the mucosa of mammalian urinary bladders has been well documented (Elbadawi and Schenk, 1966; Gosling and Dixon, 1974; Alm and Elmér, 1975; Dixon and Gosling, 1987; Wakabayashi et al., 1992). Gosling and Dixon (1974) suggested that most of the AchE-positive axons in the mucosa of the cat urinary bladder are sensory in nature, because apparently they did not associate with any recognizable neuro-effector target sites. However, the urinary bladder is also abundantly innervated by parasympathetic nerves and an experimental study is necessary to elucidate the nature of AchE-positive axons in the bladder mucosa.

To examine AchE enzyme activity in dorsal root ganglion cells that innervate the bladder mucosa of adult cats, the ganglion was labelled using gold-labelled retrograde tracer (Basbaum and Menetrey, 1987), and the enzyme activity of AchE was determined by sensitive enzyme histochemistry (Tago et al., 1986). In addition, unilateral dorsal root ganglionectomy was performed to investigate the possible changes of AchE-positive afferent axons in the bladder mucosa. In the present study, the AchE-positive afferent nerve is described and the increase of these fibres after ganglionectomy is discussed in relation to nerve sprouting as well as the biological significance of AchE enzyme activity of afferent axons.

Materials and methods

Animals

Sixteen adult cats of either sex, weighing 2.5-4.0 kg

and kept under routine laboratory conditions, were used for the present study. All experiments were performed in accordance with the *Standards of Animal Experiments and Animal Care* of our institutions.

Tracer injection

Colloidal-gold-labelled WGAapoHRP was used as a retrograde tracer, and was prepared according to the method of Basbaum and Menetrey (1987). Two cats were anaesthetized with an intramuscular injection of ketamine hydrochloride (20 mg/kg). The bladder was exposed and after opening the anterior wall of the bladder, 4-5 µl of WGAapoHRP-Au was injected into the mucosa of the left lateral lower bladder body under aseptic conditions. Care was taken to prevent leakage of the tracer by leaving the needle (glass micropipet) in place for up to 1 min after the injection. The needle was then carefully removed, and the whole area was cleaned prior to suturing.

Tissue processing

At 48 h following the above injection, animals were anaesthetized with an intramuscular injection of ketamine hydrochloride (25 mg/kg), and perfused through the left cardiac ventricle with 0.01M phosphate-buffered saline (pH 7.4) to wash out the blood. This was followed by perfusion of ice-cold fixative that was composed of 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.4). The dorsal root ganglia (L_3, and $S_{2,3}$) and the urinary bladder were removed and post-fixed for 24 h in ice-cold 0.1M phosphate buffer (pH 7.4) containing 4% paraformaldehyde. Then the tissues were immersed in 0.1M phosphate buffer (pH 7.4) containing 15% sucrose for a further 24 h at 4 ºC. Twenty µm-thick sections were cut with a cryotome. The free-floating sections were then placed in 0.1M phosphate-buffered saline (pH 7.4).

Silver enhancement and acetylcholinesterase enzyme histochemistry

The sections were placed in the silver enhancement reagent (intense SE, Amersham International plc., Buckinghamshire, England) for 15 min at room temperature in normal daylight. After the silver-intensification procedure, the sections were stained by Tago's method for AchE activity (Tago et al., 1986). Before the enzyme reaction, the sections were immersed in 0.1M phosphate-buffered saline (pH 7.4) containing 0.1% hydrogen peroxide for 20 min at room temperature to suppress the intrinsic peroxidase activity. They were then incubated in a 0.1M sodium hydrogen maleate buffer (pH 6.0) solution containing 0.05 mM sodium citrate, 0.04 mM cupric sulphate, 5 µM potassium ferricyanide and 0.026 mM acetyl-thiocholine iodide (Nacalai Tesque Inc., Kyoto, Japan) for 1 h at 37 ºC. After this enzyme reaction, the sections were washed and incubated in a 50 mM Tris-HCl buffer (pH 7.6) solution containing 0.05% 3,3'-diaminobenzidine (Dojin Pharmaceutical, Kumamoto, Japan) and 0.05% hydrogen peroxide for 5 min at room temperature to visualize the reaction products.

Dorsal root ganglionectomy

Twelve cats were anaesthetized with an intramuscular injection of ketamine hydrochloride (20 mg/kg) and laid in a prone position on an operating table. Supplementary doses of 5-10 mg/kg ketamine hydrochloride were given to maintain the state of anaesthesia. Body core temperature was measured rectally and maintained at 38 ºC. A minimal longitudinal skin incision was made in the back, the paravertebral muscles were elevated subperiosteally and the vertebral lamina was exposed. The lamina was then partially removed with an electric dental drill, and the spinal ganglia were located with the aid of a dissecting microscope. The L_{2-5} and S_{1-3} spinal ganglia, which give rise to afferent axons that innervate the cat urinary bladder (Applebaum et al., 1980), were ablated unilaterally. Great care was taken to minimize bleeding. The operated animals were then allowed to recover, and prophylactic antibiotic therapy, consisting of a daily injection of amikacin sulphate (5 mg/kg i.m.), was initiated postoperatively for 5 days. The general health status of the animals was monitored and recorded daily. The urinary bladders from two animals were removed after 1, 2, 3, 10, 21 and 60 days following ganglionectomy. The control bladders were obtained from non-operated animals.

Tissue collection

Each cat was deeply anaesthetized with an intramuscular injection of ketamine hydrochloride (25 mg/kg). The urinary bladder was then exposed and emptied by manual pressure. Animals were perfused as described earlier, and the urinary bladders were removed and post-fixed. Square samples (0.5 cm) of the bladder wall were then excised from the lateral lower bladder body of the operated side. Enzyme (AchE) reaction was performed using free-floating 80 µm-thick vibratome sections. For visualization of reaction products, 1% nickel ammonium sulphate was added in the diaminobenzidine solution as described above. Some sections were incubated for the enzyme reaction in a solution containing 10^{-5} M tetra-isopropyl-pyrophosphamide (iso-OMPA). These sections were then post-fixed with 0.1M phosphate buffer (pH 7.4) containing 1% osmium tetroxide for 1 h at 4 ºC. After fixation, the sections were dehydrated in graded alcohol, embedded in epon with the aid of propylene oxide, and mounted on siliconized glass slides. Square samples (0.5 mm) of the mucosa were excised from the slides, cut with an ultramicrotome, then stained with uranyl acetate and lead citrate, and examined under a Hitachi H-500 electron microscope.

Statistical analysis

The Chi-square test for independence and 1 x m contingency table were used; a P value of less than 0.05 was considered significant.

Results

Tracer injection into the mucosa of the urinary bladder

In sections of the WGAapoHRP-Au injected mucosa, particles visualized by silver intensification were localized among the blood vessels, and were diffusely scattered among collagen bundles and phagocytic cells.

Retrograde labelling was found in the bilateral dorsal root ganglia, where a small number of cells were labelled. The labelling was seen in the cytoplasm of the perikarya. The labelled cells were small ganglion cells (20-40 μm in diameter) and the larger cells in the ganglia displayed no labelling. One-half (48.9%, 90/184) of the labelled cells in L_3, S_2 and S_3 dorsal root ganglia contained AchE activity (Fig. 1).

Dorsal root ganglionectomy

The mucosa of the urinary bladder is divided into the epithelial layer and the lamina propria. There were numerous AchE-positive nerve fibres in the lamina propria of the bladder body forming a nerve plexus. There was no apparent difference in the density or distribution of the mucosal AchE-positive nerve fibres between controls and operated cats (Fig. 2). In the deep lamina propria, relatively thick AchE-positive nerve bundles were present which extended from the submucosal layer to form a new plexus. Some AchE-positive fibres in the lamina propria were accompanied by blood vessels, while others projected in all directions in the connective tissue, frequently reaching beneath the epithelium. All the AchE-positive nerve fibres remained intact after the addition of iso-OMPA (10^{-5} M).

The constituents of the lamina propria, including fibrocytes, collagen fibrils and blood vessels, were observed under an electron microscope. Numerous small vessels ran closely beneath the epithelium, and relatively large vessels were observed in the deep lamina propria. AchE reaction products precipitated between axons and Schwann cell sheaths. The AchE-positive nerve plexus of the deep lamina propria was composed of five or

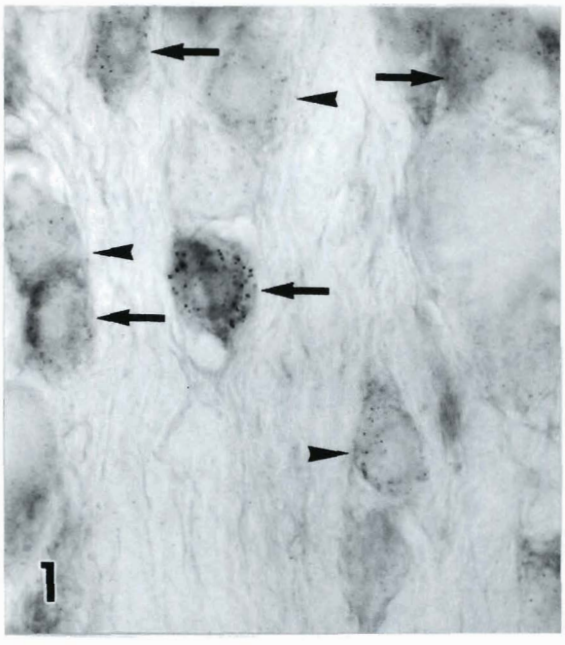

Fig. 1. S_2 dorsal root ganglion of the tracer-injection side. WGAapo-HRP-Au is injected into the mucosa of lower lateral urinary bladder wall. Au labelling is silver-enhanced. There are small WGAapoHRP-Au-labelled cells with (arrow) or without AchE activity (arrowhead). x 340

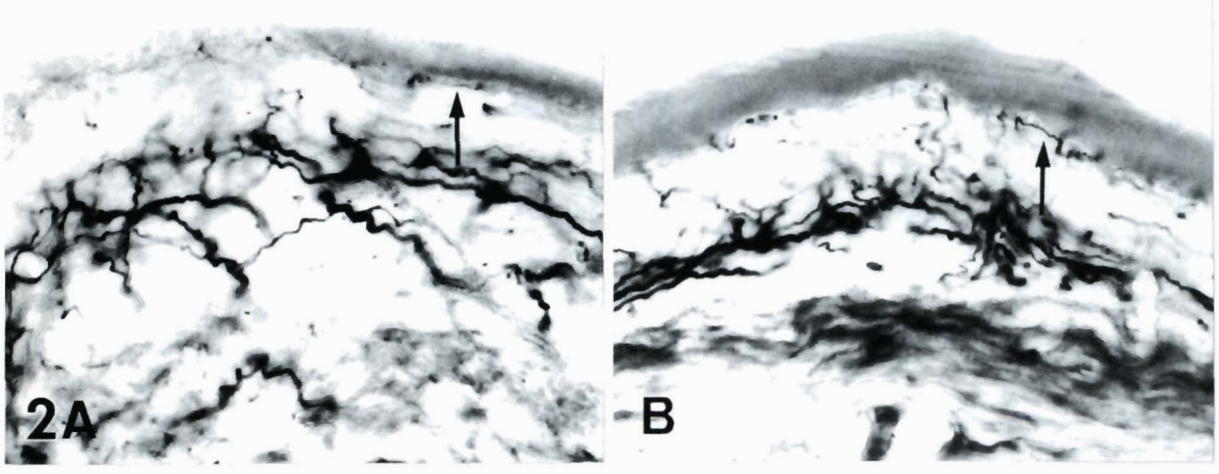

Fig. 2. AchE-positive nerve plexus in the lamina propria of the mucosa. **(A)** Control. **(B)** 10 days after dorsal root ganglionectomy. Arrows indicate subeptihelial AchE-positive nerves. There is no apparent difference between nerve plexus in A and B. Photographs taken from an electron-microsocpic osmificated specimen by AchE histochemistry with nickel ammonium sulphate. x 158

more unmyelinated axons that were surrounded by Schwann cell sheaths and some of these axons were AchE-negative. Axon bundles divided into thinner branches that contained one to five axons. Most axons within these branches partially lacked Schwann cell sheaths. Axon bundles were frequently observed around the blood vessels. No myelinated axons were seen in the mucosa of the cat urinary bladder, although they were present in the submucosal layer as well as in the muscle layers (unpublished observation).

In this study, the term «axon terminal» was defined as a varicose profile that was either alone or with a few axons. Dorsal root ganglionectomy produced various degeneration profiles of axon terminals such as loss of axoplasm, vacuolation, accumulation of osmiophilic material, mitochondrial disintegration, onion skin-like cell membrane and increase of electron density throughout the axon terminal. Degenerating axon terminals were observed in both AchE enzyme activity-positive and -negative groups (Fig. 3). Most degenerating axon terminals were apparently unrelated to specific target structures and ran freely in the

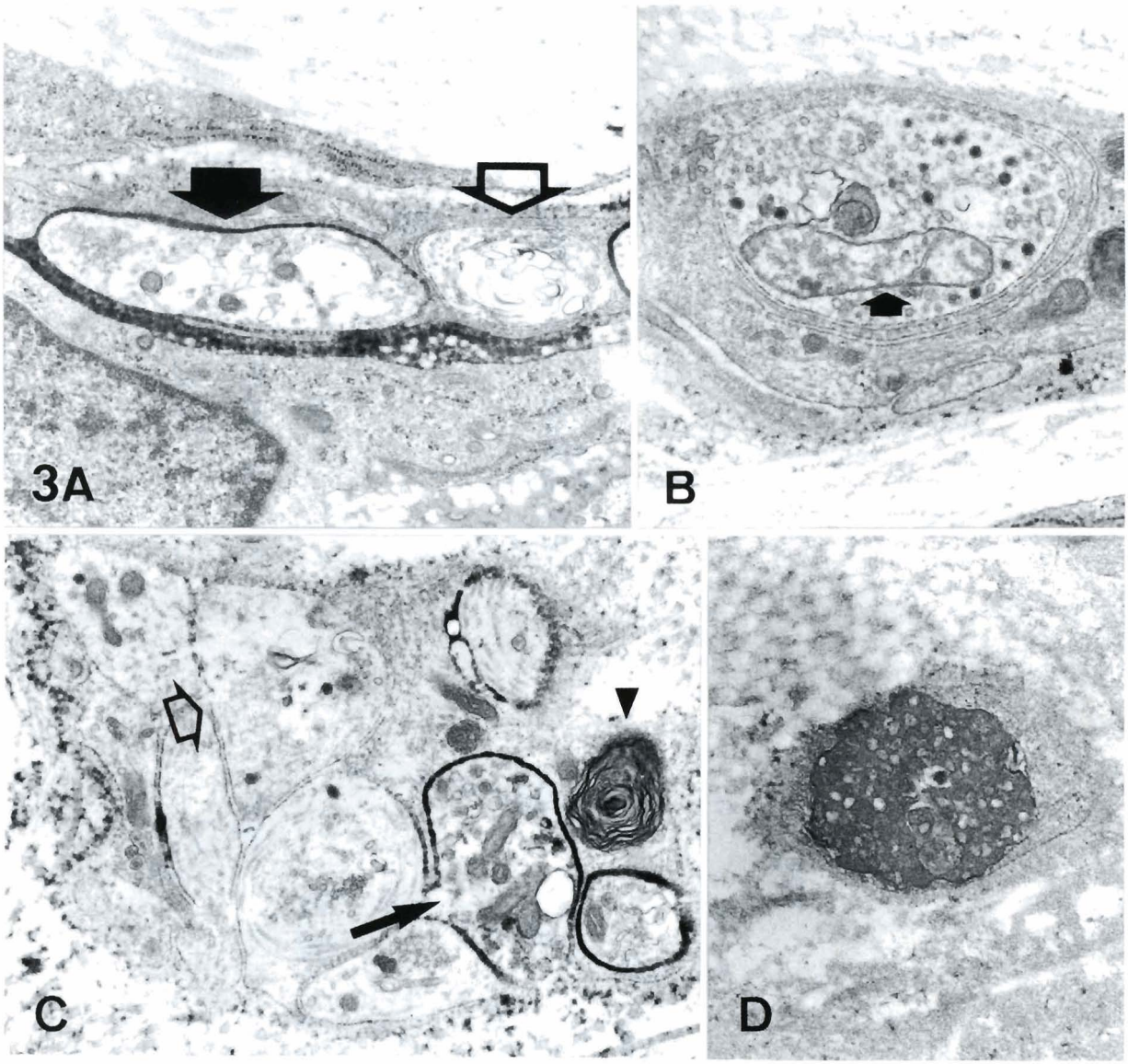

Fig. 3. Electron photomicrographs of degenerating terminals. **A.** Both the AchE-positive (arrow) and -negative (open arrow) axon terminals show loss of axoplasm. x 25,000. **B.** An AchE-negative degenerating axon terminal shows mitochondrial swelling (arrow), loss of axoplasm, and is filled with numerous granular and agranular synpatic vesicles. x 33,000. **C.** An AchE-positive degenerating axonal terminal (arrow) showing vacuolation and granular synaptic vesicles; an AchE-negative degenerating axon terminal showing an onion skin-like cell membrane (arrowhead) and loss of axoplasm (open arrow). x 27,000. **D.** An AchE-negative degenerating axon terminal displaying an increase of electron density throughout the axon terminal filled with many synaptic vesicles. x 40,000

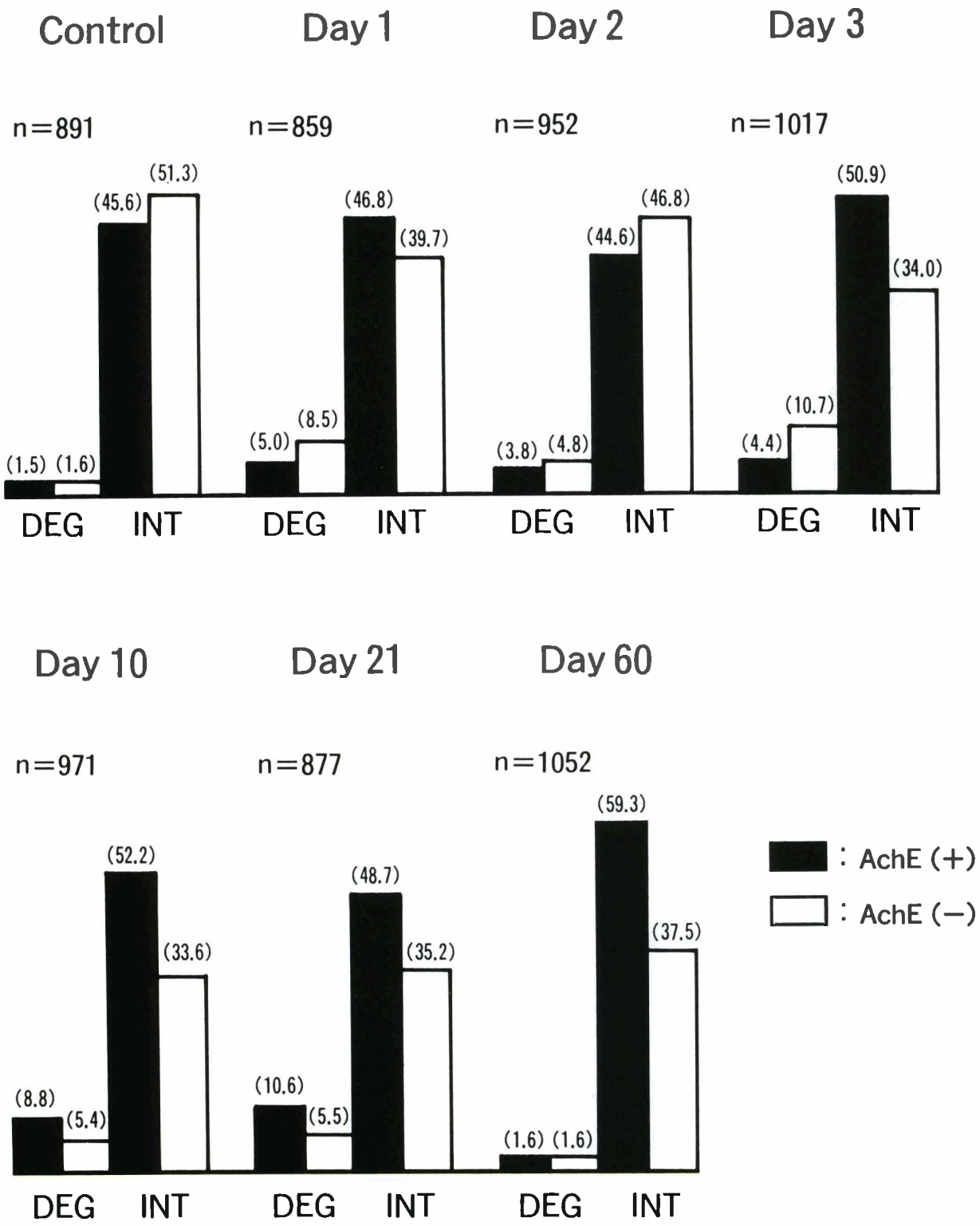

Fig. 4. Histograms showing percentage of AchE-positive and-negative axon terminals in the bladder mucosa of the operated side after unilateral dorsal root ganglionectomy (1, 2, 3, 10, 21 and 60 days). Normal urinary bladders of non-operated cats were used as controls. AchE(+): Acetylcholiesterase-positive; AchE(-): acetylcholinesterase-negative; DEG: degeneratign axon terminals; INT: intact (non-degenerating) axon terminals.

connective tissue, but some were located beneath the epithelium and around the blood vessels. Neither the degenerating axon terminals nor the intact ones (terminals that did not show any sign of degenerating profile) were seen in the epithelium.

A total of 6619 axon terminals were examined, and degeneration was found in 691. Figure 4 shows the percentages of the degenerating and the intact terminal profiles with or without AchE activity, for various postoperative intervals. Approximately one-half of all axon terminals in the control animals (47.1%) were AchE-positive. The percentages of degenerating axon terminals of controls and at 60 days after ganglionectomy (3.1-3.2%) were similar. The percentage (8.6-16.1%) of degenerating axon terminals observed after 1-21 days of ganglionectomy was significantly ($p<0.001$) higher than those of the control and the 60-day post-operative animals. When a total of 630 degenerating axon terminals were examined in the 1-21 day post-operative animals, 302 (47.9%) were found to be AchE-positive. Some degenerating axon terminals contained numerous synaptic vesicles (Figs. 3B-D).

Sixty days after ganglionectomy, the number of intact AchE-positive axon terminals (59.3%) was significantly ($p<0.001$) greater than that of controls (45.6%). In addition, the population of intact axon terminals (AchE-positive) contained few synaptic vesicles (50.6%, 315/623), and was significantly ($p<0.001$) higher than that of controls (38.2%, 155/406) (Fig. 5).

Discussion

This study shows that about one-half of the afferent axons in the mucosa of the cat urinary bladder possessed AchE enzyme activity. Gosling and Dixon (1974) suggest that most AchE-positive unmyelinated axons in the bladder mucosa belong to visceral afferents, since they are unrelated to specific neuro-effector target sites. Using retrograde tracer, we demonstrated that one-half of these labelled cells in the L_3, S_2 and S_3 dorsal root ganglia (48.9%) showed AchE activity. In the ganglionectomy study, the percentages of degenerating axon terminals in controls (3.1%) and ganglionectomized animals (60 days; 3.2%) were almost equal. In addition, the proportions (8.6-16.1%) of degenerating axon terminals in the ganglionectomized cats (1-21 days) were significantly higher than those of controls and the 60-day post-operated animals. Hence, degeneration of the axon terminals for a period of 1-21 days was judged to be caused by dorsal root ganglionectomy. In the 1-21 days ganglionectomized cats about one-half (47.9%) of the degenerating axons in the mucosa were AchE-positive. This observation supports the result of the retrograde tracing study and indicates that one-half of afferent axons in the mucosa have AchE enzyme activity. However, our recent work (Wakabayashi et al., 1992) and the present study suggest that not all of the AchE-positive axons in the mucosa are afferent (see Fig. 4, day 1-21). The present experiments show that in the 1-21-ganglionectomized animals the percentage of intact AchE-positive axon terminals was greater than that of degenerating terminals, suggesting that at least some axons are parasympathetic in nature. Taking into account that about 30% of afferent axons in the mucosa are derived from the contralateral dorsal root ganglia (Uemura et al., 1975), this still does not rule out the presence of parasympathetic innervation in the bladder mucosa.

The significant increase of AchE-positive axon terminals in the 60 days ganglionectomized animals

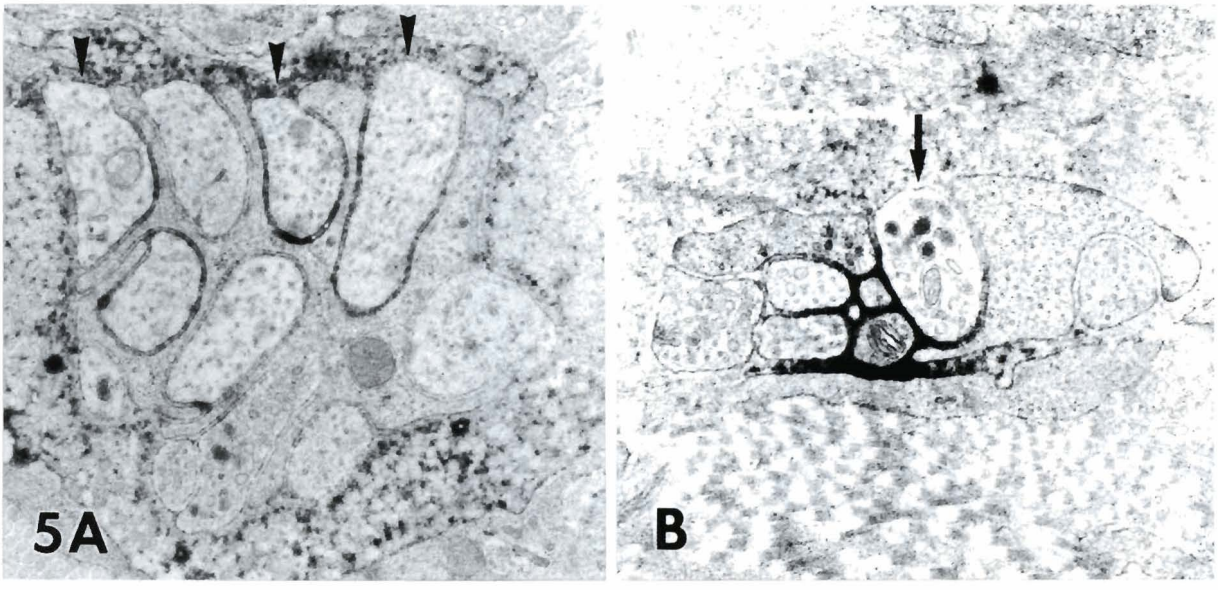

Fig. 5. At 60 days postoperation, intact AchE-positive axon terminals with few synaptic vesicles (arrowheads) **(A)** are more abundant than those with numerous synaptic vesicles (arrow) **(B)** A. x 29,000; B. x 44,000

were terminals that contained few synaptic vesicles. In the present experiments the ventral roots were preserved, while the unilateral dorsal root ganglia innervating the bladder were completely abolished. Some afferent axons in the mucosa of the cat urinary bladder are derived from the contralateral dorsal root ganglia (Uemura et al., 1975). In addition, a type of AchE-positive axon terminals that had few synaptic vesicles was particularly prominent, therefore, it is difficult to consider that these axon terminals are parasympathetic in nature. With this in mind, it is possible that AchE-positive afferent axons originating from the contralateral side might sprout after dorsal root ganglionectomy. The phenomenon of such sprouting from intact and surrounding afferent axons into denervated tissues has been extensively studied in the skin. In adult animals, it has been demonstrated that only small afferent fibres (A∂ and C fibres) were able to functionally reinnervate denervated skin by collateral sprouting (Devor and Govrin-Lippmann, 1979; Jackson and Diamond, 1983; Brenan, 1986; Snow and Wilson, 1991). Our observations suggest that AchE-positive unmyelinated afferent axons with few synaptic vesicles in the bladder mucosa of adult cats possess a greater ability to sprout than AchE-negative ones do.

Although some theories regarding non-cholinergic roles for AchE have been proposed in nervous transmission (Nachmansohn, 1970) and neurosecretion (Greenfield, 1984), the function of AchE in the dorsal root ganglia is not well known. In the present study, the AchE-positive afferent (degenerating) axon terminals can be divided into two types: terminals that contained numerous or few synaptic vesicles. Recent studies have shown hat AchE might function similarly to protease to hydrolyze neuropeptide, such as substance P (Chubb et al., 1980; Small, 1990), and that AchE activity is co-localized with substance P in some of chick dorsal root ganglion cells (Castrignano et al., 1990). Hence, afferent axon terminals that possess numerous synaptic vesicles may contain neuropeptides, such as substance P (Wakabayashi et al., 1993). In contrast, the majority of the increased AchE-positive terminals in the 60-day ganglionectomized animals were associated with terminals that contained few synaptic vesicles (see Fig. 5A). Gupta and Bigbee (1992) have demonstrated that inhibition of AchE activity retards neuritic outgrowth from cultured dorsal root ganglia. Thus, AchE of afferent axon might be related not only to hydrolysis of neuropeptides but also to nerve sprouting.

In conclusion, the present study reveals that one-half of the afferent axons in the bladder mucosa are AchE-positive. The present data also suggest that AchE-positive afferent axonal terminals that contained few synaptic vesicles in the bladder mucosa of adult cats can sprout easily after ganglionectomy. Further studies on regeneration of AchE-positive afferent axons may gain some insight into the physiological significance of the present observations.

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