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Distribution of calcitonin gene-related peptide-containing fibers in the urinary bladder of the rat and their origin

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Summary. By use of indirect immunofluorescence, this study demonstrated the presence of calcitonin gene-related peptide-like immunoreactive (CGRPI) fibers in the bladder of the rat. These fibers were abundant in the muscle layer, in which they ran parallel to the muscles, submucosa, and epithelium. No immunoreactive cells were detected. We also examined the origins of these fibers, using a method that combined biotinized retrograde tracer (biotin-wheat germ agglutinin) (B-WGA) and immunocytochemistry. Injection of the tracer into the bladder resulted in the demonstration of small to medium-sized labeled cells that contain CGRPI structures in single dorsal root ganglion cells mostly at the level of L6 and S1, but also a few at L2.

Double-staining for CGRPI and immunoreactive P-like substance (SPI) indicated that there are cells in the dorsal root ganglia at the level of L6 and S1 that react to both, but that there are many CGRPI-positive cells that contain no demonstrable SPI; most of the latter are large.

Key words: Calcitonin gene-related peptide – Substance P – Dorsal root ganglion – Urinary bladder – Rat

Calcitonin gene-related peptide (CGRP), a compound of 37 amino acids, has been demonstrated in nerve tissue by use of recombinant DNA and other techniques of molecular biology (Amara et al. 1984; Rosenfeld et al. 1983). The distribution of CGRP-like immunoreactivity (CGRPI) in central and peripheral nervous systems is extensive but irregular (Rosenfeld et al. 1983; Gibson et al. 1984; Kawai et al. 1985; Lee et al. 1985a, b; Takami et al. 1985). Because little is known concerning the occurrence of CGRPI in the lower urinary tract, we first examined the urinary bladder for its presence. After demonstrating the presence of CGRPI-immunoreactive fibers but no reactive cell bodies in the bladder, we traced the origin of the fibers to dorsal root ganglia at the level of L6 and S1. Thereafter we explored the possible co-existence of CGRPI and substance P(SP)-like immunoreactivity (SPI) in the bladder and dorsal root ganglia, as reported in single cells of sensory ganglia by Gibson et al. (1984) and Lee et al. (1985a, b).

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Materials and methods

Analysis of distribution of CGRPI and SPI in bladder and dorsal root ganglion

Preparation of tissue: Frozen sections. Six rats were used for the analysis of CGRPI and four for SPI. Half of each group received a colchicine injection intraperitoneally (3.5 mg dissolved in saline) 48 h before sacrifice. Colchicine, which inhibits axonal flow (Dalström 1968; Kreutzberg 1969), was used to increase cell-body levels of neurotransmitters. This treatment was intended to facilitate the visualization of these immunoreactive cells in the dorsal root ganglion, and of cells that would normally be difficult to demonstrate in the bladder. Under sodium pentobarbital anesthesia (10 mg/kg, i.p.), all animals were perfused transcardially with ice-cold saline (150 ml) followed by Zamboni's fixative (200 ml at 4° C) (Zamboni and De Martino 1967). The bladder and the dorsal root ganglion at the level of L6 and S1 (see below) were then removed and placed in the same fixative overnight at 4° C. After 24 h of rinsing in phosphate-buffered saline (PBS) containing 30% sucrose, serial frontal sections 5 μm thick were made on a cryostat.

Whole-mount preparations: Four rats each were used for investigation of CGRP and for SP. Two in each group were injected with colchicine and perfused with cold saline as described above. After perfusion, the bladders were removed, opened, and pinned to pieces of balsa. Following fixation in Zamboni's fixative at 4° C for 3 days, the bladders were rinsed for 24 h in 0.1 M phosphate buffer (pH 7.4) containing 30% sucrose, and then frozen and thawed. After being washed in 80% ethanol, the specimens were processed by the whole-mount method described by Costa et al. (1980).

Staining procedures: Frozen sections. Tissues were first incubated overnight at 4° C in CGRP antiserum or SP monoclonal antiserum (Sera Lab.). Tissues were then rinsed three

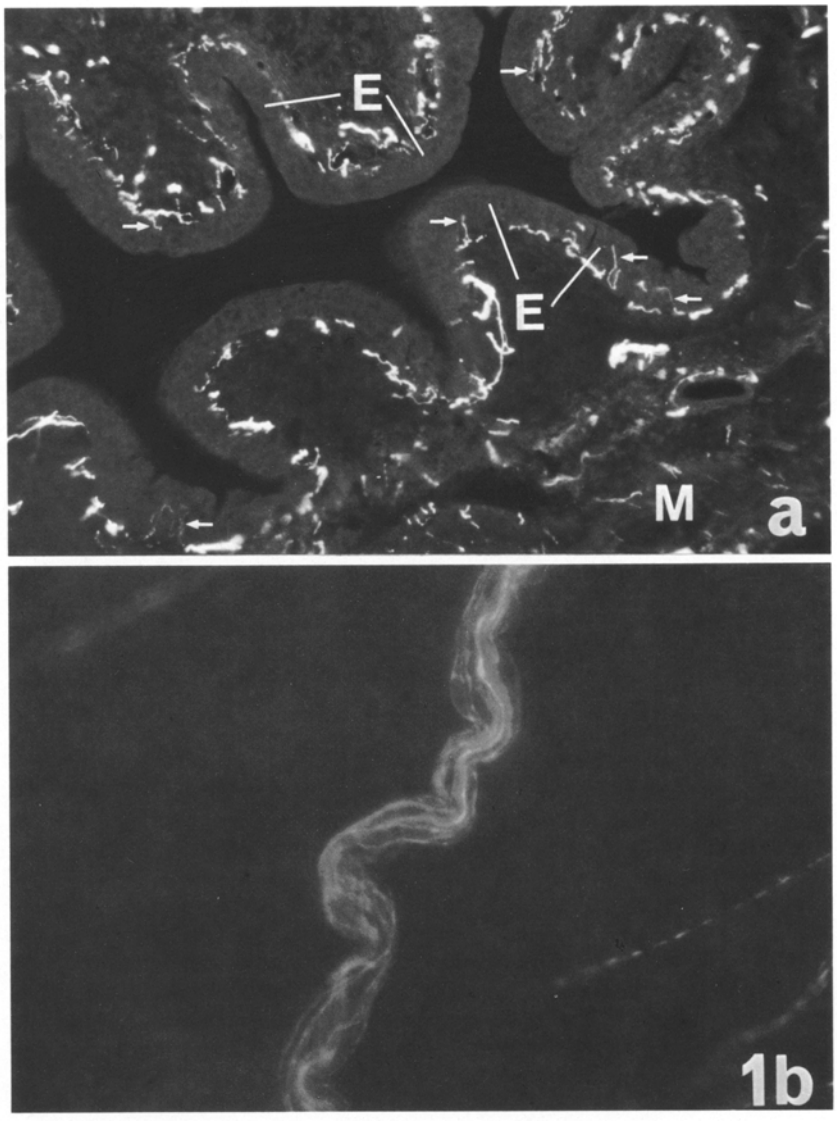

Fig. 1. a Fluorescence photomicrograph of a frozen section showing CGRPI fibers in the urinary bladder. Numerous CGRPI fibers are seen in the muscle layer (*M*) and submucosa just beneath the epithelium (*E*). ×240.
b Fluorescence photomicrograph of a whole-mount showing thick CGRPI fiber bundle entering the bladder. ×480

times for 10 min each time in PBS. Next, sections for the detection of CGRPI were incubated with FITC-conjugated goat anti-rabbit IgG (Miles) or with Texas red-conjugated goat anti-rabbit IgG (Amersham). Sections for the detection of SPI were incubated with FITC-conjugated donkey anti-rat IgG (Capel). After 24 h of incubation at 4° C, tissues were rinsed and mounted in a 1:1 mixture of glycerine and PBS.

Study of origins of CGRPI fibers in the bladder

Because sensory peripheral ganglia contained many CGRPI cells, in contrast to other ganglia which contain few or none (Lee et al. 1985a, b), we examined dorsal root ganglia at various levels after injection of B-WGA into the bladder (Shiosaka et al. 1984, 1986; Shiosaka and Tohyama 1986).

The 7 rats used in this experiment were anesthetized as describe above and the bladder was exposed. First, 3–5 µl of 5% water-solubilized B-WGA was injected into several sites of the bladder using a Hamilton syringe. After 48 h, a solution of colchicine (see above) was injected intraperitoneally with ice-cold saline (50 ml) followed by Zamboni's solution (500 ml). Dorsal root ganglia from the levels T11 to S3 were removed and immersed in the same fixative for 24 h followed by immersion for 24 h in 0.1 M phosphate buffer (pH 7.4) containing 30% sucrose. The ganglia were sectioned in a cryostat at a thickness of 5 µm and rinsed in 0.02 M PBS. The sections were first incubated in the CGRP antiserum (1:2000) and a solution of streptavidin-Texas red conjugate (1:400, Amersham) for 24 h at 15° C. After rinsing with PBS, the sections were incubated with FITC-conjugated goat anti-rabbit IgG (1:1000, Amersham). They were thereafter washed with PBS, mounted in a glycerine-PBS mixture, and observed under a fluorescence microscope. For observation of B-WGA that had reacted with streptavidin-Texas red, the fluorescence microscope was equipped with a G dichroic mirror filter and 580-nm absorption filter. For antigen that had reacted with FITC-conjugated goat anti-rabbit IgG, a B dichroic mirror filter and IF 520–545-nm interference filter was used.

To verify results from retrograde labelling by B-WGA, the conventional retrograde tracer, horseradish peroxidase (HRP), was also used in two rats. In these, 3–5 µl of 5% HRP was injected into several sites of the bladder in the anesthetized rats, after which the bladders were studied by

HRP method described by Mesulam et al. (1978) using the tetramethylbenzidine reaction.

Double-staining immunohistochemical analysis

Of the 6 rats used in this experiments 3 received a colchicine injection as described above. Frozen sections were prepared as in the analyses of distribution of CGRPI and SPI. The sections were first incubated in a mixture containing both CGRP polyclonal antibody raised in a rabbit and SP monoclonal antibody raised in a rat. After overnight incubation of these first antisera at 17° C, the tissues were rinsed 3 times for 10 min with PBS whereafter they were incubated with a mixture of the second antisera, Texas red-conjugated goat anti-rabbit IgG and FITC-conjugated donkey anti-rat IgG for 24 h at 17° C. The tissues were then rinsed and mounted in a glycerine-PBS mixture. The dilution of the first and second antisera was as described above. The tissues were examined by fluorescence microscopy. CGRPI material was red when examined under the G dichroic-mirror system and SPI material was green under the B dichroic-mirror filter system. This depends on specific differences in the two antisera and on the absence of cross-reactivities between the primary and second antisera (see also Lee et al. 1985b).

Specificity of antiserum

Specificity of the first antisera. SP monoclonal antibody (carboxyl terminal fragment) reacts with 5–8 terminals well as with intact SP. Little cross-reactivity was found using the related peptide eledoisin. Antiserum against CGRP was raised in a rabbit against tyr°CGRP28–37 conjugated to ovalbumin (Morris et al. 1984). The radioimmunoassay system showed that this antiserum crossreacts with rat and human CGRP, but not with a large series of other peptide hormones, i.e., neurotensin, ACTH, SP, enkephalins, somatostatin, oxytocin and preproopiomelanocortin (Morris et al. 1984). The specificity of the antiserum was also confirmed by the following adsorption and blocking tests: 1) replacement of the specific antiserum by normal rat (for SP) or rabbit (for CGRP) serum, 2) omission of the specific serum, and 3) adsorption of the SP or CGRP antiserum with the following peptides: CGRP, SP, somatostatin, calcitonin, neurotensin, cholecystokinin-8, enkephalins, and vasoactive intestinal polypeptide. The positive structures that satisfied all of the following criteria were regarded as being CGRP positive: immunostaining was negated in cases 1), 2), and when anti-CGRP serum was adsorbed with CGRP, but not with any other peptides. Similar criteria were also applied to judge reactions for SP.

Specificity of the second antisera. We tested the specificity of the the second antisera for the double-staining method as follows: 1) sections incubated with SP antiserum were reacted with Texas red-conjugated goat anti-rabbit IgG, 2) sections incubated with CGRP antiserum were reacted with FITC-conjugated donkey anti-rat IgG, and 3) and 4), sections incubated with CGRP antiserum and those incubated with SP antiserum were incubated with a mixture of both second antisera. No immunostaining was observed when sections incubated with SP antiserum were reacted with Texas red-conjugated goat anti-rabbit IgG, or when sections incubated with CGRP antiserum were reacted with FITC-conjugate donkey anti-rat IgG. In the sections incu-

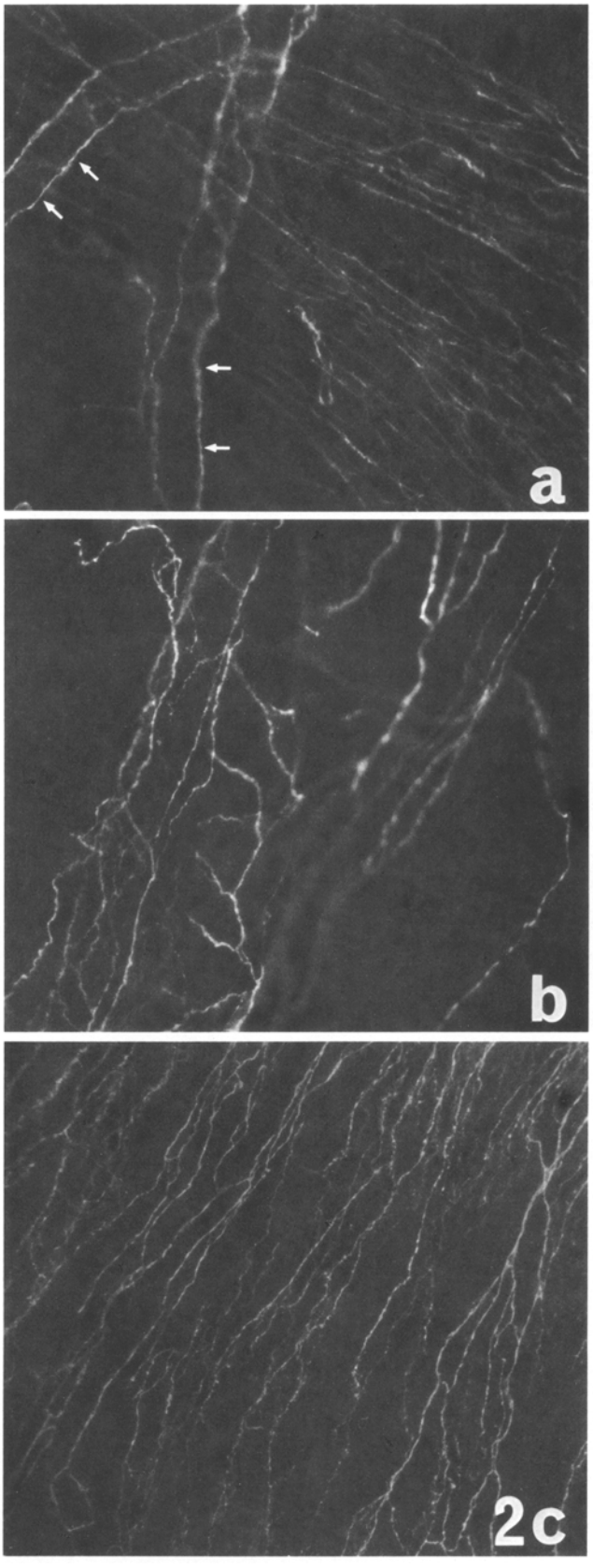

Fig. 2a–c. Fluorescence photomicrographs showing CGRPI fibers in whole-mount preparations of urinary bladder. **a** In circular muscle layer. ×120. **b** In deep portion of the submucosa. ×200. **c** In submucosa just beneath the epithelium. ×120. In the muscle layer, CGRPI fibers (*arrows*) along the blood vessels. Note the dense CGRPI fiber plexus just beneath the epithelium

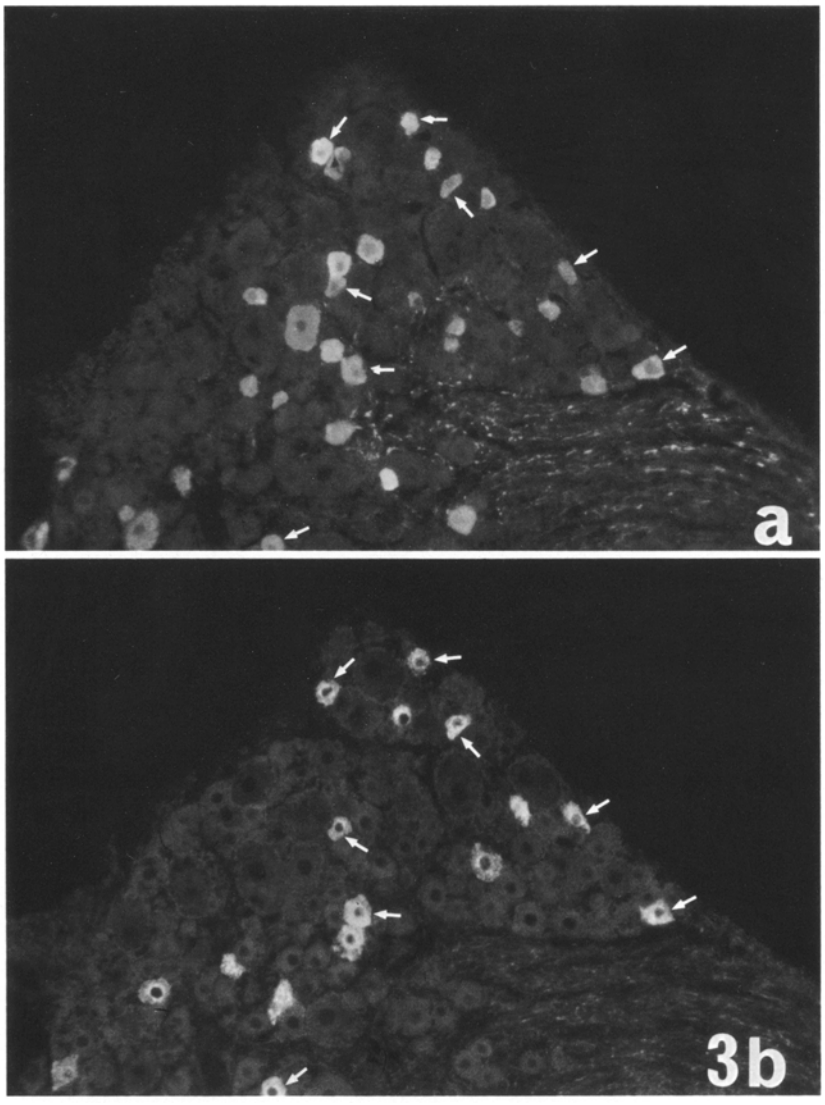

Fig. 3a, b. Fluorescence photomicrographs showing double-labeled neurons in the dorsal root ganglion at the level of L6 after injection of B-WGA into the bladder.
a CGRPI cells visualized by FITC in the dorsal root ganglion observed. B-dichroic mirror filter and IF 520–545 nm interference filter. ×120.
b B-WGA-labelled neurons in the same field of *a* visualized by streptavidin-Texas red. G dichroic mirror and 580-nm absorption filter. Double-labelled cells are indicated by *arrows*. ×120

bated first with SP antiserum and then with a mixture of both second antisera, specific fluorescence was found only with the B dichroic-mirror system. Those incubated first with CGRP antiserum and then with the mixture of second antisera were fluorescent only under the G dichroic-mirror filter.

Thus, we concluded that the two primary antisera showed specific differences, and were without cross-reactivity with the second antisera.

All of the materials were stained with cresyl violet for precise observation of the cell size in the dorsal root ganglia and urinary bladder.

Results

1. Distribution of CGRP fibers in the bladder

Frozen sections. A number of CGRPI fibers were present in the bladder in the rats not given colchicine (Figs. 1a, 4a). Circular and longitudinal muscle layer contained numerous CGRPI fibers running parallel to the muscle. In the submucosal layer, CGRPI fibers formed a dense plexus just beneath the epithelium, from which some CGRPI fibers entered the epithelium (Figs. 1a, 4a; arrows). No CGRPI-containing nerve cell bodies were detected even in rats injected colchicine.

Whole-mount preparations. CGRPI fibers entered the bladder either by a thick bundle of fibers (Fig. 1b) or by extending around blood vessels. These fibers extended to the fundic part of the bladder, branching into several thinner bundles. In the smooth muscle layer, a number of CGRPI fibers were seen running parallel to both longitudinal and circular muscles (Fig. 2a). These fibers seemed to be distributed evenly in the smooth muscle layer throughout the entire bladder. In the submucosa, CGRPI fibers formed a dense meshwork just beneath the epithelium (Fig. 2c) with some entering the epithelium. CGRPI fibers running along blood vessels were often seen in the muscle and submucosal layer (Fig. 2a, b). No CGRPI-bearing nerve cells bodies were found, even in rats given colchicine.

2. Origins of CGRPI fibers of the bladder

Injection of B-WGA into the bladder labeled a number of cells in the dorsal root ganglia at the levels of L6 (Fig. 3b)

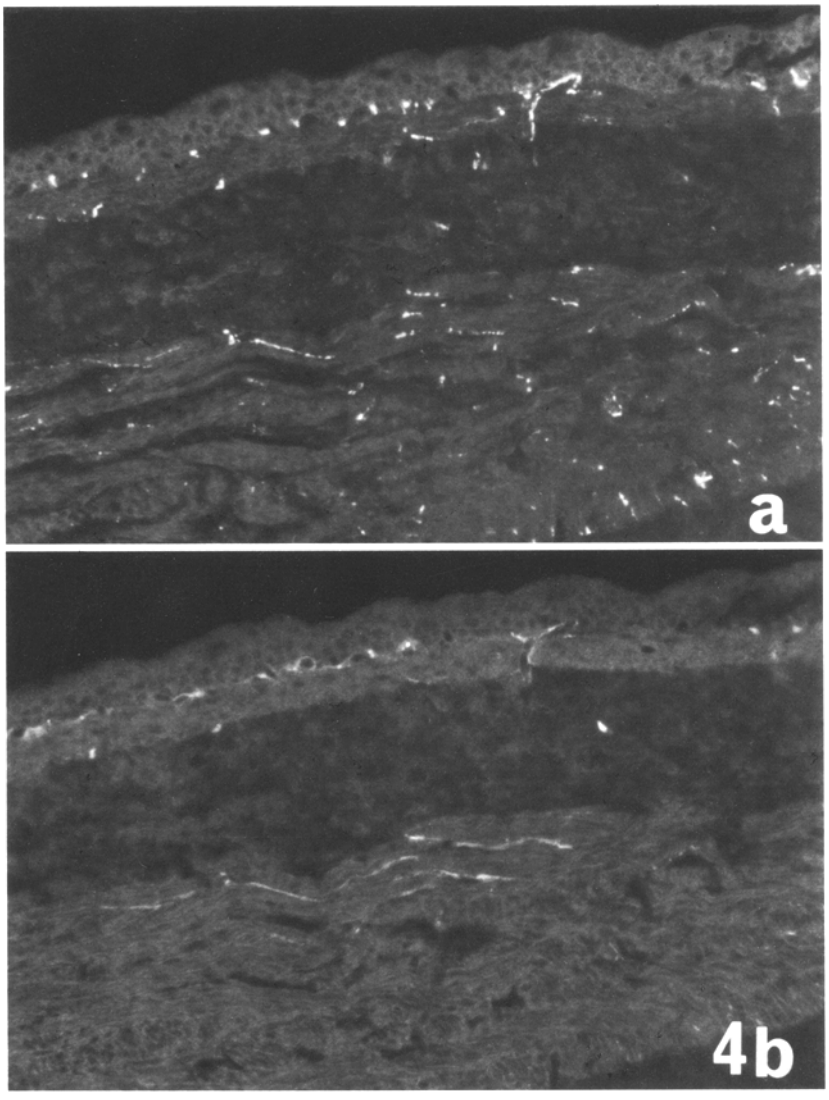

Fig. 4a, b. Double-immunofluorescence photomicrographs of the same frozen section of urinary bladder. CGRPI fibers showed red fluorescence with Texas red (**a**), and SPI fibers green FITC fluorescence (**b**). The pattern of running of the CGRPI and SPI fibers are very similar, though CGRPI fibers are more numerous. ×120

and S1, and a few cells at the level of L2. These cells were those containing red fluorescent granules in the soma when observations were made with the G dichroic mirror. No labeled cells were seen in dorsal root ganglia at other levels. The labeled cells were all small to medium-sized, about 15–40 μm in diameter; no large (about 40–45 μm in diameter) or giant (more than 45 μm in diameter) cells were labeled (Fig. 3b).

When observations of the same sections were made with the B dichroic mirror, numerous CGRPI cells with green fluorescence (Fig. 3a) were found. About 40% of the cells were CGRPI-positive. These cells ranged from small (below 20 μm in diameter) to large (40–45 μm in diameter). About 90% of the immunoreactive cells were of small to medium size; the remainder were large. Giant cells invariably lacked immunoreactivity.

By careful alternation of the two filters, we observed that some B-WGA cells in small to medium-sized cells contained CGRPI reactive structures (Fig. 3a, b, arrows). Double-labeled large cells were not detected.

When we verified the results on the retrograde labelling of B-WGA using HRP, we found that HRP-labeled cells in the dorsal root ganglia are similar to those labeled with B-WGA. When we verified the results of immunocytochemistry using FITC as a marker by using Texas red instead, a similar distribution of CGRPI cells in the dorsal root ganglia was found at the levels of L2, L6 and S1.

3. Coexistence with SP

Fig. 4 shows the distribution of CGRPI (Fig. 4a) and SPI (Fig. 4b) fibers in the bladder on the same section. Although CGRPI fibers outnumbered the SPI fibers slightly, many of these fibers had similar courses as though they were indeed single nerve fibers. However, from light microscopic observations of the immunoreactive fibers, we were not certain that CGRP and SP occurred together in single cells. We next examined this possibility at the level of the soma of the dorsal root ganglia at the levels of L6 and S1.

Fig. 5 shows CGRPI and SPI cells in the dorsal root ganglia at the level of L6 on the same section. The findings of CGRPI neurons in the dorsal root ganglia are described above. More than 20% of the ganglion cells were positive for SPI. These SPI cells also ranged from small to large. These results are same as for the sections stained for SP

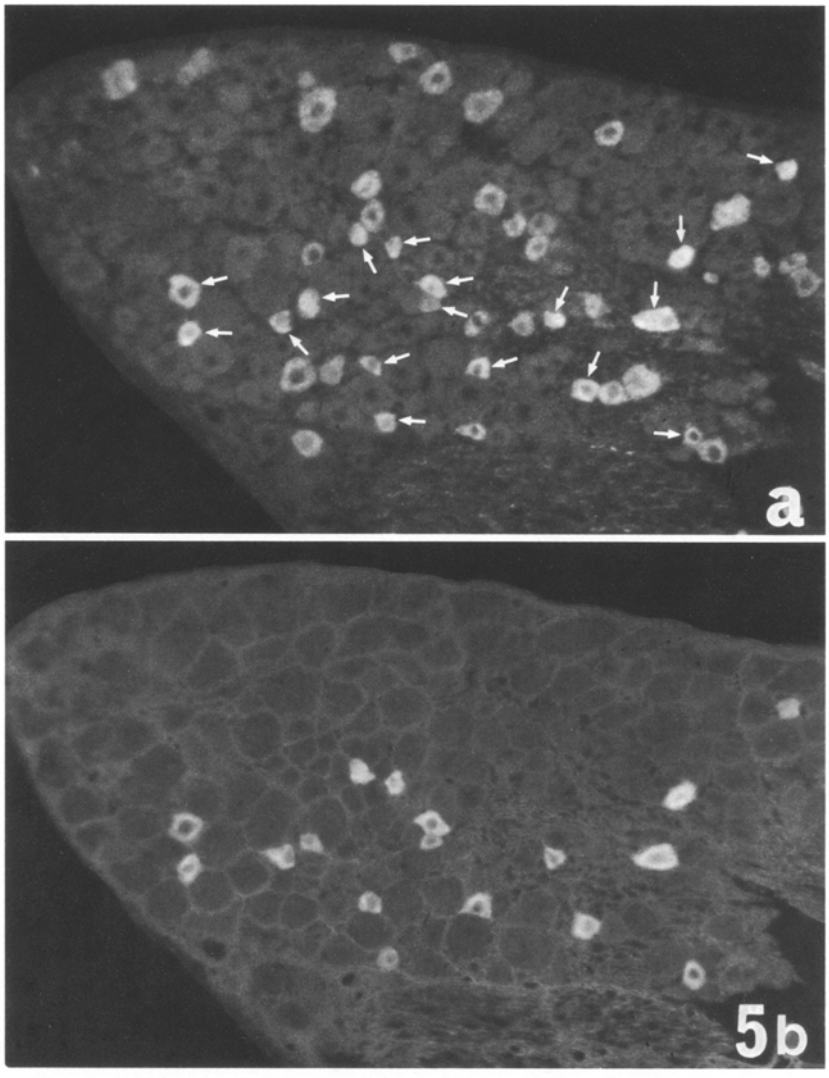

Fig. 5a, b. Double-immunofluorescence photomicrographs of the same frozen sections through the dorsal root ganglion at the level of L6. CGRPI structures displayed red fluorescence with Texas red (**a**), whereas SPI structures had weak green fluorescence (**b**). CGRPI and SPI cells were mostly small to medium-sized. Most of SPI cells were positive for CGRP. Note the presence of a few small to medium-sized CGRPI cells without SPI; large CGRPI cells also lack SPI. *Arrows* indicate double-labeled cells. ×120

only. Double-staining showed that most SPI cells were positive for CGRPI. A few small to medium-sized CGRPI cells and most large CGRPI cells lacked SP immunoreactivity.

Discussion

Distribution and origins of CGRPI fibers with reference to SP

Because there is a very high density of CGRPI fibers in the trigeminal spinal nucleus (Rosenfeld et al. 1984; Kawai et al. 1985; Lee et al. 1985b) and in the dorsal horn of the spinal cord (Gibson et al. 1984), it is likely that CGRP is transported both centrally and peripherally together with SP so that both may act as neurotransmitters or neuromodulators. Although the functional significance of the coexistence of CGRPI and SPI structures in single cells is not known, a similar pharmacological effect of these substances, vasodilation, has been reported (Edvinsson et al. 1981; Edvinsson and Uddman 1982; Fischer et al. 1983; Brain et al. 1984).

Sharkey et al. (1983) in a study using a retrograde fluorescent dye and immunocytochemistry also reported that SPI fibers in the bladder of the rat originate from the dorsal root ganglia, and that there are two main sources, Th12 to L2 and L6 and S1. Appelbaum et al. (1980), using HRP injections into the bladder, found labeled cells in the dorsal root ganglia, primarily at the levels of L6 and S1, but a few at L1 and L2. The results of our present study using B-WGA or HRP without and/or with combining immunocytochemistry agree well with those of Appelbaum et al. (1980). The differing results of Sharkey and his group, and other investigators, including us, may be due to differences in tracers. The fluorescent dye used by Sharkey et al. (1983) is less sensitive than HRP or B-WGA. Thus, to demonstrate a sufficient number of labelled cells, much dye must be injected, which might allow diffusion of the dye outside the bladder.

Our observation that only small to medium-sized cells were labeled by B-WGA is similar to that of Appelbaum et al. (1980), and of Nadelhaft and Booth (1984), who injected HRP into the pelvic nerve (see below).

Distribution of CGRPI cells in the dorsal root ganglia

CGRPI cells in dorsal root ganglia have already been reported (Gibson et al. 1984; Lee et al. 1985b). The fraction

of CGRPI cells among all immunoreactive cells in the cervical cord with respect to cell size is 60% small- to medium-sized cells and 40% large cells (Lee et al. 1985b). However, in our investigation using L6 and S1, the percentages were 90% small to medium and 10% were large. This difference may arise from the functional differences of the cervical cord and L6-S1.

Although the distribution patterns of CGRPI and SPI fibers was very similar. CGRPI fibers seemed to be slightly more numerous than SPI fibers. In addition, there were CGRPI cells lacking SPI in the dorsal ganglia; a few were small to medium-sized, but most were large. As mentioned above, only the small to medium-sized cells were labeled after injection of the retrograde tracer into the bladder. Thus, it is likely that small to medium-sized CGRPI cells lacking SPI also project to the bladder, while large CGRPI cells may project elsewhere.

Function of CGRPI in the bladder

The function of CGRP-containing fibers in the urinary bladder is not known. The urinary bladder contains many kinds of neurotransmitters and neuromodulators, such as acetylcholine, catecholamine (Alm and Elmer 1975), SP (Alm et al. 1978; Hökfelt et al. 1978; Gibbins 1983; Sharkey et al. 1983; Mattiasson et al. 1985; Yokokawa et al. 1985), neuropeptide Y (Mattiasson et al. 1985), enkephalin (Alm et al. 1981), and vasoactive intestinal polypeptide (Alm et al. 1977, Gu et al. 1984; Mattiasson et al. 1985). To explore the function of CGRP in the bladder, interactions among these bioactive substances, including CGRP, must be examined both at the terminal and somatic levels.

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