Peptide Immunoreactivity of Unmyelinated Primary Afferent Axons in Rat Lumbar Dorsal Roots

DANIEL L. McNEILL, KARIN N. WESTLUND, and RICHARD E. COGGESHALL

The present study demonstrates calcitonin gene-related peptide (CGRP), somatostatin (SOM), bombesin (BOM), and substance P (SP) at the electron microscopic level in lumbar dorsal root axons of normal rats. The highest percentages of labeled axons were for CGRP (14%) and then, in descending order, for SP (8.6%), SOM (6.8%), and BOM (3.1%). The labeled axons were exclusively unmyelinated for SP, SOM, and BOM, and predominantly unmyelinated for CGRP. These data are consistent with the data for labeled sensory cell bodies for these same compounds. We emphasized that these peptides were immunocytochemically visualized in the dorsal roots without experimental manipulation, such as colchicine or dorsal root ligation. Quantitative sampling of this type can be used to assay changes in response to physiological stimuli in numbers of sensory axons that contain identifiable concentrations of these peptides. (J Histochem Cytochem 37:1047-1052, 1989)

KEY WORDS: Calcitonin gene-related peptide; Substance P; Somatostatin; Bombesin; Sensory axons; Immunocytochemistry.

Introduction

Certain peptides have been demonstrated immunocytochemically in subpopulations of dorsal root ganglion cells. The functions of these peptides are not known, but it is probable that they are important in synaptic activity. One way to gain insight into the functions of these compounds would be to show changes in the numbers of sensory elements in response to physiologically relevant stimuli. There are two problems in obtaining such determinations. First, optimal cell body localizations often require colchicine or root ligation, and these procedures make interpretation of further manipulations difficult. Second, counting large numbers of labeled and unlabeled dorsal root ganglion cells is not completely routine. This is presumably the reason that published percentages of labeled cells are sometimes the result of impressions rather than precise counts. Accordingly, development of a simpler quantitative assay, not dependent on colchicine or root ligation for successful immunostaining, would be useful.

Determining numbers of immunostained axons in dorsal roots would be easier than numbers of immunostained cells in the dorsal root ganglia, but most axons that contain peptides are unmyelinated, and the usual immunocytochemical fixatives are not optimal for fine structural analyses. Fortunately, however, the recent introduction of sodium borohydride into immunocytochemistry is an advance (9) that permits good electron microscopic localizations without loss of immunoreactivity. Of particular benefit is the finding that dorsal root axons can be labeled without using such treatments as colchicine or root ligation to increase the concentration of antigen. Accordingly, the present study localizes calcitonin gene-related peptide (CGRP), substance P (SP), bombesin (BOM), and somatostatin (SOM) in the second, fourth, and sixth lumbar dorsal root axons (L2, L4, L6) of normal rats. These roots were chosen because they represent segments of the sympathetic (L2) and parasympathetic (L6) outflows, as well as a segment (L4) not concerned with visceral outflow. The data consist of the percentages and diameters of immunocytochemically labeled axons in these roots. The findings will be compared with the numbers and diameters of labeled sensory cells from which these axons arise. We emphasize that counts are relatively simple and that the animals are not otherwise manipulated. Furthermore, standard deviations of the percentages of labeled axons are relatively low. These counts can therefore serve as a basis for assaying changes in numbers of sensory axons that contain identifiable concentrations of the above peptides that result from physiological or behavioral stimuli.

Materials and Methods

Normal adult (200-250 g) Sprague-Dawley rats were anesthetized with...
Figure 1. Electron micrographs demonstrating calcitonin gene-related peptide (A) and substance P (B) immunoreactivity in unmyelinated (arrows) lumbar dorsal root axons. Examples of unlabeled unmyelinated axons (arrowheads) are present. Bars = 0.5 μm.
Figure 2. Bombesin- (A) and somatostatin- (B) immunoreactive unmyelinated axons (arrows) in lumbar dorsal roots. Examples of unlabeled unmyelinated axons (arrowheads) are present. Bars = 0.5 μm.
sodium pentobarbital (Nembutal; 35 mg/kg, ip). The chest was opened and normal saline at room temperature containing heparin (200 IU/100 cc) and sodium nitrite (0.02%) was perfused through the left ventricle until the right auricular effluent was free of blood. The perfusion fluid was then changed to a mixture of 3% glutaraldehyde, 3% paraformaldehyde, and 0.1% picric acid in 0.1 M phosphate buffer (PB), pH 7.4. After perfusion the second, fourth, and sixth lumbar (L2, L4, L6) dorsal roots were removed, cut into approximately 3-mm segments, and placed in PB overnight. The segments were then placed in 1.0% sodium borohydride in PB for 30 min, followed by six 5-min rinses in PB. Each segment was exposed to a graded series of ethyl alcohol solutions (10%, 25%, 40%, 25%, 10%) in PB and 1% uranyl acetate in 0.1 M maleate buffer, pH 6.0, for 1 hr, and then placed in 1% en bloc in 1% uranyl acetate for 2-3 days on a shaken table. Goat anti-rabbit IgG (1:50; 30 min) and rabbit PAP (1:100; 30 min) were then applied in succession. Pre-incubation solution was 3% normal goat serum in PBS, and diluent buffers and rinses were 1% normal goat serum in PBS. Triton X-100 was not used in these experiments. The reactions were visualized with 3,3'-diaminobenzidine hydrochloride and 0.01% hydrogen peroxide in 0.01% phosphate buffer for 6 min. Controls were treated as above except for omission of the primary antiserum. The antiserum treated as above except for omission of the primary antiserum. The antiserum treated as above except for omission of the primary antiserum. The antiserum treated as above except for omission of the primary antiserum.

### Table 1. Percentages of labeled unmyelinated axons in the L2, L4, and L6 dorsal roots of the rat

<table>
<thead>
<tr>
<th>Peptide</th>
<th>L2</th>
<th>L4</th>
<th>L6</th>
<th>L2, L4, and L6 combined</th>
</tr>
</thead>
<tbody>
<tr>
<td>CGRP</td>
<td>14.1 ± 3.4</td>
<td>15.9 ± 2.5</td>
<td>14.0 ± 4.0</td>
<td>14.0 ± 3.0</td>
</tr>
<tr>
<td>SP</td>
<td>8.2 ± 3.9</td>
<td>9.4 ± 4.1</td>
<td>8.1 ± 3.3</td>
<td>8.6 ± 3.5</td>
</tr>
<tr>
<td>SOM</td>
<td>5.7 ± 0.7</td>
<td>7.6 ± 1.7</td>
<td>6.9 ± 1.3</td>
<td>6.8 ± 1.4</td>
</tr>
<tr>
<td>BOM</td>
<td>3.6 ± 1.1</td>
<td>3.2 ± 0.9</td>
<td>2.3 ± 0.9</td>
<td>3.1 ± 1.0</td>
</tr>
</tbody>
</table>

* Percentages ± the standard deviations (mean ± SD) of labeled cells for calcitonin gene-related peptide (CGRP), substance P (SP), somatostatin (SOM), and bombesin (BOM); n = 5 for CGRP and n = 4 for other compounds.

One of our goals was to present percentages of labeled axons for the four peptides. The percentages for the unmyelinated axons in each of the roots are given in Table 1. Note that the greatest percentage of labeled axons was seen with CGRP, and then, in decreasing order, SP, SOM, and BOM. Analyses of variance indicated that the percentage of labeled CGRP axons was significantly greater than for the other compounds, whereas the percentage for BOM was significantly less. Substance P and SOM were not significantly different from one another. There were no significant differences for each antibody in terms of the percentage of labeled axons in relation to segmental levels. Note that the standard deviations are relatively small.

Some small myelinated fibers were labeled after the reaction for CGRP. The mean diameter (± SD) of these fibers was 1.90 ± 0.31 μm, and they made up 3.2% of the small myelinated fibers.

Our second major goal was to determine areas and idealized diameters for labeled unmyelinated axons. The mean diameters are given in Table 2. The mean areas and diameters of the CGRP axons at L6 were significantly greater than for the CGRP axons at L2 and L4. Other differences were not significant.

### Discussion

The present study shows that fine axons are identifiably labeled for the antibodies under investigation, even though high concentrations of glutaraldehyde are used in the fixation. This is because of the sodium borohydride treatment, which results in adequate fixation for electron microscopy without loss of immunoreactivity (9). When this procedure is used, approximately 14.0% of the unmyelinated axons are labeled for CGRP, 8.6% for SP, 6.8% for SOM, and 3.1% for BOM. In addition, some fine myelinated axons are labeled for CGRP. The conclusion is therefore that unmyelinated

### Table 2. Diameters (μm) of labeled unmyelinated axons in the lumbar dorsal roots (mean ± SD)

<table>
<thead>
<tr>
<th>Peptide</th>
<th>L2</th>
<th>L4</th>
<th>L6</th>
<th>L2, L4, and L6 combined</th>
</tr>
</thead>
<tbody>
<tr>
<td>CGRP</td>
<td>0.44 ± 0.10</td>
<td>0.43 ± 0.10</td>
<td>0.36 ± 0.17</td>
<td>0.46 ± 0.13</td>
</tr>
<tr>
<td>SP</td>
<td>0.47 ± 0.11</td>
<td>0.46 ± 0.14</td>
<td>0.39 ± 0.19</td>
<td>0.53 ± 0.12</td>
</tr>
<tr>
<td>BOM</td>
<td>0.54 ± 0.18</td>
<td>0.45 ± 0.13</td>
<td>0.37 ± 0.18</td>
<td>0.50 ± 0.16</td>
</tr>
<tr>
<td>SOM</td>
<td>0.62 ± 0.15</td>
<td>0.53 ± 0.18</td>
<td>0.44 ± 0.20</td>
<td>0.65 ± 0.22</td>
</tr>
</tbody>
</table>

* Average diameters of unmyelinated axons immunostained for calcitonin gene-related peptide (CGRP), substance P (SP), bombesin (BOM), and somatostatin (SOM); n = 5 for CGRP and n = 4 for the other compounds.

### Results

After the immunocytochemical reactions, both ends of the dorsal roots appeared light brown, whereas the rest of the segment appeared unstained. No labeled axons could be seen grossly or by light microscopy after sectioning. By electron microscopy, many unmyelinated axons could be seen labeled for CGRP, SP, SOM, or BOM at the ends of each segment (Figures 1 and 2). In addition, a few myelinated axons were labeled for CGRP. In heavily labeled axons, immunoreactive material spread throughout the axoplasm (Figures 1 and 2). With lighter labeling, the immunoreactive material was associated primarily with microtubules and the inner surface of the axonal membrane (Figures 1 and 2). No obvious cytological differences were seen in the reactions for the different peptides (Figures 1 and 2). Serial sections demonstrated that the same axons were labeled from section to section. Axonal labeling was not seen if the primary antibody was omitted.

### Table 2.

| Diameters (μm) of labeled unmyelinated axons in the lumbar dorsal roots (mean ± SD)² |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| CGRP            | SP              | BOM             | SOM             |
| L2  | 0.44 ± 0.10 | 0.43 ± 0.10 | 0.36 ± 0.17 | 0.46 ± 0.13 |
| L4  | 0.47 ± 0.11 | 0.46 ± 0.14 | 0.39 ± 0.19 | 0.53 ± 0.12 |
| L6  | 0.54 ± 0.18 | 0.45 ± 0.13 | 0.37 ± 0.18 | 0.50 ± 0.16 |

² Average diameters of unmyelinated axons immunostained for calcitonin gene-related peptide (CGRP), substance P (SP), bombesin (BOM), and somatostatin (SOM); n = 5 for CGRP and n = 4 for the other compounds.
fibers are the predominantly labeled population under the conditions of our experiments. It is of interest that for each antibody the percentages of labeled axons are not different in the various segments that we studied. A positive finding in relation to segmental differences is that the diameters of CGRP-immunoreactive axons are significantly larger in the L6 dorsal root than in the L2 and L4 dorsal roots. This conclusion does not hold for the other peptides we studied. We do not know the functional correlate of these morphological findings, but since L6 is one of the segments of the pelvic sensory inflow it is possible that slightly larger CGRP fibers are necessary for sensory input from pelvic organs. For interpeptide comparisons, the mean diameters of the axonal populations are not significantly different. Therefore, the differences in cell body diameters seen for SP and SOM by Price (18) are not mirrored in differences in axon diameter. Finally, in previous studies we determined the mean diameter of CGRP-labeled primary afferent fibers in the dorsal and dorsolateral funiculi and the tract of Lissauer (14,15). The average diameter of the CGRP-immunoreactive fibers in the lumbar dorsal roots is approximately equal to the average diameter of the fibers in the tract of Lissauer, and is considerably greater than those in the dorsal and dorsolateral funiculi. This suggests that the fibers in the dorsal and dorsolateral funiculi are collaterals of those in the tract of Lissauer.

One purpose of the present study was to compare the diameters and percentages of labeled axons with the sizes and percentages of dorsal root ganglion cells labeled with these same compounds. Substance P is the most commonly studied compound in this regard, and the usual report is that 20% of the cells are labeled, with the range of reported values being 8-33% (1,2,5-12,13, 16-18,20,21). For SOM the reported range is 4.2-20%, with 10% being the usually cited figure (4,6,12,21). For CGRP the values range from 35-50% (3,7,13), and for BOM the figure is approximately 5% (17). Most of these studies note that the small cells are exclusively labeled, but some reports indicate that, particularly for CGRP, a few larger cells are also labeled. These data are consistent with the axonal findings, in that the labeled axons are essentially unmyelinated and the labeled cells are essentially in the small-diameter category. In addition, the percentages of labeled axons are greatest for those compounds where the largest number of cells are labeled. The major difference is that relatively fewer axons are labeled. We believe that there are four possible reasons for this discrepancy. First, there are species and segmental differences between the various studies. Second, it is often necessary to give colchicine or ligate the roots for the ganglion cells to be visualized, and this may distort comparisons. An instructive example is the study of Lee et al. (13). These investigators found that 20% of rat ganglion cells were labeled when they used colchicine and 5-10% when they did not. The non-colchicine figures are in accord with our axon data. Third, there may be higher concentrations of antigen in the cell body, which in many cases is the result of root ligation or colchicine administration. Fourth, the fixation we use may not preserve as many antigenic sites as the predominantly formaldehyde fixatives (which are not, in general, suitable for electron microscopy) used in most other studies. Thus, numbers of labeled primary afferent axons would not be expected to correlate linearly with numbers of labeled cells, but this does not diminish the value of the fiber determinations as an assay. In particular, the axon evaluations show significant numbers of labeled fibers rapidly and simply without using ligation or colchicine to increase concentrations of the antigen, and the standard deviations of the axon determinations are relatively low. To conclude, our major finding is that a significant number of unmyelinated and fine myelinated axons contain enough of the peptides we are studying to be visualized by standard immunocytochemical techniques. This lends support to the idea that these compounds may act as transmitters. Furthermore, the finding that primary afferent axons in normal animals can be fixed adequately for electron microscopic study and still be labeled for these compounds offers the possibility of using the numbers of labeled axons as an assay of long-term primary afferent cell responses to physiological perturbations.

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