Correction. In the article "Precursor forms of substance P (SP) in nervous tissue: Detection with antisera to SP, SP-Gly, and SP-Gly-Lys" by Richard M. Kream, Thomas A. Schoenfeld, Robert Mancuso, Andrew N. Clancy, Walid El-Bermani, and Foteos Macrides, which appeared in number 14, July 1985, of Proc. Natl. Acad. Sci. USA (82, 4832-4836), Figs. 2 and 3 were printed with incorrect axis labels, and Fig. 3 contained incorrectly positioned internal labels. The corrected figures and their legends are shown below.

**Fig. 2.** Immunoreactivity of brain stem extract (A) and spinal cord extract (B) after fractionation by gel filtration in 2 M acetic acid on Ultrogel AcA 202 (fraction size, 5 ml). Standards were BSA, 67 kDa; cytochrome c, 12.2 kDa; aprotinin, 6 kDa; β-endorphin, 3.5 kDa; SP-G-K, 1.5 kDa (same position as SP); SP, 1.3 kDa; and tyrosine, 0.2 kDa. The equivalent of 30 ng of SP-LI (A, upper trace) and 20 ng of SP-LI (B, upper trace) were chromatographed and >90% recovered in the elution position of authentic SP as monitored by RIA before and after trypsin treatment of each fraction. Lower traces in A and B represent SP-G-K-LI after incubation of fractionated column standards (1 mg/ml; BSA, cytochrome c, aprotinin) with trypsin.

**Fig. 3.** HPLC elution profiles of SP-G-K-LI generated from a pooled spinal cord extract by trypsinization (A) and of SP-G-LI generated by carboxypeptidase B treatment of the trypsinized extract (B). Arrows denote the elution positions of the authentic peptides and their sulfoxide derivative (-O). In A, an aliquot of the trypsinzed extract containing 12 ng of SP-G-K-LI was injected onto the column and 83.7% total recovery of immunoreactivity was achieved in collected fractions (1 ml each) assayed with anti-SP-G-K. The peak of SP-G-K-LI corresponds to the elution position of authentic SP-G-K. In B, an equivalent aliquot treated with carboxypeptidase B yielded 5.6 ng of SP-G-LI (46.7% yield), of which 82.5% was recovered in collected fractions assayed with anti-SP-G. The peak of SP-G-LI corresponds to the elution position of authentic SP-G.
Precursor forms of substance P (SP) in nervous tissue: Detection with antisera to SP, SP-Gly, and SP-Gly-Lys

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Communicated by Mahlon Hoagland, March 18, 1985

ABSTRACT Antisera generated to substance P-Gly (SP-G) and substance P-Gly-Lys (SP-G-K), the likely unamidated COOH-terminally extended forms of substance P, were used to quantify and localize substance P precursor forms in hamster brain stem and spinal cord. The precursor determinant SP-G-K was liberated from larger heterogeneous forms by mild trypsinization of tissue extracts and was converted into the second precursor determinant, SP-G, by subsequent treatment with carboxypeptidase B. The basal levels of SP-G-K in brain stem and spinal cord were ~0.5 pg/mg of tissue and rose 43- to 64-fold after trypsinization. Basal levels of SP-G were comparable to those of SP-G-K and rose 10- to 29-fold after combined enzyme treatments. Immunohistochemical labeling of axons and somata with anti-SP-G-K increased dramatically after trypsinization. This labeling was eliminated by preadsorption with authentic SP-G-K but not substance P or SP-G. Gel-permeation chromatography revealed SP-G-K-like immunoreactivity in fractions corresponding to considerably higher molecular weight than mature substance P. Collectively, these results support the hypothesis that substance P is synthesized from larger precursors and demonstrate that extended precursor forms are normally present in the axons and somata of neural systems that synthesize substance P.

A plethora of recent research on the tachykinin, substance P (SP; Arg-Pro-Lys-Pro-Gln-Gln-Phe-Gly-Leu-Met-NH2), includes pharmacological and electrophysiological studies, immunohistochemical mapping, and studies on interactions with classical neurotransmitters (1). However, the number of studies on the biosynthesis of SP has been modest. Several groups have demonstrated incorporation of radiolabeled amino acids into SP in nervous tissues in vivo (2–5) and in vitro (6–10) and subsequent axonal transport to nerve terminals (2, 3, 5, 9, 10). Biosynthesis is presumed to be by conventional ribosomal mechanisms, since inhibitors of protein synthesis dramatically reduce isolate incorporation (2, 3, 5, 6–10). None of these previous studies has yielded information on precursor forms of SP representing biosynthetic intermediates in these tissues. Although small bioactive peptides typically arise from processing of larger, inactive precursors (11, 12), the evidence for larger precursor forms of SP has been circumstantial. First, in the aforesaid biosynthetic studies, lag periods of several hours preceded the appearance of radiolabeled SP, suggesting intervening processing of a large precursor. Second, substance P is amided at its COOH terminus. Recent work has shown that COOH-terminal amides are derived from the α-amino groups of adjacent glycine residues (13, 14). In precursor proteins to known peptides that have been sequenced, the COOH-terminal amino acid is always followed by glycine, and the glycine is always followed by lysine or arginine (12). Thus, it is highly probable that SP precursors may be identified by screening for the determinants substance P-Gly (SP-G) and substance P-Gly-Lys (SP-G-K). Consistent with this speculation, Nawa et al. (15) recently elucidated the sequences of two cDNAs coding for bovine brain preprotachykinins and observed that both of the determinants SP-G and SP-G-K are encoded in each cDNA. Furthermore, basic amino acids are coded at both the NH2 and COOH termini of the SP-G-K determinant, suggesting that the SP-G-K is formed through proteolysis at basic amino acid pairs by a trypsin-like enzyme, as for other neuropeptide precursors (11, 12).

In this report we describe the generation and characterization of antisera against the unamidated COOH-terminal extensions of SP, SP-G, and SP-G-K. Using these antisera, we are able to detect SP precursor-like immunoreactivity in nervous tissues after enzymatic treatment through complementary biochemical and immunohistochemical analyses.

MATERIALS AND METHODS

The analyses were performed in adult male Syrian hamsters. Reagents, radiochemicals, and sulfoxide derivatizations were as described (16). SP-G-K was custom-synthesized by Peninsula Laboratories (San Carlos, CA) and purified by ion-exchange HPLC, followed by reversed-phase HPLC. SP-G was generated by incubating 5 mg of SP-G-K with 25 μg of carboxypeptidase B in 0.1 M Na2HPO4 buffer (pH 8.0) for 15 min at 22°C and was purified with the same procedures as for SP-G-K. Purity of the analogs was assessed by amino acid analysis. Authentic SP (Sigma) was repurified with the above HPLC procedures. Reversed-phase elution was performed on a 8 × 100 mm NOVA-Pak C8 column using a 27–32% acetonitrile gradient in 0.1% CF3COOH over 30 min at a flow rate of 1 ml/min. Ion-exchange HPLC was performed on a 8 × 100 mm Radial Pak Partisil SCX-10 cation-exchange column using a gradient of 0.2 M K2HPO4/0.05 M KCl/25% acetonitrile/0.1% CF3COOH to 0.2 M K2HPO4/0.5 M KCl/25% acetonitrile/0.1% CF3COOH over 40 min at a flow rate of 1 ml/min. Fractions were monitored at 214 nm.

Generation of Antisera. Antisera were raised in 2- to 3-kg female New Zealand rabbits. SP (2 mg) was conjugated to succinylated thyroglobulin (4 mg), using 10 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide similarly to the procedure of Mroz and Leeman (17). Incorporation was 80% as monitored by HPLC elution of unconjugated peptide. SP-G-K (2 mg) and SP-G-Lys (2 mg) were conjugated to phenylmethlysulfonyl fluoride-treated bovine serum albumin (BSA; 4

Abbreviations: SP, substance P; SP-G, SP-Gly; SP-G-K, SP-Gly-Lys; [125I]BH, radiiodinated Bolton–Hunter reagent-conjugated; LI, like immunoreactivity; BSA, bovine serum albumin.

†To whom reprint requests should be addressed.
mg) in separate incubations, using 0.4% glutaraldehyde. Incorporation (>80%) was monitored by HPLC elution of unconjugated peptide. Conjugated peptides were emulsified with an equal volume of Freund’s complete adjuvant (0.7 ml final volume) and injected intradermally (200–300 μg). Animals were given 4–6 booster injections at 1-month intervals and were bled 2 weeks after each boost. Sera were screened for binding of radioiodinated peptide tracers, prepared in a modification of our procedure (16). Radioiodinated Bolton–Hunter reagent-conjugated (125I-BH) SP, SP-G and SP-G-K were purified to homogeneity by reversed-phase HPLC. The specific activities were ~2000 Ci/mmol (1 Ci = 37 GBq).

Biochemical Analyses. Ten hamsters were stunned by placing them for 20 sec in 100% CO₂ and then decapitated and exsanguinated. Spinal cords were obtained by cutting the cauda equina and applying hydraulic pressure to the back of the spinal column with a saline-filled syringe. Brain stems were obtained by making a vertical cut at the border between the posterior hypothalamus and midbrain and making a horizontal cut at the base of the cerebellar peduncles. The tissue samples were frozen quickly by placing them on dry ice and then were stored at −70°C.

Tissues were extracted in 50 volumes of 2 M acetic acid. Supernatants were lyophilized and reconstituted in distilled water. Aliquots of the extracts were assayed for SP, SP-G-K, and SP-G immunoreactivity before and after trypsin and carboxypeptidase B treatments, with the addition of 0.01 M EDTA to inhibit carboxypeptidase A. These treatments were similar to those used in opioid precursor studies (18). Separate RIAs were performed for SP, SP-G-K, and SP-G in a modification of a previous procedure (16). HPLC/RIA analyses of the SP-G-K-like and SP-G-like immunoreactivities enzymatically generated from a pooled spinal cord extract were performed using a 3.9 × 150 mm Waters C₁₈ Nova-Pak column. Elution was initially achieved under isocratic conditions (8 min) with a mobile phase of 15% (wt/vol) acetonitrile in 0.1% CF₃COOH, followed by a linear 15–40% gradient of acetonitrile in 0.1% CF₃COOH (12 min). The flow rate was maintained at 1 ml/min.

Immunohistochemical Analyses. Ten hamsters were anesthetized with pentobarbital and perfused transcardially with buffered saline followed by fixative [2% paraformaldehyde and 0.15% saturated picric acid in 0.1 M phosphate buffer (PB) at pH 7.4]. Blocks of spinal cord, medulla, pons, and midbrain were postfixed for 1–6 hr and then cut into 0.1 M PB at 30 μm on a Vibratome. Sections from one animal were processed with the peroxidase-antiperoxidase method, using procedures described previously (19). Sections from nine animals were processed with the indirect immunofluorescence method, using similar procedures except that following the incubations in primary antisera and postincubation rinses, the sections were incubated at 4°C for 2 hr in rhodamine-conjugated goat anti-rabbit immunoglobulin (Cappel Laboratories, Cochranville, PA) diluted 1:500 with 1% normal goat serum/0.02% Triton X-100/0.1 M PB. Optimal dilutions of antisera were determined over the range 1:200 to 1:10,000. Adjacent sections from each animal were subjected to the various experimental or control treatments and incubated with the various antisera in parallel. For sets of sections from seven of the hamsters, incubation in primary antiserum was preceded by mild trypsinization (0.2–10 μg of trypsin/ml of 0.1 M PB for 1 hr; optimal concentration, 5 μg/ml). Prior to trypsin treatment, sections were rinsed in 0.4% Triton X-100/0.1 M PB for 30 min. After trypsinization, sections were treated for 15 min with phenylmethylsulfonyl fluoride diluted in 0.1 M PB at 10 times the concentration of trypsin. Sections were then rinsed five times in PB and preincubated in 1% normal goat serum/0.02% Triton X-100/0.1 M PB for 1 hr prior to incubation in primary antiserum. In four cases, adsorption controls were run for each of the antisera with each of the purified antigens over the concentration range 0.01–10 μM to assess specificity and crossreactivity; trypsinized sections were included in these adsorption control runs. Adsorption controls with the two carrier proteins that had been conjugated to the antigens prior to immunization also were performed, at a concentration of 1 mg/ml. Antisera were adsorbed for 1 hr prior to introduction of tissue.

RESULTS

Radioimmunoassays. Three distinct antisera displaying specific binding to an 125I-BH peptide tracer homologous to the original antigen were generated (Fig. 1). Anti-SP bound 50% of added 125I-BH-SP (=5 fmol of peptide) at a final dilution of 1:200,000. At low dilutions (1:1000–2000), anti-SP bound 8–12% and 3–6% of 125I-BH-SP-G and 125I-BH-SP-G-K, respectively. Anti-SP-G bound 50% of added 125I-BH-SP-G at a final dilution of 1:12,000 with minimal crossreactivity to 125I-BH-SP and 125I-BH-SP-G-K at dilutions greater than 1:100. Anti-SP-G-K bound 50% of added 125I-BH-SP-G-K at a final dilution of 1:100,000. However, anti-SP-G-K displayed a 30% crossreactivity to 125I-BH-SP-G at dilutions of 1:1000–2000, which diminished to <5% at dilutions greater than 1:32,000 and was undetectable at dilutions greater than 1:100,000. Binding of 125I-BH-SP by anti-SP-G-K was ~5% at dilutions of 1:1000–2000.

Specific and sensitive RIAs were established with anti-SP, anti-SP-G, and anti-SP-G-K used at final dilutions of 1:350,000, 1:20,000, and 1:150,000, respectively, at which 35–40% of added radioactivity was bound and the respective RIA standard curves displayed dose-dependent dependence of 125I-BH-SP, 125I-BH-SP-G, and 125I-BH-SP-G-K by nonradioactive SP, SP-G, and SP-G-K. The sensitivities of the respective RIAs, defined as 10% displacement of added tracer, were 1 pg of SP, 1.5 pg of SP-G, and 2 pg of SP-G-K per tube. The relative immunoreactivities of SP, SP-G,
SP-G-K, their sulfoxide derivatives, SP COOH-terminal fragments, and a variety of other neuropeptides with three antisera were reported for high-quality tide, respectively.

peptides SP-G-K, and displayed negligible potencies (<0.0005%) were three K, after enzymatic digestion. Resistivity of SP-LI to trypsin and carboxypeptidase B digestion provided strong evidence that we have measured authentic SP in these tissues (16, 21–23). In contrast to SP-LI, levels of SP-G-K-LI rose markedly after trypsin digestion, from 0.42 to 27.0 pg/mg of tissue. Values remained unchanged after mild trypsinization, and decreased <20% after trypsin and carboxypeptidase B digestion. The RIAs for SP antisera showed high specificity for the homologous peptide and their respective sulfoxide derivatives. In the SP-G RIA, anti-SP-G exhibited 0.02–0.03% crossreactivity to SP and related COOH-terminal fragments SP(3–11) and SP(4–11), 0.09% to SP-G-K, and <0.001% to physalaemin and substance K. Conversely, SP-G was found to have 1.4% of the potency of SP-G-K in competitive binding to anti-SP-G-K, and all other SP-related peptides tested in the SP-G RIA were of minimal efficacy. Finally, the other neuropeptides displayed negligible potencies (<0.0005%) in each of these RIAs.

Tissue Levels of SP Precursor Forms. SP-like, SP-G-like, and SP-G-K-like immunoreactivities (SP-LI, SP-G-LI, and SP-G-K-LI, respectively) were measured in acetic acid extracts of hamster brain stem and spinal cord before and after enzymatic treatments (Table 2). Control SP-LI levels were ~200 pg/mg of tissue in brain stem and spinal cord, remaining unchanged after mild trypsinization, and decreased <20% after trypsin and carboxypeptidase B digestion. Resistivity of SP-LI to trypsin and carboxypeptidase B digestion provides strong evidence that we have measured authentic SP in these tissues (16, 21–23). In contrast to SP-LI, levels of SP-G-K-LI rose markedly after trypsin digestion, from 0.42 to 27.0 pg/mg of tissue for brain stem and from 0.66 to 28.3 pg/mg of tissue for spinal cord, increases of 64- and 43-fold, respectively. After carboxypeptidase B digestion, SP-G-K-LI values returned to near control levels, consistent with the removal of the COOH-terminal lysine from the antigenic determinant. In control incubations spiked with 100 pg of SP-G-K, >90% of immunoreactivity was lost after carboxypeptidase B treatment. Basal levels of SP-G-LI were 0.29 and 0.81 pg/mg of tissue in brain stem and spinal cord, respectively. These values remained unchanged after trypsin treatment and rose markedly only after treatment with trypsin and carboxypeptidase B, to 8.3 and 7.9 pg/mg of tissue (29- and 10-fold increases) for brain stem and spinal cord, respectively. In control incubations spiked with 100 pg of SP-G-K, ~80 pg of SP-G was generated after carboxypeptidase B digestion.

Table 1. Relative immunoreactivities with the three antisera

<table>
<thead>
<tr>
<th>Peptide</th>
<th>anti-SP</th>
<th>anti-SP-G</th>
<th>anti-SP-G-K</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP</td>
<td>100.0</td>
<td>0.030</td>
<td>0.005</td>
</tr>
<tr>
<td>SP-G</td>
<td>0.006</td>
<td>100.0</td>
<td>1.40</td>
</tr>
<tr>
<td>SP-G-K</td>
<td>0.100</td>
<td>114.0</td>
<td>0.730</td>
</tr>
<tr>
<td>SP-G-K-LI</td>
<td>0.030</td>
<td>0.000</td>
<td>100.0</td>
</tr>
<tr>
<td>SP(3–11)</td>
<td>0.010</td>
<td>0.010</td>
<td>96.0</td>
</tr>
<tr>
<td>SP(4–11)</td>
<td>0.010</td>
<td>0.000</td>
<td>96.0</td>
</tr>
<tr>
<td>[D-Ala²]SP(5–11)</td>
<td>45.5</td>
<td>0.004</td>
<td>&lt;0.0002</td>
</tr>
<tr>
<td>SP(6–11)</td>
<td>0.100</td>
<td>&lt;0.0005</td>
<td>&lt;0.0002</td>
</tr>
</tbody>
</table>

Values are calculated on a % molar basis, with 100% corresponding to the amount of homologous peptide needed to displace 50% of bound 125I-BH tracer: 12.0 fmol of SP for anti-SP; 15.0 fmol of SP-G for anti-SP-G; 12 fmol of SP-G-K for anti-SP-G-K.

### Table 2. Effects of enzymatic treatments

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Immunoreactivity, pg/mg of tissue (mean ± SD, n = 10)</th>
<th>Trypsin + carboxypeptidase B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain stem</td>
<td>193.8 ± 26.4</td>
<td>201.9 ± 50.5</td>
</tr>
<tr>
<td>Spinal cord</td>
<td>33.3 ± 0.03</td>
<td>203.8 ± 45.0</td>
</tr>
</tbody>
</table>

Tissue extracts were untreated (control) or incubated with trypsin (0.5 µg/ml) for 16 hr at 22°C. Aliquots of trypsinized samples were incubated with carboxypeptidase B (0.5 µg/ml) for 30 min at 22°C.

### Table 3. Immunoreactivity of SP precursors

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Immunoreactivity, pg/mg of tissue (mean ± SD, n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP</td>
<td>100.0 ± 0.03</td>
</tr>
<tr>
<td>SP-G</td>
<td>0.006 ± 0.01</td>
</tr>
<tr>
<td>SP-G-K</td>
<td>0.100 ± 0.01</td>
</tr>
</tbody>
</table>

**HPLC Elution and Characterization of SP-G-K-LI and SP-G-LI.** SP-G-K-LI generated from a pooled spinal cord extract by trypsinization was characterized by gradient reversed-phase HPLC. The major immunoreactive fraction was coeluted with authentic SP-G-K, and none of the recovered SP-G-K-LI was coeluted with the sulfoxide derivative of SP-G-K (Fig. 3A). SP-G-LI generated by carboxypeptidase B treatment of trypsinized extract was analyzed similarly (Fig. 3B). The major immunoreactive fraction was coeluted with authentic SP-G and no detectable SP-G-LI was coeluted with the sulfoxide derivative. Thus, major portions of the protease-generated immunoreactivities showed the same chromatographic behavior as the hypothesized precursor forms of SP.

### Immunohistochemical Localization.** Anti-SP produced prominent labeling (optimal dilution, 1:5000) in all of the neural systems of the spinal cord and brain stem previously shown to contain SP-LI (24). This labeling was eliminated by
preamsorption with authentic SP but not by preadsorption with SP-G-K, SP-G, thyroglobulin, or BSA. Anti-SP-G-K produced axonal labeling and fine, granular somal labeling in the same neural systems (optimal dilution, 1:1000). However, this labeling was extremely faint and confidently detectable above background only at high power (63 x objective) under immersion. The only exception to this generalization was the sensory fibers of the substantia gelatinosa in the spinal cord and medulla. The labeling in this fiber system with anti-SP-G-K was prominent, though less dense than that produced with anti-SP (Fig. 4A and B). The labeling with anti-SP-G-K was eliminated by preadsorption with authentic SP-G-K (Fig. 4C), was noticeably reduced but not eliminated by preadsorption with SP (Fig. 4D) or SP-G, and was unaffected by preadsorption with thyroglobulin or BSA (results not shown). Thus, some of the labeling with anti-SP-G-K may represent crossreactivity with SP or SP-G, but the normal presence of SP-G-K in SP-synthesizing central neural systems and in the terminal fields of C-fiber peripheral inputs is indicated. Anti-SP-G did not produce immunohistochemical labeling.

Trypsinization of adjacent sections markedly increased the density of axonal and somal labeling with anti-SP-G-K in regions where labeling was normally faint or undetectable (Fig. 5), but trypsin treatment did not affect the labeling with anti-SP or produce labeling with anti-SP-G. The trypsin-induced labeling with anti-SP-G-K was eliminated by preadsorption with SP-G-K; was not visibly affected by preadsorption with SP, thyroglobulin, or BSA; and was noticeably reduced but not eliminated by preadsorption with SP-G. The trypsin-induced axonal labeling was predominantly in segments with swellings along their extents; labeling of fine terminal arbors, as typically seen widely with anti-SP and normally in the substantia gelatinosa with anti-SP-G-K, was not augmented. The levels of more extended precursor forms thus appear to be higher in axonal segments than in terminal arbors. The distributions of densely labeled precursor forms after trypsinization correspond to the descending and ascending projections of brain stem systems previously reported to contain SP-LI (24).

**DISCUSSION**

We have demonstrated the presence of SP precursor forms in nervous tissue by using antibodies to unamidated COOH-

**Fig. 3.** HPLC elution profiles of SP-G-K-LI generated from a pooled spinal cord extract by trypsinization (A) and of SP-G-LI generated by carboxypeptidase B treatment of the trypsinized extract (B). Arrows denote the elution positions of the authentic peptides and their sulfoxide derivative (--O). In A, an aliquot of the trypsinized extract containing 12 ng of SP-G-K-LI was injected onto the column and 83.7% total recovery of immunoreactivity was achieved in collected fractions (1 ml each) assayed with anti-SP-G-K. The peak of SP-G-K-LI corresponds to the elution position of authentic SP-G-K. In B, an equivalent aliquot treated with carboxypeptidase B yielded 5.6 ng of SP-G-LI (46.7% yield), of which 82.5% was recovered in collected fractions assayed with anti-SP-G. The peak of SP-G-LI corresponds to the elution position of authentic SP-G.

**Fig. 4.** Localization of SP-LI (A) and SP-G-K-LI (B) in sensory fibers of the substantia gelatinosa, and effects of preadsorption of anti-SP-G-K with 1.0 μM SP-G-K (C) or 1.0 μM SP (D). Adjacent sections were taken from the cervical spinal cord and processed in parallel. Preadsorption of anti-SP-G-K with authentic SP-G-K eliminated the staining of sensory fibers, whereas preadsorption of this antiserum with SP did not. (Bar = 100 μm.)
Fig. 5. Effects of trypsinization (5 μg/ml for 1 hr) on SP-G-K-LI. Control (A, C, and E) and trypsinized (B, D, and F) sections were processed in parallel. The photomicrographs are from the region of the spinal canal in the cervical spinal cord (A and B), the pontine dorsal raphe nucleus (C and D), and the midbrain ventral tegmental area (E and F). Marked increases of SP-G-K-LI were produced by treatment of the sections with trypsin. (Bar = 100 μm for A, B, E, and F; 40 μm for C and D.)

terminal extensions of the peptide. We have liberated SP-G-K-LI from larger heterogeneous forms by mild trypsinization of extracts, followed by the conversion into SP-G-LI determinants by carboxypeptidase B treatment, and have shown in HPLC/RIA analyses that a major portion of the protease-generated immunoreactivity has chromatographic properties of extended forms of SP. By gel-permeation chromatography, fractions of considerably higher molecular weight than mature SP have been identified as containing SP-G-K-LI. Finally, a coordinated series of immunohistochemical analyses localized SP precursor reactivity in axons and somata of various brain areas before and after treatment with trypsin, complementing our biochemical data.

Initial chemical characterization of SP-G-K-LI indicates the presence of this determinant in a broad range of proteins of intermediate molecular weights (Fig. 2). Nawa et al. (15) calculated the molecular weights for α- and β-preprotachykinins to be approximately 13,000 and 15,000, respectively, consistent with the upper range of SP-G-K-LI in our fractionation scheme. The low molecular weight species of SP-G-K-LI that were eluted near the position of authentic SP may represent steady-state levels of SP extended at either terminus by a few amino acids—e.g., SP-Gly-Lys-Arg, as predicted from the cDNA sequences (15).

Although the measured basal levels of SP-G-K-LI represent a heterogeneous population of proteins of differing immunoreactivities, with trypsin treatment SP-G-K-LI is converted to quantifiable small determinants equivalent to total precursor forms in the tissue. Thus, the SP-G-K-LI levels of 27-28 pg/mg of tissue after trypsinization indicate that in the steady state, total SP precursor forms represent 12.5% of mature SP on a molar basis in both brain stem and spinal cord. Levels of generated SP-G-LI represent, however, only about 5% of mature SP levels. Thus, it is plausible that >50% of the generated SP-G-K-LI (equivalent to 7.5% of mature SP levels) represents a related Gly-Lys-extended tachykinin or incomplete trypsin cleavage products not quantifiable as SP-G-LI after carboxypeptidase B treatment. The sequence of the cDNA coding for β-preprotachykinin from bovine striatum indicates the SP-G-K sequence as residues 58-70 (15). In addition, residues 97-109 represent a Gly-Lys-extended form of substance K, a kassinin-like tachykinin, with a COOH-terminal sequence (Phe-Val-Gly-Leu-Met-Gly-Lys) very similar to that of SP-G-K (15). At this time, we cannot rule out that anti-SP-G-K displays some crossreactivity with substance K-Gly-Lys in our analyses.

Overall, the present results support the view that biosynthesis of mature SP involves cleavage of larger precursors at pairs of basic amino acids by a trypsin-like enzyme to generate SP-G-K, followed by hydrolysis of the Gly-Lys bond by a carboxypeptidase B-like enzyme and then amidation from the α-amino group of the glycine residue. Inhibitors of these enzymatic activities may constitute novel ways of blocking nociception, and candidate inhibitors can now be screened with the antisera and RIAs reported here.

This work was supported by New England Medical Center Grant BRS91807419 (to R. M. K.) and National Institutes of Health Grant NS-12344 (to F. M.).