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Immunohistochemical and Chromatographic Studies of Peptides With Tachykinin-Like Immunoreactivity in the Central Nervous System of the Lamprey

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Tachykinins Substance P Physalaemin Neurokinin A Kassinin Eledoisin Lamprey Spinal cord Brain

TACHYKININS (TKs) constitute a family of neuropeptides which are present in several phyla of the animal kingdom and have similar C-terminal amino acid sequences and similar spectra of biological activities [8]. The first discovered and most investigated TK is substance P (SP), which is present in the mammalian nervous system (see [30]). SP-immunoreactive (SP-IR) material has also been found in the central nervous system of birds, reptiles, amphibians, fishes and tunicates and even in *Hydra*, a coelenterate [9, 10, 17, 21, 26, 34, 35, 37, 38]. Rigorous controls are, however, necessary to demonstrate that such SP-IR material actually is SP. Several TKs and TK-like peptides other than SP have now been detected in amphibians and mammals [8, 19, 20, 23, 27–29, 39, 40, 44], some of which might cross-react with antisera raised against SP.

In the lamprey spinal cord and brain, SP-IR material has

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ABBREVIATIONS

A_1–A_3	peaks eluted at anion exchange chromatography
C_1–C_5	peaks eluted at cation exchange chromatography
CC	central canal region
DCp	dorsal cell plane
DCR	dorsal cell region
DH	dorsal horn
DLC	dorsolateral axon column
DMC	dorsomedial axon column
Dp	dorsal plane
DR	dorsal root
ELE	eledoisin
H_1–H_7	peaks eluted at HPLC
HPLC	high performance liquid chromatography
5-HT	5-hydroxytryptamine (=serotonin)
Ichth	Ichthyomyzon unicuspis
Ip	intermediate plane
KAS	kassinin
Lamp	lampetra fluviatilis
LC	lateral axon column
LCC	lateral column of cell bodies
LT_1	presumed lamprey tachykinin for which anti-SP_1 has preferential affinity
LT_2	presumed lamprey tachykinin for which anti-SP_2 has preferential affinity
LT_S	presumed lamprey tachykinin for which anti-SP_S has preferential affinity
MFR	Müller-fiber region
Mp	Müller-fiber plane
NKA	neurokinin A (=substance K, neurokinin α, neuromedin L)
NKB	neurokinin B (=neurokinin β, neuromedin K)
NPK	neuropeptide K
PHY	physalaemin
RIA	radioimmunoassay
SP	substance P
TK	tachykinin
VLC	ventrolateral column
VR	ventral root

been detected in cells and fibers with monoclonal antibodies raised against SP ([6,41] and unpublished). In single cases some of this SP-IR material was found in a few cells containing 5-hydroxytryptamine (5-HT) ([41]; cf. [5, 14, 18]). However, the demonstration of SP-like material in the lamprey has up till now been associated with some problems. For example, Homma [16], using another antiserum was not able to demonstrate SP-IR material in the lamprey CNS [16]. Further studies on the possible occurrence and character of TKs in the lamprey therefore appear of value.

An analysis of lamprey TKs is interesting also from a phylogenetic point of view, since lampreys are in many respects one of the most primitive groups of living vertebrates. The overall morphology of their body and brain have been changed remarkably little during their evolution for at least 400 million years [2,43]. Moreover, among the vertebrates, lampreys have the lowest degree of encephalization [3,31], and yet this low encephalization is not due to regression, since the basic anatomical systems are present in lampreys [1].

In order to characterize the TK-IR material in the lamprey CNS, immunohistochemical techniques were used as well as chromatographic methods combined with radioimmunoassay (RIA) based on several antisera raised against various TKs. The present findings indicate that the lamprey CNS contains possibly new, not earlier described TKs. A preliminary report of this work has appeared [42].

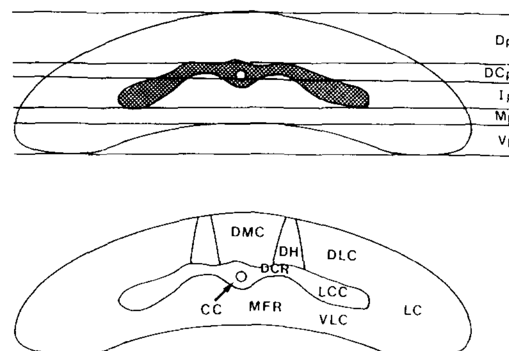


FIG. 1. Subdivision of the lamprey spinal cord. In the upper drawing of the flattened spinal cord the various dorsoventral planes are indicated as obtained when horizontal sections are made. Dotted field indicates cell body area. The lower drawing shows the nomenclature used in denoting the different regions of the lamprey spinal cord. CC=central cana., DCp=dorsal cell plane, DCR=dorsal cell region, DH=dorsal horn, DLC=dorsolateral axon column, DMC=dorsomedial axon column, Dp=dorsal plane, Ip=intermediate plane, Lc=lateral column, LC=lateral axon column, LCC=lateral column of cell bodies, MFR=Müller-fiber region, Mp=Müller-fiber plane, VLC=ventrolateral axon column, Vp=ventral plane.

METHOD

Animals

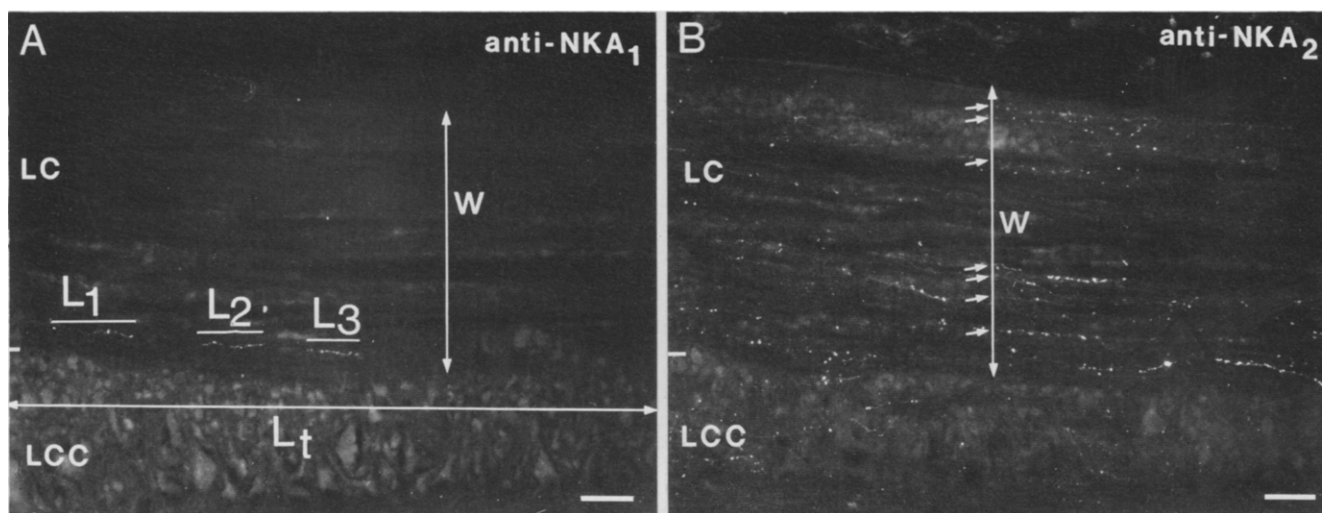
Adult lampreys of two species were used, the river lamprey (*Lampetra fluviatilis;* 15–40 cm, labeled *Lamp*), and the silver lamprey (*Ichthyomyzon unicuspis;* 15–25 cm, labeled *Ichth*). They were caught in Sweden and Iowa (USA) respectively, and kept in aerated aquaria at a temperature of 5–10°C. In the text, both species will be referred to as "lamprey," while the species will be mentioned in the figure legends. They were anaesthetized with tricaine methanesulfonate (MS 222, Sandoz). The spinal cords were subdivided in horizontal planes and in regions as shown in Fig. 1 (cf. [36,41]).

Immunohistochemistry

Immunohistochemistry. Spinal cords were dissected and fixed for 1–2 hr in an ice-cold solution of para-formaldehyde (40 g/l) in 0.16 M phosphate buffer, pH 6.9 with picric acid (2 g/l). After fixation, the tissue was rinsed at least 24 hr in 5% sucrose in 0.1 M phosphate buffer, pH 7.2, containing 0.01% Na-azide and 0.02% Bacitracin (Sigma) at 4°C. The sectioning (8 or 14 μm sections), the tissue processing, the microscopic equipment and the photographic procedures have been described earlier [12, 13, 15, 41]. Fiber counts were made in horizontal sections of the rostral and caudal parts of the spinal cords (both species). The density of fibers in the various regions was measured in two different ways (cf. Fig. 2). (1) For regions with a low fiber density (less than about 200 fibers/mm²), the sum of the fiber lengths over a given stretch of a homogeneous region was determined, as was the length of this stretch and the width of the region (Ip, LC in Fig. 2A). (2) for regions with a higher fiber density, the number of fibers transected by an imaginary line (formed by the ocular micrometer) was counted for a homogeneous region (Ip, LC in Fig. 2B), and the width of this region was determined.

Pre-incubation ("absorption") experiments. The various antisera were diluted with 0.3% Triton in 0.1 M phosphate

less than 200 fibers / mm^2 **more than 200 fibers / mm^2**



Density: $D = \dfrac{L_1 + L_2 + L_3}{L_t\ w\ t}$ $D = \dfrac{N}{w\ t}$

(t = thickness of the section ; N = number of crossings)

FIG. 2. Survey of two methods of fiber counting. These and other micrographs have been taken with relatively long exposure times to visualize also autofluorescent neuronal elements, in order to show the relative position of TK-LI cells and fibers. A. Applied for regions with fiber density less than 200 fibers/mm^2. Horizontal section through the intermediate plane of *Ichth* treated with anti-NKA$_1$. Density (D) = (L_1 + L_2 + L_3) L_t^{-1} w^{-1} t^{-1}, where L_i=length of segment number i, L_t=length of whole region, t=thickness of the section, usually 0.014 mm; w and t expressed in mm gives number of fibers per mm^2. B. Applied for regions with fiber density more than 200 fibers/mm^2. Adjacent section treated with anti-NKA$_2$. D = N w^{-1} t^{-1} (where N is the number of fibers crossed by an imaginary line, here N=7). Note that in micrographs of 14 μm thick sections only a limited proportion of the fibers can be in focus and visible, but by focussing up and down at the microscope more, fibers were usually visible. Bars indicate 50 μm.

TABLE 1

CHARACTERISTICS OF THE ANTISERA USED FOR RIA, TESTED WITH RADIOIMMUNOASSAY

Antisera	Sensitivity m.d.a. (fmoles/tube)	Dilution	Cross-Reactivity (%) SP	PHY	NKA	NKB	KAS	ELE	Reference
anti-SP$_2$	1.0	1:350,000	100	0.002	<0.001	<0.001	<0.001	<0.001	a (SP2)
anti-SP$_{10}$	1.5	1:500,000	100	89	5	0.6	2	11	a (SP10)
anti-KAS$_7$	0.4	1: 25,000	<0.1	<0.1	0.1	0.1	100	0.1	b (K7)
anti-KAS$_{12}$	1.3	1: 14,000	<0.1	<0.1	173	28	100	119	b (K12)
anti-ELE$_2$	0.2	1:400,000	<0.1	<0.1	8	9	9	100	b (E2)
anti-ELE$_7$	0.3	1:140,000	<0.1	<0.1	37	34	41	100	b (E7)

m.d.a. is minimum detectable amount. At the references, a=[4], b=[40]; the numbers between parentheses refer to the names these antisera are given by these authors.

TABLE 2

THE DEGREE OF REDUCTION OF THE NUMBER OF IMMUNOREACTIVE FIBERS AS DETECTED WITH THE VARIOUS ANTIBODIES PRE-INCUBATED WITH VARIOUS TACHYKININS

			Incubation peptides:				
	Dilution	Reference	SP	PHY	NKA	KAS	ELE
anti-SP_{RRF}	1:200	a (RRF)	7	8*	5	5	6
anti-SP_1	1:200	b (SP1)	6	6	5	5	6
anti-SP_2	1:200	b (SP2)	8	9*	7	3	4
anti-SP_{10}	1:200	b (SP10)	9	9	6	6	8
anti-SP_C	1:250	c (NC1/34)	3	3	2	3	2
anti-SP_S	1:200	d—	4	4	4	5*	5*
anti-PHY_2	1:100	e (PS-2)	6	7	6	5	6
anti-NKA_1	1:200	b (SK1)	4	6	6	6	6
anti-NKA_2	1:200	b (SK2)	2	2	6	9*	6
anti-KAS_7	1:200	f (K7)	3	3	6	6	6
anti-KAS_{12}	1:200	f (K12)	2	3	8	9*	8
anti-ELE_2	1:200	f (E2)	5	6	9	9	9
anti-ELE_7	1:200	f (E7)	4	6	9	9	9

The reduction of fiber staining was expressed on a 9-points scale (see the Method section: 1=no apparent reduction at 50 μg/ml, 3=strong reduction at 50 μg/ml, 5=complete abolishment of immunostaining at 50 μg/ml, 7=complete abolishment at 5 μg/ml, 9=complete abolishment at 0.5 μg/ml peptide concentration). When preincubation with another TK than that against which the antiserum is raised caused a stronger reduction of the immunoreactivity, this antiserum-peptide combination is labeled with an *. For the references: a=[33]; b=[4]; c=[6]; d=Singer, unpublished; e=[22]; f=[40]; the codes between parentheses refer to the original names given to these antibodies.

buffer, pH 7.2, containing 0.01% sodiumazide and 0.02% Bacitracin, and incubated with one of the 5 TK peptides tested (SP, PHY, NKA, KAS or ELE) at an antiserum dilution of 1:10 and peptide concentrations 0.5, 5.0 or 50 μg/ml. These antisera were kept at 4°C for 16–20 hours and thereafter diluted to their final concentrations (Table 2). Antisera treated the same way, except that no peptide was present, were used as controls. For each section treated with preincubated antiserum, an adjacent section was treated with control antiserum. The effects of preincubation at each peptide concentration were expressed on a 5-point scale, and the three partially overlapping 5-point scales (for the 3 different peptide concentrations) were combined to one 9-point scale (cf. Table 2).

Chromatography and Radioimmunoassay

Tissue collection and extraction. Spinal cords of 86, and the brains of 50 large (30–40 cm) river lampreys (*Lampetra fluviatilis*) were used for chromatographic studies. The spinal cords and brains together with some perimeningeal tissue were rapidly dissected, frozen in liquid nitrogen, weighed in the frozen state (i.e., wet weight), and stored at −70°C for less than two weeks. (The average wet weight of a lamprey brain is 50 mg and of a spinal cord 250 mg.) Tissues were extracted as described earlier [40]. In short, the frozen tissues were minced, heated in neutral water (10 ml per g tissue) in a bath of boiling water for 10 min, homogenized (Polytron), centrifuged (1000 × g for 15 min), and the supernatants were taken off and stored (−20°C). In one case the minced tissue was heated in 1.0 M acetic acid instead of neutral water ("primary acetic acid extract"). In some cases, the pellets were resuspended in 1.0 M acetic acid, homogenized and treated as described above ("secondary acetic acid extract").

Ion-exchange and gel-permeation chromatography. Ion-exchange and gel-permeation chromatography were performed as described earlier [40]. Briefly, samples (10 ml, containing the extracts of 1 g nervous tissue) of acid or neutral water extracts were applied onto a CM-Sephadex C-25 column (2.6×20 cm) for cation-exchange chromatography, or a DEAE-Sephadex A-25 column (2.6×20 cm) for anion-exchange chromatography. The samples were eluted with a gradient made by having 250 ml of 0.025 M ammonium bicarbonate (pH 8.3) in the mixing chamber, and 250 ml 0.25 M ammonium bicarbonate (pH 8.3) in the reservoir. For gel-permeation chromatography, a Sephadex G-25 superfine column (2.6×140 cm) was used, and the sample was eluted with 0.05 M ammonium acetate (pH 5.3). The columns were calibrated for various TKs in separate runs. The eluted fractions were lyophilized and redissolved in 100 μl of distilled water immediately before RIA. Every third fraction was assayed with the same antiserum.

High performance liquid chromatography (HPLC). A nucleosil C18 5 μm (Merck), 4.6×300 mm column was used for reversed-phase chromatography. It was eluted with a 40 min linear gradient of 20% eluent A to 40% eluent B. Eluent A was double-distilled ultrafiltrated water containing 0.1% trifluoroacetic acid (TFA) (Merck). Eluent B was 0.1% TFA (v/v) in acetonitrile (Rathburn Chemicals, HPLC grade S). Two LDC/Milton Roy constametric pumps were controlled by a Sinclair Spectrum/Bercol microcomputer. The samples were passed through Millipore filters (0.22 μm) to remove

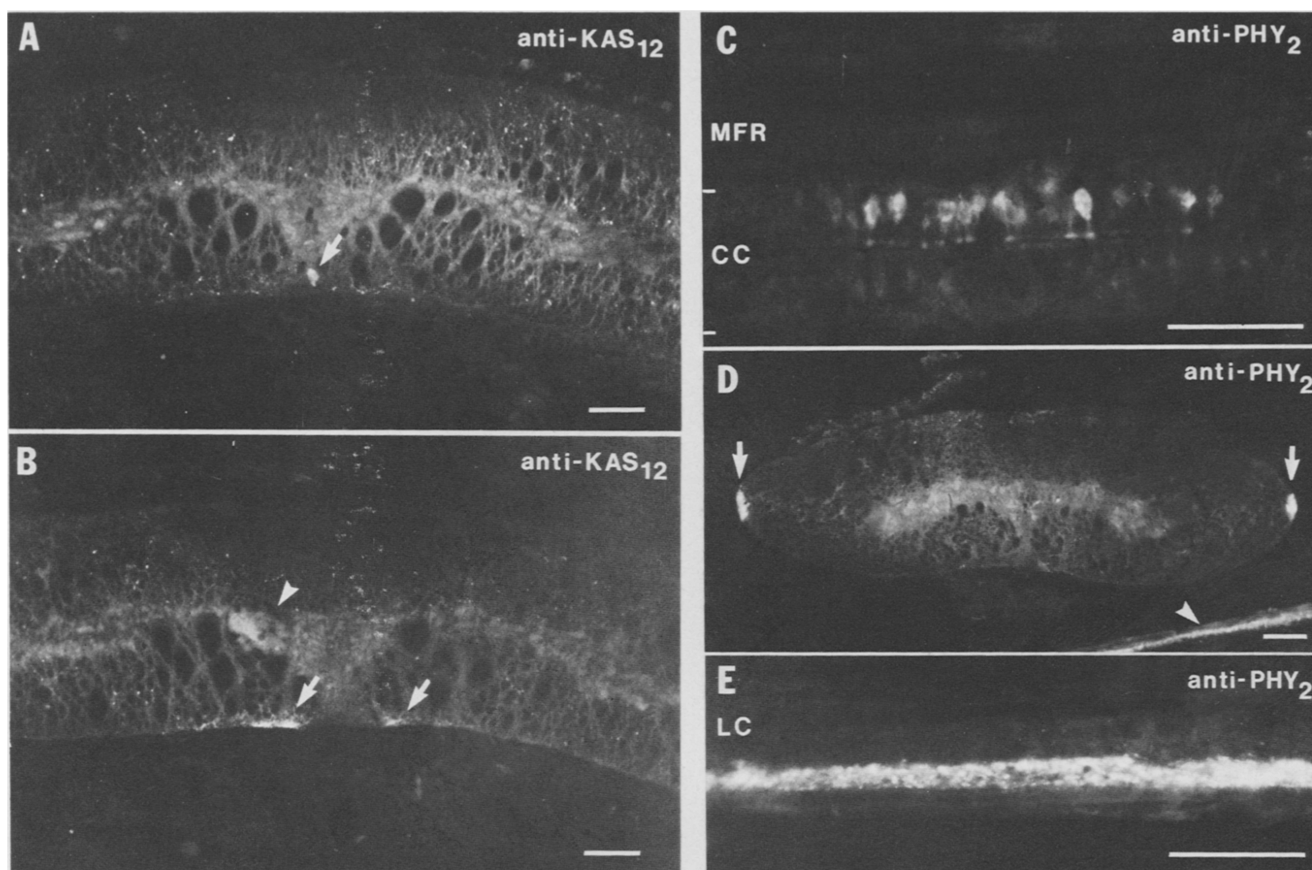


FIG. 3. TK-immunofluorescence in the spinal cord of *Ichth*. A. Transverse section treated with anti-KAS_{12} showing a single fluorescent cell body in a ventromedial position (arrow). Note also the many fibers containing TK-LI material seen as white dots. B. Transverse section treated with anti-KAS_{12} showing a bilateral ventromedial fluorescent plexus (arrows), and an autofluorescent (staining not specific for TKs) sensory dorsal cell (arrow-head). C. Horizontal section through the intermediate plane treated with anti-PHY_2, showing fluorescent cell bodies contacting the CSF. D. Transverse section treated with anti-PHY_2 showing a bilateral plexus of fluorescent material at the lateral edges of the spinal cord (arrows). Below in the figure (arrowhead) the same plexus is shown in horizontal section. E. Horizontal section through the intermediate plane treated with anti-PHY_2 showing a continuous plexus of fluorescent varicosities along the lateral edge of the spinal cord. Bars indicate 50 μm.

particulate matter before chromatography. Samples of 100 μl were injected using a Rheodyne injector (715s). Fractions (0.5 ml) were collected at an elution rate of 1.0 ml/min. Each fraction was lyophilized, redissolved in 100 μl of distilled water, and RIA was performed in the tubes used for collection of the fractions.

Radioimmunoassay. RIA of tissue extracts and chromatography eluates was performed as described earlier [40] using six antisera raised against SP, KAS and ELE (Table 1). Synthetic SP, KAS and ELE were used as standards respectively. For the nomenclature and abbreviations, the recommendations of the nomenclature committee of the IUPHAR satellite symposium on substance P (August 1984) will be followed.

RESULTS

Immunohistochemistry

Antisera. Immunohistochemical tests were done with 34 different antisera raised against SP, PHY, NKA, KAS or ELE. Of these antisera 10 did not give rise to staining of nerve fibers, and from the remaining 24 antisera, 13 were selected (Table 2) for the following reasons. (1) They gave clear staining of fibers, (2) some of them gave rise to different distributions of immunoreactive material or, (3) they appeared to be selective antisera in RIA (cf. Table 1). The distribution of neurons and fibers containing TK-immunoreactive material was similar in *Lampetra fluviatilis* and *Ichthyomyzon unicuspis*.

Cell bodies. Immunofluorescent cell bodies were found with 5 out of the 13 antisera raised against TKs. The fluorescence was generally weak and not seen in all animals suggesting that TK-like immunoreactivity (LI) in cell bodies could only be visualized under optimal conditions. With the use of anti-PHY_2, small (10–14 μm) cell bodies were found between the ependymal cells (Fig. 3C). The cells had short (about 8 μm) processes with an enlargement at the lumen of the central canal (Fig. 3C). Of the other TK-antisera, anti-SP_C, anti-KAS_{12}, anti-ELE_2 and anti-ELE_7 stained cell bodies (Fig. 3A), but they were few and only weakly fluorescent. These cell bodies were small and located in a midline position just ventral to the central canal, and they were in shape and location identical to the 5-HT-containing cells [41]. As is evident from Figs. 2 to 6, many cell bodies of

other neurons in the lamprey spinal cord showed a weak background fluorescence, which was not specific for TKs, since it was also visualized with TK antisera previously preincubated with an excess of TK peptides, and with antisera against 5-HT and neuropeptide Y [41]. It cannot be excluded that this unspecific fluorescence prevented detection of TK-IR material in some of these cells.

Overall fiber distribution. Fine varicose fibers containing TK-IR material were visualized in the lamprey spinal cord; they had similar shapes as seen with the various antisera and were mainly longitudinal (Figs. 4–6). In the regions of the cell bodies of the medium-sized and large cells (i.e., in the dorsal cell region and lateral column of cell bodies) only few fibers containing TK-IR material were found and they did not have a preferential orientation. Sometimes fibers were associated with small cells in the dorsal cell region or with small or medium-sized cells in the lateral column of cell bodies (Figs. 5F, 6H, I, J, K). Fiber counts were performed for regions with predominantly longitudinal fibers (cf. Fig. 7).

Types of distribution. Four patterns of distribution were found (cf. Fig. 7) and they are termed on the basis of one of the representative antisera (SP_C-, SP_1-, SP_S- and SP_2-type; see Tables 1 and 2).

SP_C-type of distribution. This type is described more extensively in Van Dongen *et al.* [41]. Overall, the density of the immunoreactive fibers was high (often >1000 fibers/mm^2), while the highest density was always present in the dorsal horns (always >5000 fibers/mm^2). This pattern was found with the antibodies anti-SP_C, anti-SP_{RRF}, anti-NKA_2, anti-KAS_7 and anti-ELE_7, although with the anti-KAS_7 antiserum the fibers were only weakly stained.

SP_1-type of distribution. This type of innervation was characterized by a high density of fibers in the dorsal horns and in the lateral axon column, and a low density (29–94 fibers/mm^2) in the dorsal axon columns, medial to the dorsal horns (Figs. 4D, E, F, I), while the overall density was rather high but still lower than that of the SP_C type, The highest density of fibers was present in the dorsal horns. This pattern was found with the antisera anti-SP_1, anti-SP_{10}, anti-NKA_1, anti-KAS_{12} and anti-ELE_2.

SP_S-type of distribution. This type was characterized by a high density of fibers (>4800 fibers/mm^2) in the dorsal horns and in the dorsomedial axon columns (Figs. 4A,H), while the density of fibers in other regions was low (<250 fibers/mm^2). This pattern was found with the antisera anti-SP_S and anti-PHY_2, although with anti-PHY_2 additional TK-IR elements have been found (cell bodies, see above, and lateral plexus, see below).

SP_2-type of distribution. This type was characterized by a low density of fibers in the dorsal horns (<50 fibers/mm^2)

FACING AND FOLLOWING PAGES

FIG. 4. TK immunofluorescence in horizontal sections through the lamprey spinal cord. A. Section treated with anti-SP_S *(Ichth)*, showing many fibers in the dorsal horns (DH) and several in the dorsomedial axon column (DMC). B. Section treated with anti-SP_{RRF} *(Lamp)*, showing many fibers in the dorsal horn (DH). C. As in B, but at higher magnification to show the shape of the fibers. Note the presence of many fibers in the DMC. D. Section treated with anti-KAS_{12} *(Ichth)*, showing fibers in the DH but few in the DMC. E. Section treated with anti-SP_1 *(Ichth)*, showing fibers in the DH. Note the presence of only few fibers in the DMC. Lateral to the spinal cord some autofluorescent perimeningeal tissue is visible. F. Section treated with anti-SP_{10} *(Ichth)*, showing many fibers in the DH and only few in the DMC. G. Section treated with anti-SP_C *(Lamp)*, showing fibers in the DH and in a dorsal root (DR). Note the presence of many fibers in the DMC. H. Section treated with anti-PHY_2 *(Ichth)*, showing many fibers in the DH and several in the DMC. I. Section treated with anti-NKA_1 *(Ichth)*, showing many fibers in the DH and only few in the DMC. J. Section treated with anti-SP_2 *(Lamp)*, showing the absence of fibers in the DH and DMC, and a few fibers in the dorsolateral axon column (DLC). Bars indicate 50 μm.

FIG. 5. TK immunofluorescence in horizontal sections through the dorsal cell plane of the spinal cord, showing also autofluorescent sensory dorsal cells (arrows). A. Section treated with anti-SP_{10} *(Ichth)*, showing a single longitudinal fiber. B. Section treated with anti-SP_S *(Ichth)*, showing a single fiber in the dorsolateral axon column (DLC). C. Section treated with anti-SP_2 *(Ichth)* showing longitudinal fibers in the DLC. D. Section treated with anti-SP_{RRF} *(Lamp)*, showing fibers in the dorsal cell region (DCR). E. Section treated with anti-NKA_1 *(Ichth)*, showing a single longitudinal fiber. F. Section treated with anti-KAS_{12} *(Ichth)*, showing immunofluorescent fibers around a small cell body (arrow head). G. Section treated with anti-ELE_2 *(Lamp)*, showing several fibers in the DLC. H. Section treated with anti-ELE_7 *(Lamp)*, showing many longitudinal fibers in the DLC. Bars indicate 50 μm.

FIG. 6. TK immunofluorescence in horizontal sections through the intermediate plane. A. Section treated with anti-SP_S *(Ichth)*, showing a single transverse fiber. B. Section treated with anti-PHY_2 *(Ichth)* showing a longitudinal fiber in the lateral axon column (LC). C. Section treated with anti-SP_{RRF} *(Lamp)*, showing a longitudinal fiber. D. Section treated with anti-NKA_1 *(Ichth)*, showing a single longitudinal fiber. E. Section treated with anti-NKA_2 *(Ichth)*, showing many longitudinal fibers in the LC. F. Section treated with anti-SP_2 *(Lamp)* showing some longitudinal fibers. G. Section treated with anti-ELE_7 *(Lamp)*, showing several longitudinal fibers. H. Section treated with anti-NKA_1 *(Ichth)*, showing an immunofluorescent fiber around a cell body (arrow). I. Section treated with anti-KAS_7 *(Ichth)*, showing a fiber in the lateral column of cell bodies (LCC). J. Section treated with anti-KAS_{12} *(Ichth)*, showing immunofluorescent fibers near a medium-sized cell body (arrows). K. Section treated with anti-ELE_2 *(Lamp)*, showing an immunofluorescent fiber around a small cell body (arrow). Bars indicate 50 μm.

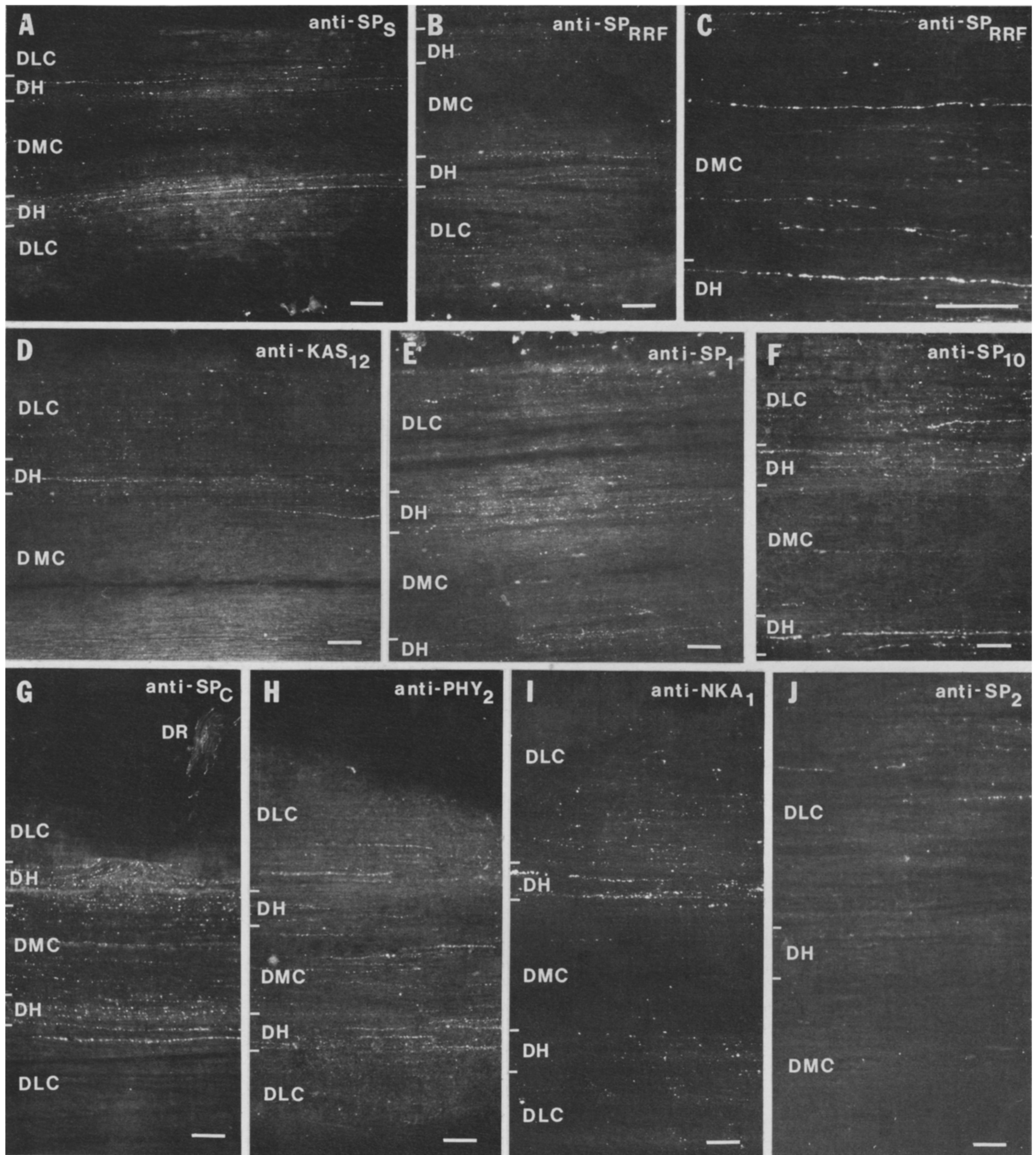


FIG. 4.

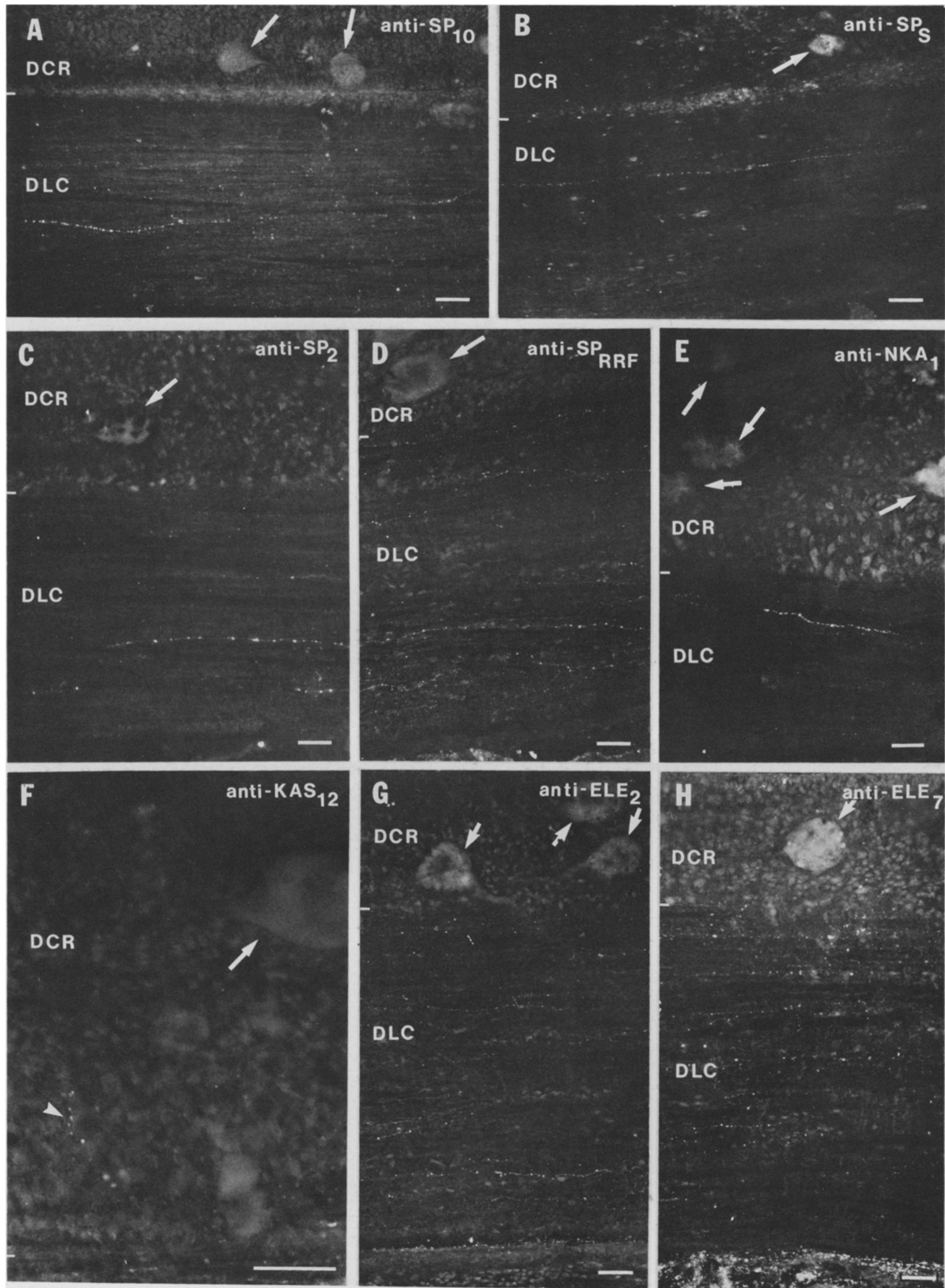


FIG. 5.

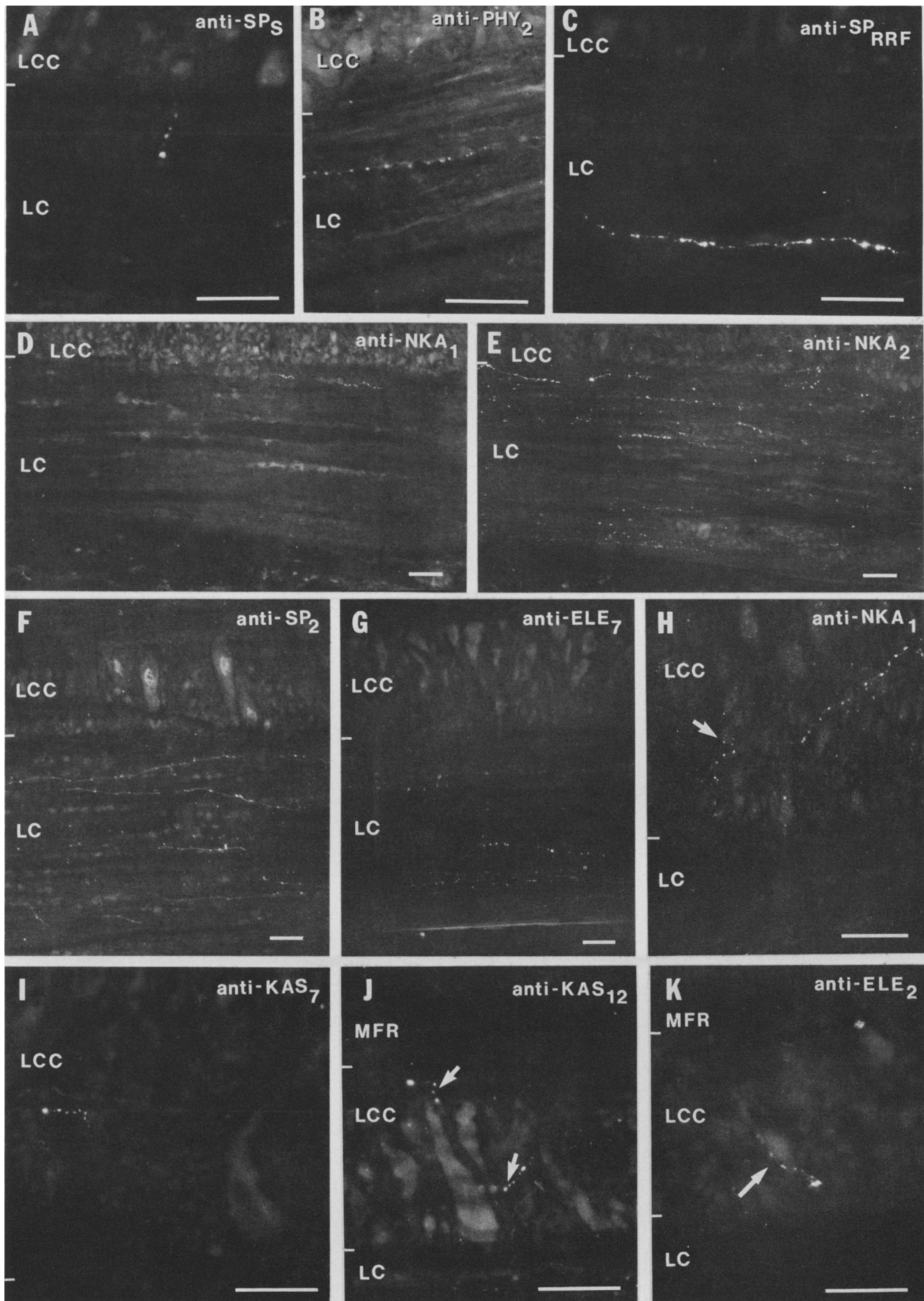


FIG. 6.

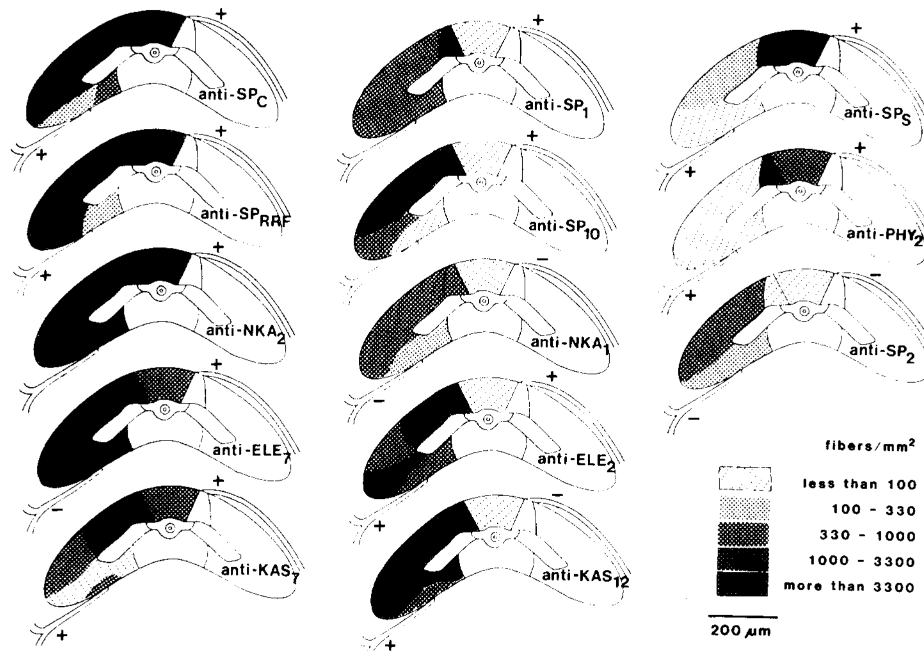


FIG. 7. Schematic survey of the density of longitudinal fibers found with the various antisera. A plus sign (+) at dorsal or ventral roots indicates the presence of immunofluorescent fibers, a minus sign (−) the absence, and no sign indicates that their presence is uncertain. At this scale, the rectangular boxes for the density codes are 0.01 mm^2, which implies that a density of less than 100 fibers/mm^2 corresponds to less than one fiber in the area of one box.

TABLE 3

CONTENTS OF TK-LI MATERIAL IN THE VARIOUS PEAKS OF THE ION-EXCHANGE CHROMATOGRAMS

	A_1	A_2	A_3	C_1	C_2	C_3	C_4	C_5
peak-frac.	35	84	136	21	67	116	160	191
spinal cord, water extract								
anti-SP_2	46	<13	<6	<7	26	<7	<8	35
anti-KAS_7	<3	<5	<3	<3	<3	<3	<3	<3
anti-KAS_{12}	202	315	?*	1398	43	36	4	68
anti-ELE_2	44	305	71	133	49	18	49	56
anti-ELE_7	15	444	<2	320	127	201	40	82
spinal cord, acetic acid extract								
anti-SP_2	—	—	—	<7	20	<7	65	59
anti-KAS_{12}	—	—	—	24	<10	7	53	48
anti-ELE_7	—	—	—	<2	<3	<2	158	69
brain, water extract								
anti-ELE_2	—	—	—	2583	726	51	15	87

For the various peaks, the contents of the following fractions have been summed: A_1 22–45; A_2 58–97; A_3 121–140; C_1 16–36; C_2 47–70; C_3 106–126; C_4 151–174; C_5 175–196. The codes for the peaks are referred to in Figs. 9–11. The amounts are expressed in fmoles/gram tissue, where fmoles refer to molar equivalents of the TK against which the antiserum is raised. With anti-KAS_{12} measurable amounts of TK-LI material were present in the fractions 121–140 of the anion-exchange chromatograms, but no peak was found at A_3; therefore it is labeled ?*. —Indicates "not determined."

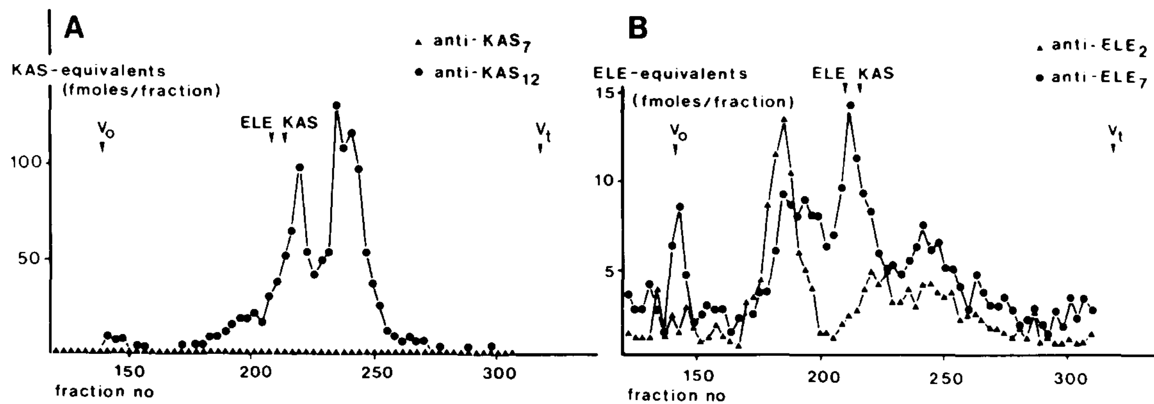


FIG. 8. Gel permeation chromatography (Sephadex G-25) of a neutral water extract of lamprey spinal cord. A. Radioimmunoassay with anti-KAS$_7$ and anti-KAS$_{12}$. B. Radioimmunoassay with anti-ELE$_2$ and anti-ELE$_7$. The column was calibrated with blue dextran (V_o), ^{22}Na (V_t) and synthetic ELE and KAS. The amounts are expressed in molar equivalents of the TKs against which the antisera were raised.

and the dorsomedial axon column (<10 fibers/mm^2), while the density in the dorsolateral and lateral axon columns was higher than 300 fibers/mm^2 (Fig. 4J). This pattern was found only with anti-SP$_2$, the most SP-selective antibody used.

Summarizing, four patterns of distribution were found with 13 different antibodies raised against various TKs. The observed pattern was unrelated to the TK against which the antiserum was raised.

Plexus of TK-LI varicosities. A dense bilateral plexus of varicosities was found along the ventral margin of the spinal cord with the antisera anti-KAS$_{12}$, anti-ELE$_2$ and anti-ELE$_7$ (Fig. 3C). This plexus extended over some 50 μm, and was continuous over the whole length of the spinal cord. With the use of anti-PHY$_2$, a dense bilateral plexus was found at the lateral margins of the spinal cord (Fig. 3C,D), and also this plexus extended over the whole length of the spinal cord.

Pre-incubation of the antisera with TK peptides. Pre-incubation of the antisera with TK peptides in appropriate concentrations reduced or abolished the immunostaining (Table 2). The antisera could be divided in two subgroups. The immunostaining by antisera raised against NKA, KAS or ELE was more markedly reduced by pre-incubation with NKA, KAS or ELE than by pre-incubation with SP of PHY. The immunostaining by antisera raised against SP (except anti-SP$_8$), on the other hand, was more markedly reduced by pre-incubation with SP or PHY than by pre-incubation with NKA, KAS or ELE. Pre-incubation of the TK antisera with the corresponding TKs in a concentration of 50 μg/ml or less abolished the immunostaining completely, except for anti-SP$_C$; pre-incubation of anti-SP$_C$ with SP or PHY caused a strong reduction, but several fibers were still present in the dorsal horns and the dorsomedial axon column, and a few in the ventrolateral axon column. The immunostaining by some antisera was more markedly reduced by pre-incubation with another TK than that against which the antiserum was raised (Table 2).

Chromatography and Radioimmunoassay

Gel-permeation chromatography. TK-IR material analyzed with anti-KAS$_{12}$, anti-ELE$_2$ and anti-ELE$_7$ eluted as several components at gel-permeation chromatography (Fig. 8). With anti-KAS$_7$, the most selective KAS antiserum (Table 1), no KAS-LI material was detected (the amount was less than 0.4 fmole KAS-equivalents per fraction in all fractions investigated).

Ion-exchange chromatography. The results of the ion-exchange chromatography of the lamprey spinal cord and brain are presented in Figs. 9–11 and in Table 3. In the anion-exchange chromatogram analyzed using anti-ELE$_2$, the TK-IR material formed three peaks (labeled A$_1$, A$_2$ and A$_3$, Fig. 9E). With anti-SP$_2$, anti-KAS$_{12}$ and anti-ELE$_7$ peaks were found in one or two of these positions (Fig. 9A, C, E). In the cation-exchange chromatograms analyzed with anti-KAS$_{12}$, anti-ELE$_2$ and anti-ELE$_7$, the TK-IR material formed four peaks (labeled C$_1$, C$_2$, C$_3$ and C$_5$, Fig. 9D,F) and in several of these positions (C$_2$ and C$_5$) peaks were also found with anti-SP$_2$ and anti-SP$_{10}$. No TK-IR material could be detected with anti-KAS$_7$, i.e., the levels were below 0.4 fmole KAS-equivalents per fraction. Cation-exchange chromatography of lamprey brain extracts analyzed with anti-ELE$_2$ showed peaks at the same elution volumes as found for the spinal cord (Fig. 10), but especially the peaks C$_1$ and C$_2$ of brain tissue contained much more (15–20 times) TK-IR material than the same peaks of the spinal cord tissue. Cation-exchange chromatography of the acetic acid extracts of the spinal cord and subsequent RIA with anti-SP$_2$, anti-KAS$_{12}$ and anti-ELE$_7$ revealed peaks in the same region as those obtained using the water extracts plus one additional peak, C$_4$ (between the fractions 150 to 175) (Fig. 11). However, the amounts of immunoreactivity eluted in these peaks were rather low (Table 3). Using antiserum SP$_{10}$, which cross-reacts markedly with physalaemin, peak C$_1$ was very marked and peaks were also found at position C$_3$ and C$_5$ (Fig. 9B). Some material at C$_1$ was also present in the water extracts (Figs. 9F, 10).

High performance liquid chromatography. The results of HPLC and RIA with anti-SP$_2$ and anti-KAS$_{12}$ are presented in Fig. 12. The SP-IR material from the spinal cord extracts detected with anti-SP$_2$ eluted in a single small peak; the SP-IR material in the neutral water and in the acetic acid extracts eluted at the same position, i.e., at the same position as neuropeptide K (NPK, Tatemoto *et al.* [39]), and remote from SP (Fig. 12A). No SP-LI material was detected in the fractions following HPLC of extracts of the brain, i.e., the amount was less than 1 fmole/fraction. The TK-IR material of the spinal cord extracted with neutral water and detected with anti-KAS$_{12}$ eluted in two main peaks (H$_1$ and H$_4$) just before the positions of NKA and NPK, respectively (Fig. 12B). Less material was detected in the acetic acid extracts, but it eluted at the same positions. The brain TK-IR material

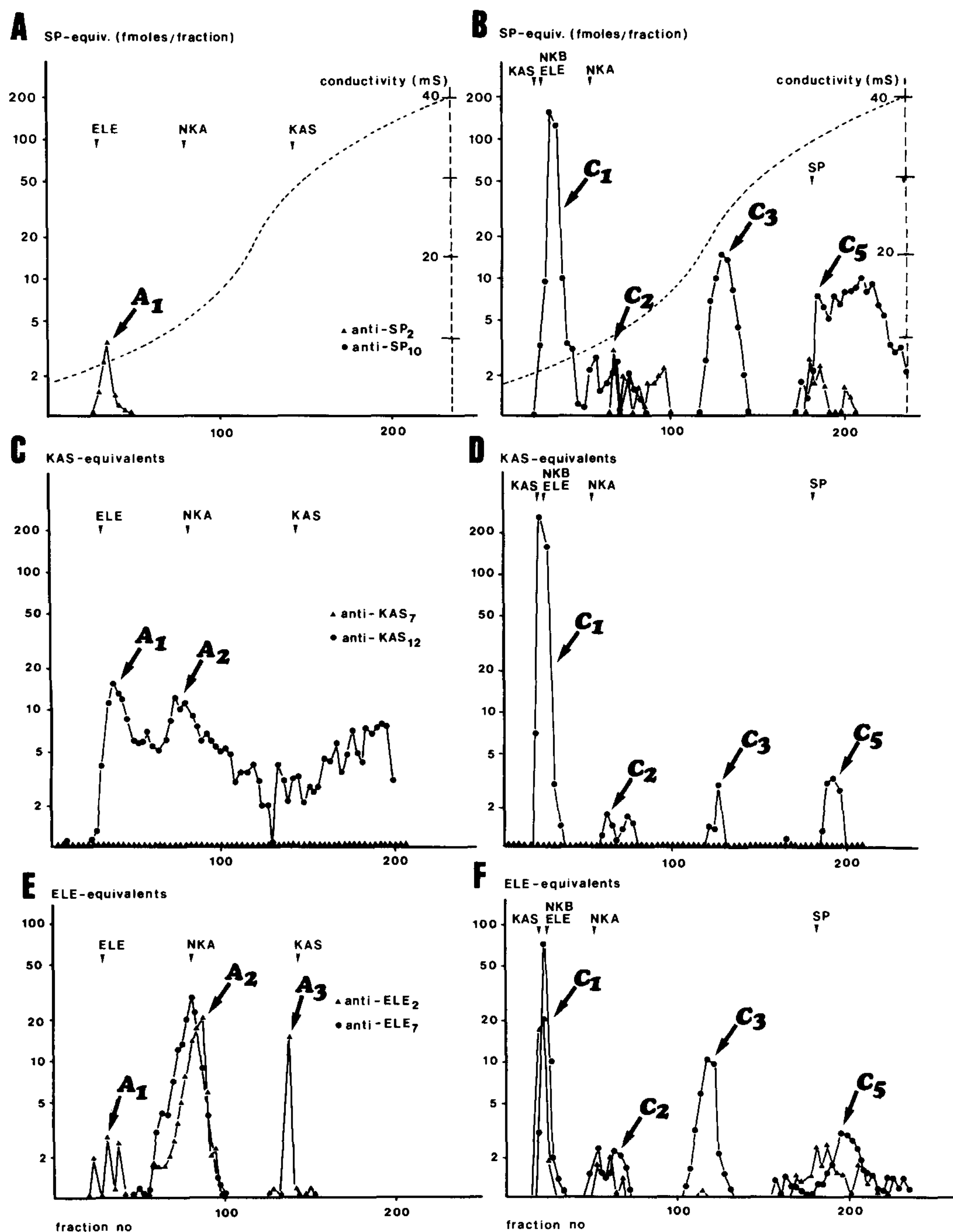


FIG. 9. Ion-exchange chromatograms of neutral water extracts (A–F) and an acetic acid extract (chromatogram analyzed with antiserum anti-SP_{10}) (B) of 1 g lamprey spinal cord. Note that antisera given in A, C and E also apply to, respectively, B, D and F. Left (A, C, E): Anion-exchange chromatography with DEAE-Sephadex A-25; this column was calibrated with synthetic NKA, KAS and ELE; synthetic SP did not leave the column. The codes A_1, A_2 and A_3 were used to label the various peaks. Right (B, D, F): Cation-exchange chromatography with CM-Sephadex C-25; this column was calibrated with synthetic SP, NKA, NKB, KAS and ELE. The codes C_1 to C_5 were used to label the various peaks. In A and B the conductivity of the eluents is indicated. *; This molarity gradient applies to all graphs of Figs. 9–11. A and B show analysis with anti-SP_2. C and D show analysis with anti-KAS_7 and anti-KAS_{12}. E and F show analysis with anti-ELE_2 and anti-ELE_7. The line indicating the detection limit is about the same as the horizontal axis. The amounts are expressed in molar equivalents of the TKs against which the antisera were raised. Note that a logarithmic scale is used on the abscissa.

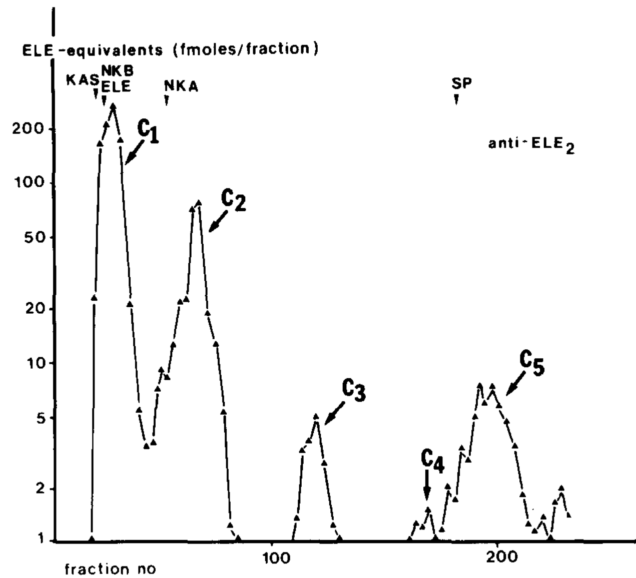


FIG. 10. Cation-exchange chromatogram (CM-Sephadex C-25) of a neutral water extract of lamprey brain, analyzed with anti-ELE_2. Note that a logarithmic scale is used on the abscissa. The codes C_1 to C_5 and the gradient used are the same as in Fig. 4.

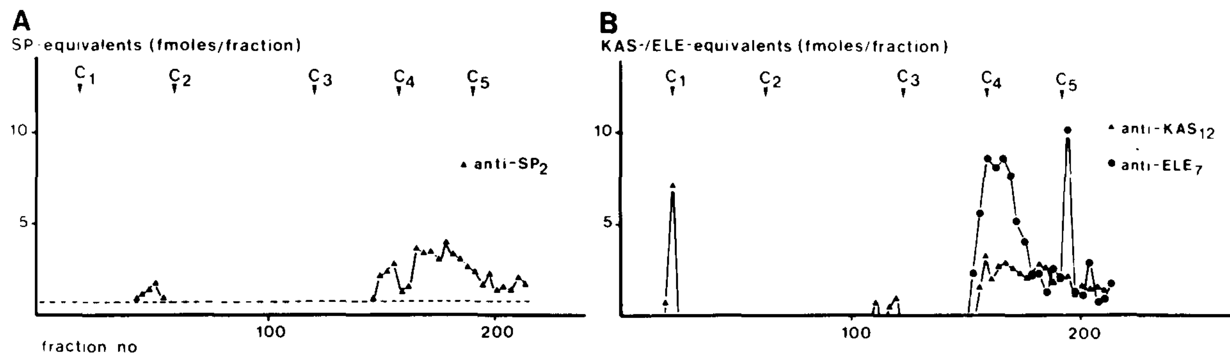


FIG. 11. Cation-exchange chromatograms (CM-Sephadex C-25) of an acetic acid extract of lamprey spinal cord previously extracted with neutral water. A. Analysis with anti-SP_2. B. Analysis with anti-KAS_{12} and anti-ELE_7. The codes C_1 to C_5 and the gradient used are the same as in Fig. 9.

with affinity to anti-KAS_{12} eluted in many peaks (H_1 to H_7, Fig. 12C); two prominent peaks of these (H_1 and H_4) were found at the same position as those in the chromatograms of spinal cord. The material of the acetic acid extracts eluted partly with the same peaks as the neutral water extracts. One peak was at the position of KAS, and another at the position of SP.

DISCUSSION

TK-LI Material in Immunohistochemistry

Four different patterns of distribution of TK-IR fibers were found in the present study (Fig. 7). Some preliminary data indicate that fibers stained by one antiserum are often also stained by other antisera; this applies at least to the following antisera combinations: anti-SP_1 together with anti-SP_{RRF}, anti-SP_2 together with anti-ELE_7, anti-SP_{10} anti-ELE_7, anti-PHY_2 together with anti-KAS_{12}, but not anti-SP_1 with anti-PHY_2, and not anti-SP_{10} with anti-PHY_2. The simplest explanation for these data is to assume the presence of at least three different TK peptides or, perhaps more likely, subgroups of peptides.

One group of peptides is stained most selectively by anti-SP_S and anti-PHY_2, but not by anti-SP_1, anti-SP_{10}, anti-NKA_1, anti-ELE_2 and anti-KAS_{12}. This peptide group is tentatively labeled LT_S, i.e., presumed *L*amprey *T*achykinin for which antisera of the SP_S-group (Fig. 13) have rather selective affinity. The cell bodies containing this peptide might be mainly around the central canal with processes towards the lumen, and the fibers are mainly present in the dorsal horns and the dorsomedial axon tracts. Sometimes a dense plexus of varicosities has been found along the lateral edge of the spinal cord.

Another group of TK peptides is stained most selectively

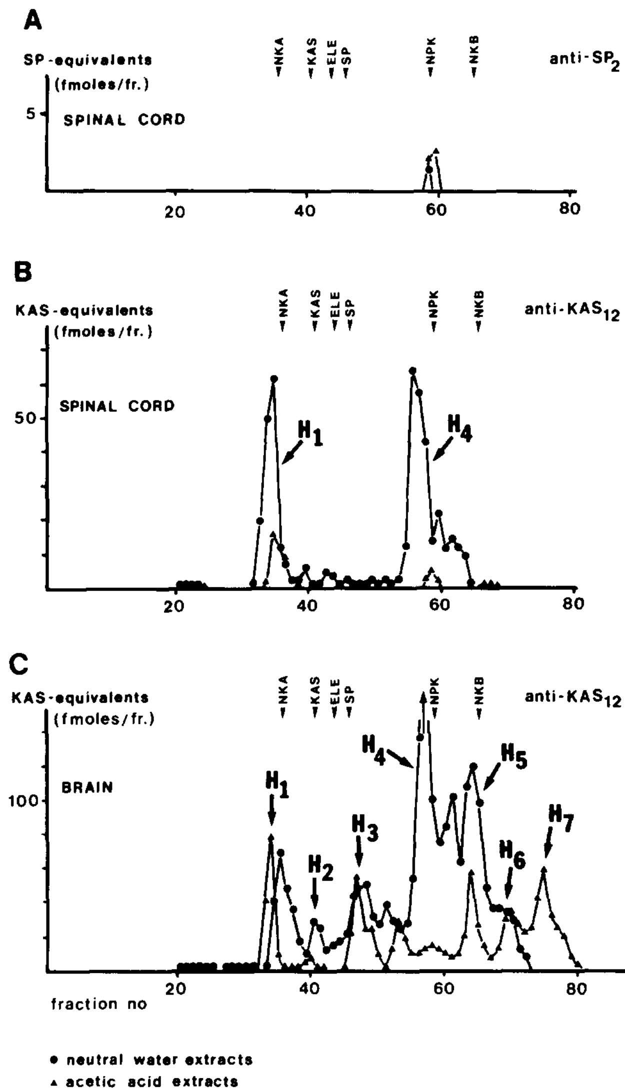


FIG. 12. HPLC analysis (nucleosil C18) of neutral water (filled circles) and acetic acid (triangles) extracts of the lamprey spinal cord and brain. A. Extracts of the spinal cord analyzed with anti-SP_2. No immunoreactivity could be detected using anti-SP_2 following chromatography of lamprey brain. B. Extracts of the spinal cord, analyzed with anti-KAS_{12}. C. Extracts of the brain, analyzed with anti-KAS_{12}.

by anti-SP_1, anti-SP_{10}, anti-NKA_1, anti-ELE_2 and anti-KAS_{12}, but not by anti-SP_S and anti-PHY_2. This peptide may be labeled LT_1, i.e., presumed *L*amprey *T*achykinin for which antisera of the SP_1-group (Fig. 13) have rather selective affinity. A few cell bodies containing this peptide were found ventral to the central canal, and the fibers were mainly present in the dorsal horns, the dorso-lateral, the lateral axon columns and sometimes in a ventromedial plexus.

A third group of TK peptides is stained most selectively by anti-SP_2 (Fig. 13) and may be labeled LT_2. No cell bodies have been found with this antiserum. Of the remaining antisera, anti-SP_C, anti-SP_{RRF}, anti-NKA_2, anti-KAS_7 and anti-ELE_7 have affinity for LT_1 and LT_S, and at least partly also for LT_2. The antisera of the SP_1-group (or some of them) have probably also affinity for LT_2.

In an earlier study, the coexistence in some fibers of 5-HT

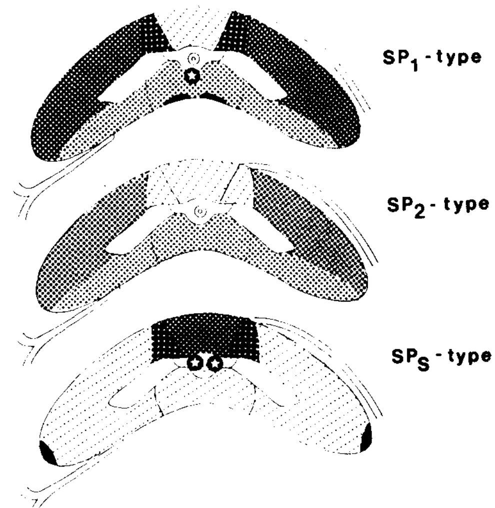


FIG. 13. Schematic survey of the distribution of fibers and cell bodies (open stars) of the three presumed lamprey tachykinins, which are postulated to explain the distribution pattern found with 13 different antibodies raised against TKs. The density codes used here are not based on actual fiber counts, but have been selected to give a general impression of fiber density.

and material with affinity to anti-SP_C has been reported [41], but anti-SP_C has probably affinity for several lamprey TKs. The TK which is coexistent with 5-HT, probably is LT_1 rather than LT_S or LT_2, since the location of cell bodies is similar, and sometimes a ventromedial plexus of TK-LI varicosities was found with anti-KAS_{12} (SP_1-type) at the location of a 5-HT plexus.

TK-IR Material in Chromatographic Studies

General findings. TK-IR material was found both in extracts of the lamprey spinal cord and brain. At gel permeation chromatography, a portion of the TK-IR material in neutral extracts of the spinal cord eluted in a similar position as ELE and KAS. Thus it may be concluded that the lamprey spinal cord contains TK-IR material with similar apparent molecular weights as previously described TKs. Cation-exchange chromatography revealed the presence of at least five components of TK-IR material in the lamprey CNS. The positions of the peaks were similar for brain and spinal cord extracts. However, cation-exchange chromatography did not separate NKB, KAS and ELE, but these compounds were separated at anion-exchange chromatography and HPLC. At HPLC the lamprey TK-IR material eluted in several components as seen at ion exchange chromatography. TK-IR material in brain extracts, however, was found to be more heterogenous than the spinal cord material; the components H_1 and H_4 being present both in the spinal cord and brain.

The brain components H_2, H_3 and H_5 might be present in the spinal cord, but in low amounts. The brain components H_6 and H_7 were prominent in the acetic acid extracts, which implies that they consist of basic peptides; in the positions of H_6 and H_7 no spinal cord TK-IR material was found.

The TKs of the rat CNS have been investigated with the same ion-exchange chromatographic systems and with the same antisera: the patterns of TK-IR peaks of rat CNS were completely different from the lamprey patterns [4, 25, 40].

Both at cation-exchange chromatography and at HPLC, the lamprey TK-IR material appeared to be more heterogeneous than the rat TK-IR material [4, 25, 40].

Substance P. With the most SP-selective antiserum (anti-SP_2), a small but a detectable amount SP-IR material was found at the same elution volume as synthetic SP at cation-exchange chromatography (peak C_5). However, this component must contain (also) some other material than SP, since also a detectable amount of KAS- and ELE-IR material was found at C_5 using antisera with low cross-reactivity (<0.1%) with SP. At HPLC, no detectable amount of immunoreactivity was found at the position of SP using anti-SP_2. Therefore it is concluded, that the lamprey CNS probably does not contain SP.

With the less specific SP-antiserum, anti-SP_{10}, two additional peaks were found at position C_1 and C_3. The elution pattern obtained using this antiserum thus resembled those found using anti-KAS_{12} and anti-ELE_2 which also revealed the same type of distribution (SP_1-type) at immunohistochemical examination.

Physalaemin. Indirect immunohistochemical evidence indicate that the lamprey TK-LI material does not contain considerable amounts of PHY. Thus, the physalaemin antiserum anti-Phy_2 and the SP-antiserum anti-SP_{10} which shows nearly full crossreactivity (89%) with physalaemin, gave rise to different patterns of distribution. However, such comparison of RIA and immunohistochemical data should be made with great caution.

Neurokinin A. With the most NKA-selective antiserum (anti-KAS_{12}) peaks were found at the same position as NKA at anion-exchange chromatography (A_2) and HPLC (H_1), but not at cation-exchange chromatography. Therefore it is concluded that NKA in any case does not constitute a major portion of the lamprey TK-LI material.

Neurokinin B. At HPLC of the spinal cord extracts and RIA with anti-KAS_{12}, which has 28% cross-reactivity with NKB, no peak was found at the position of NKB (Fig. 12B). Some material of the brain extracts, however, eluted at the position of NKB (Fig. 12C, Peak H_5). Therefore it is concluded that the lamprey spinal cord probably does not contain NKB, while it cannot be excluded that it is present in the brain.

Kassinin. No KAS was detected in the spinal cord extracts with the most KAS-selective antiserum (anti-KAS_7, Fig. 8C,D), and no peak was present in the position of KAS at HPLC (with anti-KAS_{12}, Fig. 12B). Therefore it is concluded that the lamprey spinal cord probably does not contain KAS. It is possible, however, that the brain-component H_2 contained KAS.

Eledoisin. At anion-exchange chromatography, some spinal cord material eluted at the position of ELE (A_1), but the content of this peak was low (3–10% of the total ELE-IR material). HPLC analysis of brain and spinal cord extracts using anti-KAS_{12}, which has 129% cross-reactivity with ELE (as compared to KAS), did not reveal a peak at the position of ELE (Fig. 12B,C). Therefore it is concluded that the lamprey CNS probably does not contain ELE.

Are the lamprey TK-IR peptides TKs? In the lamprey CNS, TK-IR peptides are present, but are probably different from SP, PHY, NKA, NKB, KAS and ELE. The structures of at least some of the TK-IR peptides in the lamprey CNS are probably similar enough to those of the known TKs to classify them also as TKs. This is concluded for the following reasons. (1) The overall pattern of peaks found in the ion-exchange chromatograms was consistent for several antibodies raised against different TKs. (2) The patterns of distribution of TK-LI fibers tested with even more antisera raised against different TKs showed a limited number of patterns of distribution. (3) After pre-incubation with TKs the immunostaining was reduced or abolished; therefore it is likely that the immunostaining is due to affinity of the antisera to TK-like peptides ("method specificity," see [32]). (4) The TK antisera used are probably mainly directed toward the carboxy- (C-)terminus [40], so they probably bind to compounds with a similar C-terminus, and similarities in the C-terminus are defining characteristics for TKs (cf. [8]). For these reasons we assume that the lamprey TK-LI peptides are TKs, but definitive conclusions on this issue await elucidation of their amino acid sequence.

No serum specificity. This study is unorthodox, since we have made use only of the (demonstrated) cross-reactivity of lamprey TKs with antisera raised against SP, NKA, KAS or ELE ("serum unspecificity," see [32]). An antiserum with absolute specificity for SP, NKA, KAS or ELE—and probably also for PHY—would probably give negative results in the lamprey, both with RIA and with immunohistochemistry. TK-positive staining by some antisera was more reduced by pre-incubation with other TKs than that against which the antisera was raised (Table 2), and similar results were obtained with radioimmunoassay (anti-KAS_{12}, Table 1). This corroborates the conclusion [32] that pre-incubation ("absorption") experiments cannot be used to conclude that an antiserum stains what it is supposed to stain. With the pre-incubation method no positive identification of the stained compound(s) can be obtained.

Comparison of biochemical and histological analyses. Results of immunohistochemistry and chromatography together with RIA, even when the same antisera have been used, cannot be compared directly for three reasons.

The tissues are treated differently. Fixatives for histological investigations (including formalin and picric acid) react with proteins and peptides, thereby changing their structure and possibly their immunoreactivity, while the tissues for RIA have been frozen and extracted in boiling water or acetic acid, which probably has less influence on peptide structure.

In immunofluorescence studies typical antiserum dilutions are between 1:100 and 1:1000, while in RIA typical dilutions range from 1:10,000 to 1:400,000 (cf. Tables 1 and 2).

In chromatograms, the peptides are separated on the basis of their chemical properties, while no information about their relationships (precursors or degradation products) can be obtained. In histochemical analyses, cell groups containing more than one peptide with affinity to a given antiserum (e.g., a TK, its precursors and its degradation products) are detected as single systems.

The clearest discrepancy between the RIA results and the immunohistochemistry was that anti-KAS_7 gave negative results in the RIA, but stained many fibers albeit weakly.

Which peptides elute where? The existence of three lamprey TKs (LT_S, LT_1 and LT_2) was postulated to explain the histological results. In the chromatograms, the TK-LI material was found in 5–7 peaks. Attempts to relate the hypothetical peptides from the histological results with the peaks in chromatograms are inevitably very speculative, but we will try to relate them nevertheless.

Anti-SP_2 visualized axons in the dorsolateral and lateral axon columns, and it had affinity to the material eluting as peaks C_2, C_4 and C_5 at cation exchange chromatography. Therefore it is speculated that either (1) LT_2 is homogeneous

and then it could be present in either C_2, C_4 or C_5, or (2) LT_2 is heterogeneous and then it could be present in more than one of these peaks.

The two antisera which with immunohistochemistry gave rise to the SP_S-type of distribution were not used for RIA and therefore no direct information is available on the components which they bind to. It is speculated, however, that C_3 contains LT_S, since anti-ELE_7 (which is suggested to have affinity both to LT_1 and LT_S) gave a considerable C_3 peak (200 fmoles ELE equivalents per gram tissue), while anti-ELE_2 and anti-KAS_{12} (which have rather selective affinity to LT_1) showed only a small C_3 peak. If this is correct, LT_S, which is mainly present in the dorsomedial axon column and in the dorsal horns, is a rather basic peptide, but not as basic as SP.

The third hypothetical peptide, LT_1, is probably present in C_1 and C_2, since these peaks contain 90% of the TK-LI material, which would then imply that LT_1 is somewhat acidic. The antisera anti-KAS_{12}, anti-ELE_2 and anti-ELE_7 showed both C_1 and C_2 peaks, and therefore it is not known whether the peptides constituting C_1 and C_2 are present in the same or in different neurons, and whether C_1 and C_2 contain TKs with different action, or that one is the precursor or degradation product of the other.

Comparison with other chordates. In the mammalian CNS, four different TKs, SP, NKA and NKB and an elongated form of NKA, NPK, have been found up till now [11,39], in amphibians seven [8], and now in lampreys at least three. It would be interesting to know if comparably extensive chromatographic analyses of species of other chordate classes also would indicate content of several different TKs, and whether or not SP-LI material found in tunicates, fishes, amphibians, reptiles and birds is actually SP (cf. [9, 17, 21, 26, 34, 35, 38]).

New TKs in the lamprey spinal cord. The amino acid sequence of fifteen naturally occurring TK-like peptides has been elucidated as far as we know [8, 11, 39, 44]. The presence of six of them (SP, PHY, NKA, NKB, KAS and ELE) in the lamprey CNS has been investigated, and their amounts are probably low or even negligible. Thus, the large majority of lamprey TK-LI peptides probably are other types of TK peptides. The presence of some of the other identified TKs in the lamprey CNS cannot be excluded at the moment. However, lampreys have an independent phylogenetic history from the other vertebrates for some 500–600 million years [2]. It is therefore not unexpected that several proteins and peptides in the lamprey are different from those found in other vertebrates. In fact, studies on some lamprey proteins have demonstrated clear differences as compared to the corresponding vertebrate proteins [7,45]. It is therefore not unlikely that the presently found lamprey TKs are new, not earlier described TKs. The relevance of such finding is still unclear. It would be interesting to know the structures of these lamprey TKs, and whether the lamprey TKs interact with specific receptors (cf. [24]).

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